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**Prise en compte des attentes sociétales et
professionnelles dans l'innovation et le développement
de solution pour la recherche sur les rodenticides et
l'enseignement de la nutrition clinique vétérinaire**

Sébastien Lefebvre

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Université Claude Bernard  Lyon 1

**MÉMOIRE d'HABILITATION à la DIRECTION des
RECHERCHES**

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Sébastien Lefebvre

**Prise en compte des attentes sociétales et professionnelles dans
l'innovation et le développement de solution pour la recherche sur
les rodenticides et l'enseignement de la nutrition clinique
vétérinaire**

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Pr STORCK Fanny Professeur, VetAgro Sup	Examineur
Dr LATTARD Virginie Directrice de recherche, INRAe	Examineur

Le Bret.

Si tu laissais un peu ton âme mousquetaire
La fortune et la gloire. . .

Cyrano.

Et que faudrait-il faire ?
Chercher un protecteur puissant, prendre un patron,
Et comme un lierre obscur qui circonvient un tronc
Et s'en fait un tuteur en lui léchant l'écorce,
Grimper par ruse au lieu de s'élever par force ?
Non, merci. Dédier, comme tous ils le font,
Des vers aux financiers ? Se changer en bouffon
Dans l'espoir vil de voir, aux lèvres d'un ministre,
Naître un sourire, enfin, qui ne soit pas sinistre ?
Non, merci. Déjeuner, chaque jour, d'un crapaud ?
Avoir un ventre usé par la marche ? Une peau
Qui plus vite, à l'endroit des genoux, devient sale ?
Exécuter des tours de souplesse dorsale ? . . . [...]
Non, merci ! Chez le bon éditeur de Sercy
Faire éditer ses vers en payant ? Non, merci !
S'aller faire nommer pape par les conciles
Que dans les cabarets tiennent des imbéciles ?
Non, merci ! Travailler à se construire un nom
Sur un sonnet, au lieu d'en faire d'autres ? Non,
Merci ! Ne découvrir du talent qu'aux mazettes ?
Être terrorisé par de vagues gazettes,
Et se dire sans cesse : « Oh, pourvu que je sois
Dans les petits papiers du Mercure François ? » . . .
Non, merci ! Calculer, avoir peur, être blême,
Préférer faire une visite qu'un poème,
Rédiger des placets, se faire présenter ?
Non, merci ! non, merci ! non, merci ! Mais . . . chanter,
Rêver, rire, passer, être seul, être libre,
Avoir l'œil qui regarde bien, la voix qui vibre,
Mettre, quand il vous plaît, son feutre de travers,
Pour un oui, pour un non, se battre, – ou faire un vers !
Travailler sans souci de gloire ou de fortune,
À tel voyage, auquel on pense, dans la lune !
N'écrire jamais rien qui de soi ne sortît,
Et modeste d'ailleurs, se dire : mon petit,
Sois satisfait des fleurs, des fruits, même des feuilles,
Si c'est dans ton jardin à toi que tu les cueilles !
Puis, s'il advient d'un peu triompher, par hasard,
Ne pas être obligé d'en rien rendre à César,
Vis-à-vis de soi-même en garder le mérite,
Bref, dédaignant d'être le lierre parasite,
Lors même qu'on n'est pas le chêne ou le tilleul,
Ne pas monter bien haut, peut-être, mais tout seul !

Cyrano de Bergerac
Edmond Rostand

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À la mémoire de Jacqueline Lefebvre

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Introduction

À la lecture du titre de ce mémoire, l'absence apparente de lien entre la nutrition et la gestion des populations de rongeurs peut sembler intrigante. En effet, les liens sont ténus. Cependant, cette conjugaison originale entre deux thématiques si différentes s'explique par un contexte particulier dans lequel mon objectif a été de servir au mieux la communauté. Ces deux domaines abordent des enjeux majeurs pour le bien-être animal, le bien-être humain, l'efficacité d'utilisation des ressources alimentaires, leur préservation, ainsi que la conservation de la biodiversité. Malgré la diversité des méthodes employées dans ces deux axes respectifs, deux points communs existent dans leur mise en œuvre : l'un technique et l'autre philosophique.

Sur le plan technique, mon intérêt personnel pour l'informatique m'a incité à développer et à mettre en œuvre des solutions logicielles et numériques pour relever les défis qui se présentent. Cette démarche permet d'introduire de nouvelles approches complémentaires en recherche et de faciliter la transmission des connaissances dans le domaine de l'enseignement. Toutefois, ce n'est pas cet aspect qui a principalement guidé mes choix depuis l'obtention de mon doctorat. Ces décisions sont plutôt le fruit d'une volonté profonde de servir la société et de répondre à ses attentes, malgré leur complexité et leur ambiguïté. Cette aspiration m'a conduit à embrasser la carrière d'enseignant-chercheur, dans le but de fournir les connaissances nécessaires au débat public, de participer à la création de solutions répondant aux préoccupations des citoyens, et d'assurer la formation de professionnels compétents. Elle m'a également poussé à exercer pleinement ma liberté académique afin de garantir l'indépendance et l'efficacité du service public, quitte à refuser, par un simple "non merci", les voies tracées par les volontés politiques locales. J'espère ainsi que ce travail, accompli sans souci de gloire ou de fortune, ait contribué au service de l'État et du bien commun.

L'objet de ce mémoire n'est pas de reprendre un à un les données, protocoles et discussions des recherches auxquelles j'ai pu participer. Ils sont décrits dans les publications (pages grisées du mémoire) et les paraphraser n'aurait que peu d'intérêt. Ici, mon souhait est de présenter les choix stratégiques, leur réalisation et d'analyser les succès et les erreurs qui m'ont appris à mieux encadrer et mener les projets. Ce mémoire reprendra le développement de l'ensemble de mes activités, qu'il s'agisse d'enseignement ou de recherche. En effet, même en l'absence de laboratoire de nutrition, il m'a fallu innover et encadrer des étudiants dans cette thématique afin de satisfaire aux demandes statutaires d'élaborer et de transmettre les connaissances. Et l'expérience tirée de ces deux thématiques est devenue complémentaire l'une pour l'autre.

La première partie est consacrée à la nutrition clinique. Elle débute par l'analyse des raisons qui ont conduit à une désaffection de la matière à VetAgro Sup, puis traite de la stratégie mise en place pour réimplanter cette formation tout en innovant dans les

outils mis en œuvre. Enfin, elle aborde l'émergence d'une modeste activité de recherche et d'expertise en nutrition des monogastriques (hors animaux de rente).

La seconde partie est consacrée à l'activité de recherche sur la gestion des populations de rongeurs. Elle présente le choix de cette thématique et ma perception de trois enjeux sociétaux sur lesquels j'ai basé mon activité de recherche : les risques liés à l'utilisation des AVKs, l'étude de solutions innovantes dans la gestion des populations de rongeurs et la prise en compte du bien-être animal dans la recherche sur cette thématique. Elle est suivie par la reprise des articles organisés par thématique, puis d'un chapitre analysant ce parcours et comment ces deux thématiques si différentes se sont nourries mutuellement.

Première partie

Réponse aux attentes sociétales dans le domaine de la nutrition des animaux domestiques

Chapitre 1

État des lieux

Rien ne naît du néant ; l'histoire exerce une influence considérable sur les pratiques universitaires contemporaines, tout comme le contexte plus large de la discipline. Afin d'initier cet état des lieux de l'enseignement de la nutrition clinique et d'introduire les choix stratégiques de développement, un bref historique est présenté. Ce dernier met en lumière le désengagement progressif de la nutrition dans la pratique médicale, tant humaine que vétérinaire. Ce point sert de prélude à une analyse approfondie des défis, des contextes et des enjeux associés à la nutrition clinique en France, et plus spécifiquement à VetAgro Sup. Dans de nombreuses sections de ce chapitre, la médecine humaine et la médecine vétérinaire sont abordées conjointement, en raison notamment du manque de données sur cette dernière dans des domaines plus sociologiques que médicaux.

1.1 Rapide historique

1.1.1 Du serment d'Hippocrate aux serments médicaux modernes, ou de l'effacement de la nutrition du champ médical

Dans la conscience collective, la nutrition et sa corrélation avec la médecine sont souvent reliées à Hippocrate (V^e siècle avant notre ère) et à la maxime, fréquemment attribuée de manière erronée, "que l'aliment soit ton médicament". Dans son serment originel, Hippocrate met en effet l'accent sur la diététique comme l'une des composantes essentielles des pratiques médicales : "J'utiliserai le régime pour le bien des malades selon mon habileté et mon jugement"¹. Le terme grec $\delta \iota \alpha \tau \eta \mu \alpha \varsigma \acute{\iota}$ est généralement traduit par "régime", et constitue la racine étymologique du mot "diététique", symbolisant l'ensemble des règles alimentaires et des principes d'hygiène de vie adaptés au bien-être du patient, ce qui correspond à la nutrition clinique dans l'acception contemporaine.

Le serment d'Hippocrate, en discordance avec les évolutions de la déontologie et de l'éthique médicales, a été graduellement révisé ou abandonné par les institutions acadé-

miques modernes. Cependant, l'héritage d'Hippocrate demeure une constante dans les milieux universitaires occidentaux. Ses descendants idéologiques incluent le serment de Genève, formulé et ultérieurement modifié par l'Association Médicale Mondiale depuis 1948, et en France, le serment de l'Ordre des médecins. D'autres variantes, comme le serment ou la prière de Maïmonide pour les médecins et dentistes juifs, existent également. Remarquablement, toute allusion à la diététique ou à la nutrition est absente des versions modernes du serment. Cette absence est-elle symptomatique d'un relèvement progressif de la nutrition, éclipsée par des solutions thérapeutiques plus immédiates telles que la chirurgie ou la pharmacologie ? Est-ce avec son entrée dans la science moderne, sous l'impératif de nourrir le monde, que cet art a quitté le champ médical pour rejoindre celui des agronomes et des chimistes ? Cette question mérite une attention particulière. En effet, le développement de la chimie agricole et de l'agronomie, notamment de la révolution agricole du XIX^e siècle à sa mécanisation au milieu du XX^e siècle, a peut-être contribué à éloigner la nutrition de son ancrage médical initial. Dans cette période cruciale, l'accent a été mis sur l'optimisation des rendements agricoles et la création de méthodes de production alimentaire plus efficaces, plutôt que sur les implications médicales de l'alimentation. Ainsi, le domaine de la nutrition semble avoir bifurqué, devenant une préoccupation principalement agronomique et industrielle, au détriment de l'étude de son lien avec la santé humaine et animale.

1.1.2 La lutte contre la faim

La nutrition a établi ses fondements scientifiques au cours des XVIII^e et XIX^e siècles. En effet, à l'instar de nombreuses autres disciplines, la nutrition a bénéficié des avancées révolutionnaires de la chimie. Les contributions de Lavoisier aux transformations de la matière ont ouvert la voie à une meilleure compréhension du métabolisme et des processus de digestion². Ce même Lavoisier, renommé pour ses travaux en chimie, a également mené des recherches sur des phénomènes physiologiques tels que la respiration et la transpiration³.

Dans les décennies qui ont suivi, la recherche en nutrition s'est focalisée sur un objectif clair : nourrir la population mondiale. L'étude de Magendie, réalisée à la demande de l'Académie des Sciences, est exemplaire à cet égard : il y a tenté de substituer la viande par de la gélatine extraite d'os⁴. Outre les efforts visant à valoriser tous les produits animaux, une importance particulière a été accordée à l'optimisation de la nutrition pour minimiser le gaspillage alimentaire. Ceci a été rendu possible par une meilleure connaissance des aliments et des besoins nutritionnels des animaux. Cette évolution a été soutenue par l'application d'approches prédictives et quantitatives, comme la méthode d'analyse proximale de Weende⁵, qui permet une estimation des cinq principales classes de nutriments. Grâce à ces données, la quantité d'énergie apportée par un aliment peut être calculée à l'aide de l'équation d'Atwater⁶. En outre, les besoins énergétiques au repos peuvent être estimés

selon la loi de Kleiber^{7,8}. Ces équations et méthodes, élaborées entre 1850 et 1920, demeurent pertinentes dans les systèmes nutritionnels contemporains. Accompagnées, bien sûr, de nombreuses autres découvertes qui ont profondément transformé le monde agricole, cette meilleure compréhension de la nutrition et la rationalisation de l'alimentation ont contribué à nourrir de manière adéquate et en quantité suffisante la quasi-totalité de la population des pays riches, notamment depuis la fin de la Seconde Guerre mondiale.

1.1.3 Les défis de la nutrition moderne

Mais ce succès ne doit pas faire oublier que les carences nutritionnelles persistent dans les sociétés occidentales. Ainsi, des groupes vulnérables tels que les sans-abri et les migrants demeurent exposés à des carences mortelles, telles que la carence en vitamine C^{9,10}. Ils ne sont cependant pas les seuls concernés. Une étude de 2003 a révélé que près de 50% des patients hospitalisés en médecine interne présentaient une concentration sérique en acide ascorbique inférieure aux normes, et que 16,9% étaient carencés¹¹. Ces données mettent en lumière non seulement la persistance du risque de carence, mais aussi celle du scorbut clinique dans les populations occidentales. De plus, les cas de scorbut chez les enfants ou les patients atteints de troubles psychiatriques soulignent les lacunes des systèmes de santé en matière d'intégration de la nutrition dans une approche médicale préventive et éducative^{12,13}. Plus largement, les enquêtes relatives aux apports alimentaires indiquent que les populations occidentales sont en deçà des recommandations en termes d'apports en vitamines et en oligoéléments. Ces déficits d'apport sont corrélés aux conditions socioéconomiques des sous-populations de ces pays^{14,15}.

Outre les problèmes de carence à plus large échelle, la sous-nutrition a cédé la place à une suralimentation tout aussi délétère. Ainsi, les populations occidentales sont confrontées à une augmentation des cas de diabète et d'obésité¹⁶⁻¹⁹. Une étude européenne de 2017 a estimé à 20% la proportion de décès où l'obésité est une cause sous-jacente²⁰. Ces chiffres témoignent du retard accumulé par la médecine et l'éducation en matière de nutrition humaine.

En matière de nutrition vétérinaire, particulièrement en ce qui concerne les animaux de compagnie, l'histoire a été significativement influencée par l'émergence de l'industrie alimentaire spécialisée. Remontant aux années 1860 en Angleterre, cette industrie a vu le jour avec la production des premiers biscuits pour chiens, une innovation due à James Spratt²¹. Le secteur s'est ensuite élargi par l'introduction du premier aliment complet, développé par Gaines dans les années suivant cette première avancée²¹. En 1922, la technique d'appertisation, un procédé de stérilisation à haute température, a été introduite avec la commercialisation des aliments sous la marque *Ken-L Ration*. Cette innovation avait pour objectif de valoriser la viande de cheval, illustrant ainsi la tendance de l'industrie à capitaliser sur les co-produits plutôt qu'à générer de nouvelles matières premières. Au cours

des années 1950, Purina a révolutionné le marché en inventant la première croquette extrudée, qui a connu un succès retentissant²¹. À l'heure actuelle, une grande majorité des chiens en France sont alimentés avec des croquettes, dont la plupart sont produites par extrusion^a. Des gammes complètes d'aliments sont maintenant disponibles, ciblant divers stades physiologiques et conditions médicales. Le Dr Mark Morris a ouvert la voie à cette spécialisation en créant, en 1948, le premier aliment diététique industriel destiné aux chiens souffrant de maladie rénale chronique. La simplicité d'utilisation et la standardisation de ces produits ont considérablement facilité les progrès en nutrition clinique vétérinaire, notamment dans le traitement de la maladie rénale chronique, de l'hyperthyroïdie féline et des urolithes²². Toutefois, tout comme en médecine humaine, des échecs notables persistent en matière de prévention de l'obésité et du diabète²³⁻²⁶.

Ces constatations nous poussent à nous interroger sur les raisons sous-jacentes à ces échecs en matière de nutrition, tant chez l'Humain que chez les animaux.

1.2 Les obstacles au développement de la nutrition

La nutrition clinique en médecine vétérinaire est confrontée à trois freins majeurs. Le premier frein concerne le manque de valorisation académique de la discipline par rapport à d'autres domaines jugés plus pertinents dans un contexte médical. Le second obstacle, en partie tributaire du premier, est la vulnérabilité de la nutrition aux pseudosciences. Le dernier obstacle réside dans le rôle ambivalent de l'industrie agroalimentaire, qui représente à la fois une opportunité et un frein à l'engagement des vétérinaires en nutrition clinique.

1.2.1 La dévalorisation académique de la nutrition en médecine vétérinaire

Suite aux problématiques croissantes de la nutrition clinique, on pourrait s'attendre à ce que les équipes médicales aient accru leur expertise dans ce domaine au cours des dernières années. Toutefois, diverses enquêtes et retours d'expérience montrent que la prise en charge nutritionnelle demeure souvent suboptimale dans les unités hospitalières et de soins intensifs²⁷⁻³⁴. Le déficit de prise en charge nutritionnelle en médecine vétérinaire est corroboré par deux études^{35,36}, qui révèlent que moins de 30% des animaux hospitalisés reçoivent les apports énergétiques quotidiens recommandés. De plus, l'une de ces études établit un lien entre cette sous-nutrition et une mortalité accrue durant l'hospitalisation³⁶. Malgré l'existence de protocoles standardisés en médecine humaine^{27,37} et en médecine vétérinaire³⁸, leur intégration dans la pratique médicale courante reste

a. 65% des chiens sont nourris exclusivement avec des croquettes (hors friandises) et 90% reçoivent tout ou une partie de leur ration en croquettes

limitée³⁹, avec des niveaux de compétence en nutrition qui demeurent insuffisants depuis de nombreuses années^{40,41}.

Ce déficit peut en partie s'expliquer par des lacunes dans la formation médicale en nutrition. Plusieurs études et éditoriaux pointent en effet l'insuffisance des heures d'enseignement consacrées à cette discipline et la faible intégration de la nutrition dans les programmes de formation médicale, tant du point de vue des étudiants que des enseignants⁴²⁻⁴⁶.

1.2.2 La porte ouverte aux pratiques pseudoscientifique

La pratique médicale est souvent sujette à des discours pseudoscientifiques, des mythes, et des opinions variées sur les traitements appropriés. Bien qu'Internet ait amplifié ce phénomène, il n'est pas nouveau. Ceci est illustré par la prière de Maïmonide, qui sert d'équivalent au serment d'Hippocrate pour les médecins et dentistes juifs. Cette prière est souvent attribuée à Moïse Maïmonide, médecin du XII^e siècle, mais il est plus probable qu'elle ait été rédigée vers 1790⁴⁷. Elle met également en lumière la violence verbale à laquelle les médecins peuvent être confrontés de la part des patients et du public.

« Fais que mes malades aient confiance en moi et mon Art pour qu'ils suivent mes conseils et mes prescriptions. Éloigne de leur lit les charlatans, l'armée des parents aux mille conseils, et les gardes qui savent toujours tout : car c'est une engeance dangereuse qui, par vanité, fait échouer les meilleures intentions de l'Art et conduit souvent les créatures à la mort. Si les ignorants me blâment et me raillent, fais que l'amour de mon Art, comme une cuirasse, me rende invulnérable, pour que je puisse persévérer dans le vrai, sans égard au prestige, au renom et à l'âge de mes ennemis. Prête-moi, mon Dieu, l'indulgence et la patience auprès des malades entêtés et grossiers. » *extrait de la Prière de Maïmonide, XII^{ème} siècle (attribué), plus probablement 1790*⁴⁷

Les pratiques pseudoscientifiques semblent être particulièrement prévalentes en nutrition et en nutrition clinique. On y observe régulièrement l'émergence de régimes ou de compléments alimentaires présentés comme miraculeux pour soigner divers maux, du cancer⁴⁸ à la COVID-19⁴⁹. Cette propension à offrir des conseils nutritionnels, émanant tant de professionnels que de particuliers non formés, semble découler de plusieurs facteurs. **i/** Premièrement, l'alimentation, contrairement aux médicaments, n'est pas soumise à une réglementation stricte, et les erreurs en la matière entraînent rarement des effets délétères immédiats. **ii/** Deuxièmement, un effet Dunning-Kruger manifeste se note en matière de nutrition. De nombreux professionnels de la santé ou du bien-être, humain ou animal, ont tendance à surestimer leurs compétences en nutrition⁵⁰⁻⁵³. **iii/** Troisièmement, la nutrition bénéficie d'un statut scientifique, ce qui permet d'utiliser des extraits d'articles

hors contexte pour crédibiliser des conseils nutritionnels auprès du grand public. **iv/** Enfin, l'alimentation, en raison de son caractère "naturel", est susceptible aux sophismes de l'appel à la nature.

Dans le domaine vétérinaire, cette tendance a conduit à la création de divers régimes alimentaires pour carnivores domestiques basés sur des prémisses non fondées : alimentation sans céréales, proies entières, régimes crus, régimes végétariens, etc. Ces régimes peuvent avoir des conséquences graves sur la santé des chiens et des chats, pouvant aller jusqu'à la mort⁵⁴⁻⁵⁶. Malgré ces évidences, une méfiance envers les vétérinaires est souvent observée chez les propriétaires qui nourrissent leurs animaux avec ces régimes non conventionnels⁵³. Cette méfiance, amplifiée par les réseaux sociaux, a même conduit au cyberharcèlement d'une consœur vétérinaire nutritionniste par un administrateur d'un groupe *Facebook* anti-croquettes⁵⁷.

1.2.3 L'incidence de l'industrie sur la pratique de la nutrition par les vétérinaires

L'une des distinctions majeures entre la nutrition humaine et animale, notamment en ce qui concerne les animaux de compagnie, réside dans l'importance prédominante de l'industrie agroalimentaire. En France, plus de 90% des chiens et chats sont principalement nourris avec des aliments industriels, tels que des croquettes ou de la pâtée^b. Une caractéristique singulière de ces régimes alimentaires industriels est leur qualité d'aliment "complet", fournissant tous les nutriments essentiels à la santé de l'animal. Ainsi, un chien ou un chat n'a besoin, pour subvenir à ses besoins nutritionnels, que de cet aliment en quantité adéquate et d'un accès illimité à de l'eau.

Cette industrie représente une opportunité significative, notamment en facilitant la conduite d'études et en permettant l'ajustement rapide de formulations alimentaires pour un grand nombre d'animaux. Cela a été démontré, par exemple, dans le cas de la découverte du besoin en taurine chez le chat⁵⁸, dont la carence était responsable de cardiomyopathies dilatées. Suite à cette révélation en 1987, les formulations alimentaires ont été revues, entraînant une diminution rapide et significative de la prévalence de cette pathologie mortelle⁵⁹. Par ailleurs, les aliments diététiques industriels ont contribué à plusieurs réussites en nutrition vétérinaire qui n'ont pas d'équivalent en médecine humaine, tant en termes de preuves cliniques que de populations animales concernées²².

Cependant, cette dépendance vis-à-vis de l'industrie agroalimentaire présente des inconvénients notables, notamment en termes d'indépendance scientifique et clinique. Nombre d'études en nutrition clinique sont réalisées en collaboration avec cette industrie qui les finance. Plus problématique encore est la question de la latitude clinique du vétérinaire : est-il en mesure de proposer des alternatives aux aliments industriels lorsque

b. Données de l'auteur, dérivées des enquêtes KANTAR/FACCO 1997-2022

le propriétaire ou l'animal s'y oppose, ou de personnaliser un régime alimentaire en présence de comorbidités⁶⁰ ? En effet, la complexité des autres approches est significative par rapport à la prescription d'un aliment qui porte le nom de l'affection.

Un autre obstacle à surmonter pour accéder à une réelle indépendance vis-à-vis de l'industrie agroalimentaire concerne la capacité des vétérinaires à analyser quantitativement les apports nutritionnels des rations alimentaires. Actuellement, la confiance accordée à l'industrie, associée à la complexité du calcul des apports nutritionnels, freine cette initiative. Pourtant, qu'il s'agisse d'une évaluation critique des aliments commerciaux ou de la prescription de rations ménagères, cette compétence est indispensable.

1.3 Contextes Locaux

Suite à l'introduction du contexte général de la nutrition et de ses défis associés, cette section vise à clarifier le contexte local spécifique à ma mission d'enseignant-chercheur en nutrition à VetAgro Sup. Elle retrace les questionnements et le raisonnement qui ont sous-tendu l'élaboration de ma stratégie de développement académique.

1.3.1 De l'Enseignement de la Nutrition à VetAgro Sup en 2015

La nutrition animale se segmente en plusieurs domaines qui n'ont pas tous bénéficié du même développement au sein des différentes écoles vétérinaires en France. Par exemple, l'École Nationale Vétérinaire de Toulouse a privilégié une spécialisation de ses enseignants-chercheurs sur un domaine spécifique, que ce soit les animaux de rente ou les animaux de sport et de loisir. À VetAgro Sup Lyon, l'approche a été différente : l'équipe de nutrition a choisi de ne pas imposer de spécialisation à travers les fiches de poste, permettant à chaque enseignant de développer progressivement son domaine de prédilection.

De ce fait, ma fiche de poste était d'une grande amplitude, couvrant un large éventail de la nutrition animale, depuis les fourrages jusqu'à la nutrition du chat, en passant par celle des poulets de chair et sans omettre la toxicologie végétale. Cependant, il est irréaliste d'aspirer à un enseignement d'excellence sur une si vaste gamme de sujets. C'est pourquoi, tout en remplissant les obligations liées à ma fiche de poste, j'ai opté pour une spécialisation dans un domaine particulier de la nutrition.

En 2015, j'ai procédé à une analyse rapide des domaines qui nécessitaient une attention particulière. Un sujet m'a paru extrêmement urgent : la nutrition des carnivores domestiques. Ce déficit peut être attribué à une pénurie d'enseignants en nutrition ces dernières années, mais également à un désintérêt croissant de la profession vétérinaire pour ce domaine. Actuellement, seulement 8,25 heures sont allouées à cet enseignement au cours de l'ensemble du cursus, réparties en 3,75 heures de cours magistraux et 4,5 heures de travaux dirigés. Ce volume est nettement en deçà de la moyenne des autres écoles vétérinaires

européennes, où il atteint en moyenne 19 heures⁴⁴ (tableau 1.1). De plus, les experts en nutrition européens estiment qu'environ 47 heures seraient nécessaires pour satisfaire aux exigences de la nutrition clinique⁴⁴. Accroître ce volume d'enseignement constitue un défi majeur, d'autant plus que les emplois du temps des étudiants sont déjà fort chargés, mais il est impératif pour assurer un enseignement de qualité.

Domaine	Heures à VetAgro Sup	Heures en Europe	Heures souhaitées par les enseignants européens
Base de la nutrition	20.25	37	49
Nutrition des animaux de compagnie	8.25	19	47
Nutrition des grands animaux	20.5	29	49

TABLE 1.1 – Heures de cours en nutrition (magistraux et travaux dirigés) à VetAgro Sup en 2015 par rapport aux heures des autres cursus vétérinaire d'Europe rapportées par Becvarova⁴⁴

Sur le plan qualitatif, la formation en nutrition pour les carnivores domestiques était limitée à l'alimentation de l'animal à l'entretien ; la nutrition clinique, quant à elle, était absente tant dans l'enseignement théorique que dans l'enseignement pratique. Une consultation en nutrition était certes organisée par un intervenant extérieur, mais sans l'implication d'étudiants. Cette lacune représente à mes yeux une problématique significative pour la formation de vétérinaires cliniciens, qui nécessitent des compétences pour prescrire des régimes alimentaires adaptés à leurs patients.

Bien que d'autres domaines, tels que la nutrition des équidés ou des monogastriques de rente, présentent également des insuffisances, il m'est apparu que l'urgence se situait plutôt dans la nutrition des carnivores domestiques. Cette perception s'appuie sur le nombre considérable d'étudiants qui envisagent une pratique vétérinaire exclusive ou partielle dans ce domaine. De plus, contrairement à d'autres domaines, celui des carnivores domestiques souffre d'un manque de spécialistes formés en nutrition pour orienter les propriétaires et les différents acteurs de la filière.

1.3.2 De l'intérêt de la profession et de la société pour l'alimentation des carnivores domestiques

Pour qu'un changement de cursus puisse avoir lieu et que le développement d'enseignement d'alimentation des carnivores domestiques soit un succès, il est nécessaire de s'assurer d'une attente de la profession vétérinaire française et plus largement d'une attente des propriétaires d'animaux. De plus, il est nécessaire d'estimer le niveau attendu

par la profession. Est-ce d'avoir quelques vétérinaires spécialistes référents ? Ou un niveau plus ou moins avancé en sortie d'école ? De plus, il faudra aussi mesurer le potentiel de créer des formations continues en nutrition clinique ?

En effet, à mon arrivée en poste, la question de l'intérêt de la profession pour la nutrition pouvait se poser. En 2015, le cursus préparant au Certificat d'Études Spécialisées en Diététique canine et féline (formation postuniversitaire) recevait sa dernière promotion de vétérinaires suite au départ à la retraite du Pr Paragon à l'école de Maison Alfort. Face au retard de la nutrition à VetAgro Sup, la question ne se posait pas de reprendre ou non une formation à laquelle nous étions déjà la seule école francophone de France et de Belgique à ne pas participer. Cependant, comme aucune autre école n'a repris le flambeau, se pose la question du besoin, ou non, d'assurer l'acquisition de compétences en nutrition clinique au sein de l'école vétérinaire de VetAgro Sup. Ou, y a-t-il un désintérêt général de la profession vétérinaire et des institutions universitaires françaises sur cette matière ? Si ce désintérêt était confirmé, les programmes de résidence (formation de 3 ans permettant l'accès au titre de spécialiste) assurée aux écoles vétérinaires de Toulouse et Nantes seraient suffisants pour couvrir les besoins de la profession. Lors de ce début de poste, face à la décroissance des heures de nutrition et les difficultés locales à les défendre lors des arbitrages pour les heures face à d'autres matières jugées plus "clinique" comme l'oncologie, la cardiologie ou la chirurgie, c'est un sentiment de solitude qui m'habitait ainsi qu'une remise en question de l'axe que j'avais choisi.

Cependant, en parallèle de mes premières années en tant que maître de conférences, les événements ont marqué l'intérêt de la profession et plus largement de la population française pour l'alimentation des carnivores domestiques. En effet, entre 2015 et 2020, on a pu voir émerger des vétérinaires influenceuses en nutrition clinique des carnivores domestiques (notamment Charlotte Devaux et Mathilde Thierry) qui rassemblent autour d'elles des communautés de consœurs et de confrères et assurent une transmission vulgarisée des connaissances nutritionnelles. En parallèle et face au vide laissé par l'arrêt du CES, des formations privées se sont mises en place. De plus, ce regain d'intérêt n'a pas uniquement touché la profession vétérinaire, mais aussi les propriétaires d'animaux de compagnie.

L'un des moments importants, non pas du début de la prise en compte de l'alimentation par les propriétaires d'animaux, mais plutôt de la prise de conscience par l'industrie et la profession vétérinaire que l'alimentation pour animaux de compagnie était un enjeu pour les propriétaires d'animaux est l'émission : "Quelles croquettes pour nos bêtes ?" ^c. Cette émission reprend les poncifs communs sur internet : la dangerosité des glucides dans les croquettes, le parallèle avec l'alimentation humaine ultra-transformé... Si le contenu avancé dans cette émission n'est pas original, la place qu'elle a prise de par sa diffusion sur une grande chaîne nationale et a un horaire de grande écoute a agit comme un séisme dans la

c. Émission de Marion Baillot et Mathieu Sarfati diffusée sur France 5 le 8 octobre 2017 à 20h50

profession, faisant remonter des questions de la part des propriétaires vers les vétérinaires, et des vétérinaires vers les enseignants des ENV. Cette mise en lumière de l'alimentation a pointé aussi les lacunes de la profession sur cette matière. D'autant plus que cette émission a mis en avant un intervenant porteur de raisonnement pseudoscientifique tel que ceux discutés en section 1.2.2, et qui a réussi à regrouper autour de lui une centaine de milliers de personnes francophones sur les réseaux sociaux.

Cette préoccupation de la population française peut s'expliquer entre autres par le changement de profil du propriétaire de chien et de chat en France. La figure 1.1, venant d'une étude, présentée au chapitre 3, montre que l'âge des propriétaires de chien a tendance à diminuer avec une population de chien qui était majoritairement détenue par des personnes de plus de 35 ans dans les années 2000 alors qu'aujourd'hui c'est la population des moins de 35 ans qui est majoritaire. Bien que plus légère, cette baisse de l'âge des propriétaires est aussi visible pour les chats. Ce changement de la population d'adoptant est en accord avec les études de sociologie qui montrent l'émergence de "PetParents"^{61,62}. Cette nouvelle population considère l'animal de compagnie comme un substitut aux enfants ou comme un "test" avant d'avoir des enfants. Cette dernière vision de l'animal est en accord avec nos résultats qui semblent montrer que l'adoption de l'animal est antérieure à la naissance des enfants. Ce changement de statut, plus uniquement membre de la famille, mais enfant de la famille, change aussi les attendus de cette partie de la population pour l'alimentation des animaux de compagnie en les rapprochant de ceux pour les jeunes enfants.

Un point illustrant ce propos est la crise qu'a subi l'entreprise "Purina Nestlé" suite au rappel d'aliment pour chat à cause d'une contamination au 2-Chloro-Ethanol (2-CE)⁶³.le rappel étant motivé par un risque de fraude à l'utilisation d'oxyde d'éthylène dont le 2-CE est un marqueur, mais, dans ce cas, le 2-CE était généré par un nouveau procédé de fabrication du gluten⁶⁴. L'avis de l'ANSES, saisie pour les aliments à destination humaine contaminés par ce gluten, a conclu à son innocuité aux doses impliquées⁶⁴. Cependant, malgré l'absence de risque, un avis de rappel volontaire a été émis pour les croquettes pour chat.Celui-ci a entraîné de nombreux articles et reportages par des médias nationaux et régionaux sur des morts de chatons prétendument causés par ces aliments⁶⁵⁻⁶⁷, avant que le lien soit discrètement écarté⁶⁸. Ainsi, la population est devenue très sensible sur la question des animaux de compagnie en général et sur l'alimentation en particulier. Cette sensibilité s'illustre aussi par une volonté de certains propriétaires de faire adopter par leur animal des régimes particulier proche de leur vision de leur animal de compagnie : ration ménagère, tout cru, sans-céréales, végétarienne... Ce qui augmente les questions concernant l'alimentation à destination des vétérinaires traitants et, par conséquent, les exigences en termes de compétences attendues en nutrition pour ces mêmes vétérinaires.

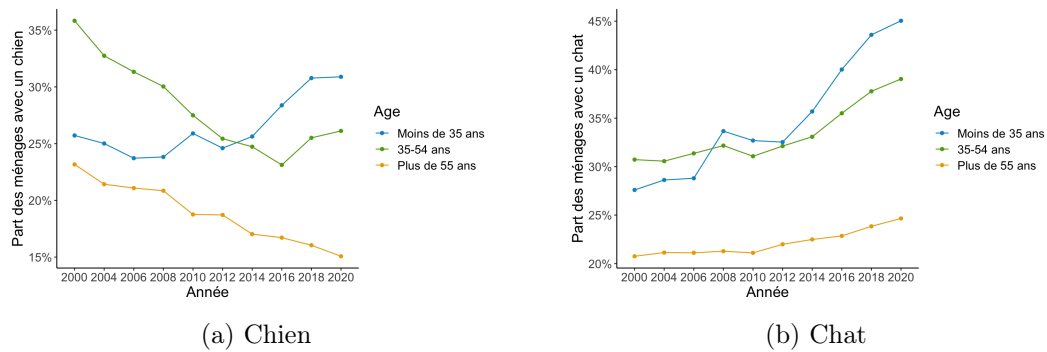


FIGURE 1.1 – Pourcentage de foyers français possédant un chien ou un chat selon l'âge du chef de famille (définition INSEE) et en fonction de l'année.

En conséquence, une divergence notable s'est manifestée au fil des années entre les attentes sociétales en matière d'alimentation des carnivores domestiques et l'expertise vétérinaire, notamment celle dispensée au sein de VetAgro Sup. Ce décalage a offert un terrain propice à l'ascension de discours pseudoscientifiques, faute d'alternatives fiables pour le conseil en nutrition animale. Il devient donc impératif de pallier ce manque en assurant une formation adéquate aux futurs vétérinaires généralistes et en favorisant l'émergence de référents spécialisés dans ce domaine. À cette fin, il est essentiel d'élaborer une stratégie efficace permettant de surmonter les obstacles évoqués dans ce chapitre.

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Chapitre 2

Développement d'outils pour la nutrition

Pour lever les obstacles identifiés dans le chapitre précédent et faciliter le développement de la nutrition clinique des carnivores domestiques au sein de mon établissement, tout en répondant aux attentes de la profession, j'ai élaboré une stratégie proactive. Cette stratégie s'appuie sur divers outils conçus pour renforcer l'enseignement, la pratique, l'expertise, et la recherche en nutrition clinique. L'ensemble de ces initiatives est guidé par un idéal sous-jacent qui oriente nos actions.

2.1 Idéaux de la stratégie

L'avantage de partir de peu est que l'on peut établir ses propres règles sans subir excessivement le fardeau des pratiques antérieures. Ces idéaux, qui incarnent ma vision de la fonction publique de l'enseignement supérieur, servent non pas de lois immuables, mais de repères pour prioriser une mission ou faire un choix. Les trois principaux sont les suivants : indépendance, accessibilité et innovation.

L'indépendance représente sans doute le défi le plus ardu, notamment dans le domaine de la nutrition. Comme exposé dans le chapitre précédent, il existe une problématique de dépendance de la nutrition des carnivores domestiques à l'industrie de l'alimentation animale (*petfood*). À mon sens, la société et la profession attendent des référents neutres. Toutefois, cette neutralité ne doit pas être contre-productive en rompant le dialogue avec le secteur privé. L'équilibre que je maintiens consiste à ne percevoir aucun avantage personnel (en nature ou monétaire) en dehors de mes rémunérations du secteur public, et à veiller à ce que mes activités ne soient pas conditionnées par la participation d'acteurs privés. Cette posture est facilitée par l'absence de laboratoire spécialisé en nutrition des carnivores au sein de mon établissement.

L'accessibilité constitue, à mes yeux, l'essence même du service public de l'enseigne-

ment supérieur. Nous produisons et diffusons le savoir sans entraves, en le rendant accessible au plus grand nombre. Ainsi, les productions d'un fonctionnaire ne devraient-elles pas appartenir à la collectivité? Cette démarche est soutenue par la loi pour une république numérique de 2016 et par le "Plan d'action logiciel libre" du gouvernement en 2021. En pratique, je garantis cette accessibilité en déposant l'ensemble de mes écrits dans des archives ouvertes, telles que HAL, et en regroupant mes productions liées à la nutrition (cours, diagrammes, logiciels, vidéos) sur un site personnel accessible gratuitement : vetbrain.fr.

Enfin, l'innovation consiste à repousser les limites du possible en explorant de nouvelles approches. Pour maintenir cette dynamique, il est crucial de rester ouvert à diverses disciplines afin d'en tirer inspiration pour ses propres pratiques. Par ailleurs, cette démarche implique également la prise de risques et l'acceptation de l'échec comme une occasion d'apprentissage. Grâce au statut que nous confère l'État, les échecs ont pour principale conséquence une perte de temps relative, compensée par l'expérience ainsi acquise. Je m'efforce donc d'intégrer cet élément d'innovation dans l'ensemble de mes missions.

2.2 Structurer la connaissance

Avec le peu d'heures disponibles à l'emploi du temps pour traiter de la nutrition des carnivores domestique (8.25 h en tout sur l'ensemble du cursus vétérinaire de Lyon), il n'était pas possible de transmettre correctement des compétences en nutrition clinique. Les discussions avec la direction de la formation de l'époque n'ayant pas permis d'augmenter ce volume, j'ai fait le choix de mettre en place à partir de 2018, un enseignement optionnel (devenu personnalisé après une réforme du cursus) en nutrition clinique.

2.2.1 Réflexion initiale

Cet enseignement est constitué de 12 séances de travaux dirigés axés sur la résolution de cas cliniques en utilisant les outils d'analyse présentés ultérieurement. Lors de la conception de ce programme, une interrogation sur le mode de transmission des connaissances s'est imposée. Traditionnellement, le cours magistral sert de vecteur à cette transmission. Toutefois, cette méthode ne semblait pas optimale. D'une part, pour des raisons temporelles, cet enseignement optionnel m'offrait 18 heures supplémentaires à consacrer à l'acquisition de compétences pratiques en nutrition clinique. D'autre part, en raison de la limitation du nombre d'étudiants, seuls 30 d'entre eux pourraient bénéficier de ce cours chaque année, malgré un vivier de 80 à 100 candidatures. Un enseignement en présentiel restreindrait donc l'accès au reste de la promotion. De surcroît, un tel cours aurait un impact limité sur la formation professionnelle, qui exprime un besoin dans ce domaine. Enfin, sur un plan plus philosophique, le cours magistral laisse peu de place à la critique par les pairs.

Or, afin de limiter l'influence des pseudosciences et éviter la propagation de mythes, il est essentiel de privilégier un mode de transmission mettant en avant les sources scientifiques et permettant la critique.

La tendance actuelle favorise la réalisation de MOOC en vidéo^a, ce qui répond aux problématiques d'accessibilité mais pas nécessairement à la mise en avant des sources. J'ai expérimenté la vidéo pour de petits formats de vulgarisation : <https://www.youtube.com/@nutritionveterinaire1268>. Cependant, ce format requiert un investissement initial significatif et ne peut pas être facilement corrigé ou mis à jour. Le support écrit traditionnel demeure donc le plus adapté pour ce cahier des charges.

Le programme optionnel est donc structuré en 12 chapitres sourcés, un pour chaque séance, que les étudiants sont incités à lire au préalable. Chaque séance commence par un court QCM mettant en exergue les connaissances essentielles du chapitre, suivi d'une discussion sur les avancées récentes ou les points sujets à controverse. Il reste ensuite les deux tiers de la séance d'une heure trente pour développer des compétences pratiques à travers des cas cliniques.

Outre cet avantage pédagogique, ce document permet d'aller au-delà des attentes purement pédagogiques, servant ainsi de référence pour la pratique de la nutrition clinique et aidant à retrouver des articles de recherche pour les thèses d'exercice.

2.2.2 Mise en œuvre et évolutions

Après la rédaction des chapitres, le plan initial était de les compiler dans un livre afin d'en assurer une diffusion plus large. Conformément aux idéaux évoqués précédemment, ce livre devait être disponible gratuitement en ligne tout en offrant une version imprimée pour plus de praticité. La contrainte d'une version numérique sous Licence Creative Commons By^b n'étant pas compatible avec les exigences des éditeurs contactés, l'auto-édition s'est avérée nécessaire pour produire une version physique. Cependant, cela signifiait renoncer au soutien de professionnels pour la création d'un document de qualité. Pour pallier cette lacune, et en accord avec la philosophie de l'accès libre, je me suis formé au langage d'édition \LaTeX . Ce langage est largement utilisé dans les domaines de la mathématique, de la physique et de l'informatique, mais demeure peu connu en biologie. Il offre des fonctionnalités avancées pour la mise en page des publications et constitue l'un des facteurs expliquant la grande quantité de revues en accès libre dans ces disciplines.

En janvier 2020, l'édition numérique de "Nutrition vétérinaire du chien et du chat" était mise en ligne sur mon site personnel et sur le site VétoSapiens^c. Cette initiative m'a valu de recevoir un prix Veto Sapiens en 2020. Pour la version physique, le choix s'est

a. Massive Open Online Course

b. Cette licence permet à d'autres de distribuer, de modifier, d'adapter et d'exploiter votre travail, même à des fins commerciales, tant qu'ils vous attribuent la paternité de l'œuvre originale.

c. Site communautaire de l'association Vetonet : <https://www.vetosapiens.net/>

porté sur le système d'autoédition d'Amazon. Ce système est basé sur l'impression à la demande et ne nécessite pas d'investissement initial de la part de l'autoéditeur. Il permet également des ventes à prix coûtant, réduisant ainsi le prix d'un exemplaire à 5€ pour cette première édition.

Toutefois, la pandémie de COVID-19 a modifié les plans initiaux. La première édition ne comprenait que les cours optionnels. Cependant, pour maintenir la qualité de l'enseignement pendant la pandémie et en complément des cours en ligne, une seconde édition a été créée en avril 2020. Cette édition, qui couvre l'ensemble du programme de nutrition des carnivores domestiques, a été téléchargée 10 000 fois et s'est vendue à 1 035 exemplaires en version physique. Elle présente l'avantage d'être plus didactique et contient un formulaire ainsi que des tables de besoins nutritionnels, ce qui en fait un outil pratique.

Cependant, la détermination de ces besoins nutritionnels a été complexe à réaliser. En effet, les minima et maxima proposés par les guides nutritionnels de la FEDIAF^{1 d} et du NRC^{2 e} assurent la sécurité de l'animal mais ne fournissent pas nécessairement une ration optimisée. À l'aide de l'analyse de la littérature effectuée lors de la rédaction ainsi que de l'expérience acquise, des optimaux minimum et maximum sont proposés pour la majorité des nutriments en complément des valeurs de références. Ces optimaux n'ont pas pour objectif de remplacer celles issues de sources bien mieux qualifiées et qui ont profité d'un travail collégial, mais de fournir, en complément des autres valeurs, un intervalle cible plus restreint pour faciliter la création de ration par les vétérinaires.

2.3 Permettre l'analyse nutritionnelle

Au-delà de l'aspect des connaissances, il est également indispensable de permettre aux vétérinaires et aux étudiants de les mettre en pratique de manière simple, objective, et indépendante. La principale difficulté dans la pratique nutritionnelle réside dans la gestion d'un volume conséquent de données. Pour chaque aliment, il est nécessaire de les identifier, de les comparer par rapport aux autres aliments afin de sélectionner le plus adapté, puis de calculer les apports nutritionnels et les comparer aux références établies. Ces tâches requièrent de nombreuses manipulations de données et des calculs souvent fastidieux. La pratique nutritionnelle peut ainsi sembler davantage être un exercice de tableur plutôt qu'une pratique médicale, occultant le fait qu'un animal est au cœur de ces chiffres.

Il me paraissait donc indispensable qu'un vétérinaire puisse choisir et analyser une ration en moins de cinq minutes. À mon avis, cela est possible grâce à l'utilisation de logiciels facilitant les calculs et les analyses, mais aussi grâce à une approche graphique

d. Fédération européenne des syndicats nationaux des fabricants d'aliments pour animaux de compagnie

e. Travail de synthèse réalisé par le Conseil national de la recherche des États-Unis et faisant référence dans le domaine

des aliments commerciaux.

2.3.1 VetNutri 1

Dès ma première année d'enseignement, et face au constat de la nécessité d'un outil logiciel, la principale solution existante était fournie par l'Université de Munich : Diet Check Munich³. Il s'agit d'un logiciel hors-ligne, disponible en allemand et en anglais, qui permet l'analyse de tout type de rations. Ce logiciel fonctionne exclusivement sur le système d'exploitation Windows et coûte 590€, avec des mises à jour à 70€. Outre l'incompatibilité avec d'autres systèmes d'exploitation (notamment macOS, très répandu parmi les étudiants en médecine vétérinaire), c'est surtout le coût qui m'a dissuadé. Il aurait certes été possible de négocier des licences gratuites dans le cadre de la formation avec l'Université de Munich. Toutefois, cela aurait contraint les étudiants à utiliser un logiciel payant à long terme s'ils souhaitaient continuer en nutrition clinique.

VetNutri a donc été initié avec un cahier des charges initialement simple : le logiciel devait être gratuit, compatible avec n'importe quelle plateforme, contenir une base de données d'aliments domestiques et commerciaux, et permettre d'analyser les apports en nutriments. Pour assurer la gratuité, le logiciel fut développé sur mon temps libre et sous licence Creative Commons BY-NC-SA^f. Ce choix me fut plus tard reproché par mon administration locale, qui souhaitait monétiser ce travail, en contradiction flagrante avec les directives gouvernementales sur la contribution des administrations aux logiciels libres. Par conséquent, j'ai pris la précaution d'intégrer des bibliothèques (fragments de code produits par d'autres programmeurs) dont les licences imposent la non-commercialisation du logiciel. Ainsi, VetNutri serait soit distribué gratuitement, en accord avec les principes d'accessibilité et d'ouverture, soit pas du tout. Cette précaution s'est avérée nécessaire, la question de la monétisation du logiciel ayant été soulevée au moins trois fois entre 2016 et 2023.

Pour assurer la compatibilité multiplateforme de VetNutri, deux options étaient envisageables : créer un site web ou utiliser un langage de programmation multiplateforme. La première option était sans doute la plus simple et la plus pratique. Cependant, elle posait deux problèmes majeurs : la confidentialité des données et le coût de fonctionnement. Le choix d'une solution en ligne aurait engendré des coûts supplémentaires, incompatibles avec la gratuité du projet. De plus, elle aurait nécessité la sécurisation des données des utilisateurs, une responsabilité légale et morale difficile à assurer dans le cadre d'un projet gratuit et individuel. Par conséquent, VetNutri a été développé en Java, permettant ainsi de stocker toutes les données localement sur la machine de l'utilisateur.

Les derniers points, concernant la base de données d'aliments et le calcul des apports, ont été résolus grâce à l'utilisation de bases de données publiques, telles que celle de

f. Attribution - Pas d'Utilisation Commerciale - Partage dans les Mêmes Conditions

l'ANSES⁴ ou de l'USDA⁵, pour les aliments ménagers et les données disponibles sur internet ou dans les clefs produits pour les aliments commerciaux. VetNutri comprend ainsi plus d'une dizaine de milliers d'aliments, dont deux mille sont des aliments commerciaux. Les apports recommandés sont ceux du NRC², avec quelques modifications mineures.

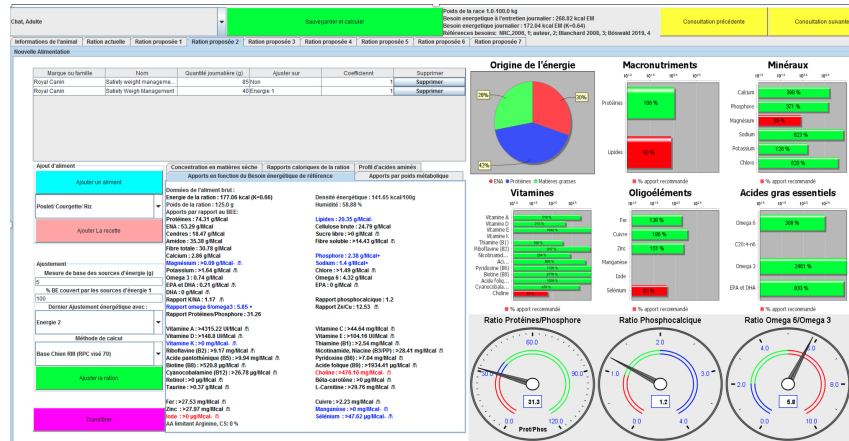


FIGURE 2.1 – Capture d'écran de la première version de VetNutri

En avril 2016, après quatre mois de programmation, la première version de VetNutri devint utilisable pour les étudiants. Au cours des cinq années suivantes, une trentaine de mises à jour ont été implémentées, ajoutant des histogrammes et diverses autres fonctionnalités (voir figure 2.1). Tous les nutriments sont affichés sur un seul écran et sont accompagnés d'un code couleur pour indiquer la couverture des besoins nutritionnels. Ces deux caractéristiques facilitent non seulement l'analyse, mais aussi l'encadrement pédagogique en réduisant le besoin d'interventions de l'enseignant pour afficher des informations. De plus, pour chaque nutriment, les normes et leurs sources sont accessibles via une bulle de texte explicative.

Ce logiciel a été téléchargé 800 fois par des utilisateurs non-étudiants. Cependant, bien qu'il soit capable d'analyser n'importe quelle ration alimentaire, VetNutri ne permettait pas aux étudiants et aux vétérinaires de choisir facilement et objectivement entre deux aliments commerciaux ou plus, sauf à les tester tous...

2.3.2 Diagramme des aliments

Grâce à VetNutri, une consultation en nutrition accueillant des étudiants a pu être créée dès 2017. Cependant, cette expérience m'a permis de constater que les étudiants étaient incapables de prescrire de manière raisonnée un aliment commercial diététique. Si l'animal était obèse, ils proposaient un aliment ciblant l'obésité d'une marque spécifique, souvent celle ayant organisé le dernier buffet, une pratique courante avant la pandémie de COVID-19. Toutefois, ils étaient incapables de justifier ce choix ou d'explorer d'autres alternatives. Les tableaux reprenant les données analytiques des produits, même multimarques, ne

résolvaient pas ce problème. Submergés par les chiffres, ils se contentaient souvent de sélectionner sur la base d'un seul critère, souvent le taux de protéines.

Dans le cadre de la thèse vétérinaire d'Agathe Champetier, nous avons comparé des aliments à l'aide de graphiques pour déterminer les plus adaptés pour des cas de cancer. Il est apparu que cette méthode pouvait être systématisée pour tous les aliments commerciaux. Utilisant la base de données déjà créée pour VetNutri et les compétences acquises en \LaTeX et R, un document de 300 pages analysant 500 aliments des gammes vétérinaires des cinq marques les plus utilisées par les confrères et consœurs fut élaboré. Pour chaque indication (stade physiologique ou affection), les aliments sont présentés dans une série de graphiques : origine de l'énergie, densité énergétique, rapport calorique en minéraux, etc. (exemple en figure 2.2).

Cette approche permet aux étudiants de comparer les aliments entre eux et d'initier une réflexion basée sur des facteurs nutritionnels clés. Pour chaque cas, ils doivent identifier les nutriments essentiels, ce qui leur permet de choisir les graphiques pertinents pour sélectionner quelques aliments à tester dans VetNutri. Un exemple de cette intégration a été détaillé dans le poster présenté dans la section 3.3.1. En analysant quelques graphiques, ils peuvent déterminer quels aliments sont les plus adaptés sans être influencés par la marque ou le nom. Les aliments sont uniquement identifiés par un numéro dans les graphiques. Une table de correspondance à la fin de chaque section permet de retrouver l'aliment en question. Les retours des étudiants sur les premières versions des diagrammes sont positifs ; ils les trouvent pédagogiques et estiment que cela leur permet de mieux appréhender la diversité du marché. Au-delà de leur utilité pédagogique, ces graphiques sont également très utiles pour démontrer aux propriétaires que le choix de l'aliment est réfléchi et indépendant des marques.

Ces outils, à savoir les diagrammes et le logiciel, incitent les étudiants à structurer leur réflexion en matière de nutrition. Sans cela, ils se trouvent submergés par un excès d'informations. Cela représente également un gain de temps, libérant ainsi davantage de place pour l'analyse clinique et permettant d'expérimenter virtuellement des rations hypothétiques. Néanmoins, ces graphiques, qu'ils soient en format papier ou PDF, présentent l'inconvénient de ne pas pouvoir être mis à jour par l'utilisateur pour y intégrer, par exemple, l'aliment actuellement reçu par l'animal ou une nouvelle marque sur le marché. Il est devenu évident que ces deux outils étaient destinés à fusionner.

2.3.3 VetNutri 2

La première version de VetNutri a été développée sur le tas, sans une réflexion substantielle concernant son évolution future. Par conséquent, la maintenance du logiciel devenait de plus en plus ardue à mesure que les mises à jour étaient déployées, principalement en raison de choix initiaux malavisés. De plus, alors qu'en 2016 peu de logiciels ou sites

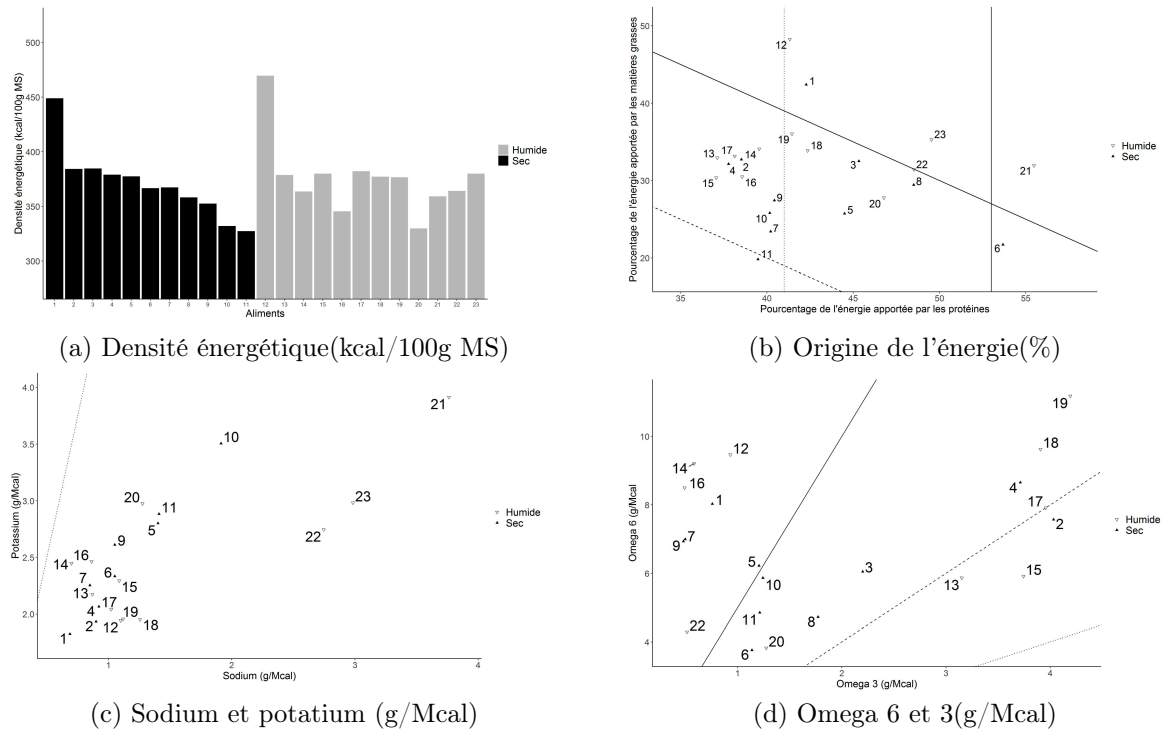


FIGURE 2.2 – Exemples de graphiques de comparaison des aliments à destination du chat souffrant d'obésité et de surpoids.

de calcul de rations étaient disponibles, le paysage a considérablement changé en 2021 avec l'émergence de trois plateformes couramment utilisées en France : Fetchy, Crokit et Cuisine à Crocs. Ces dernières sont toutes en ligne et requièrent un abonnement payant. Bien qu'il n'existe pas de véritable concurrence avec une solution gratuite, la nécessité de moderniser VetNutri en améliorant sa maintenabilité et en rectifiant ses erreurs initiales s'est imposée.

En mars 2021, un plan sur deux ans a été élaboré, identifiant les étés 2021 et 2022 comme les principales périodes de développement, étant donné que le logiciel constitue une activité secondaire en regard des obligations statutaires. L'été 2021 a été consacré à la phase de préproduction, une étape cruellement absente de la première édition. Cette période a permis l'élaboration d'un cahier des charges exhaustif, conservant les fonctionnalités appréciées de VetNutri 1 (comme la centralisation des informations essentielles sur un seul écran), identifiant les aspects nécessitant des améliorations (telles que la compatibilité avec diverses tailles d'écran, le design et la facilité d'installation) et spécifiant les nouvelles fonctionnalités à implémenter (voir tableau 2.1). Il est devenu évident que, pour surmonter certaines des difficultés rencontrées, un changement de paradigme serait nécessaire, impliquant le recours à des technologies plus modernes. Par conséquent, la modularité s'est ajoutée aux exigences du projet.

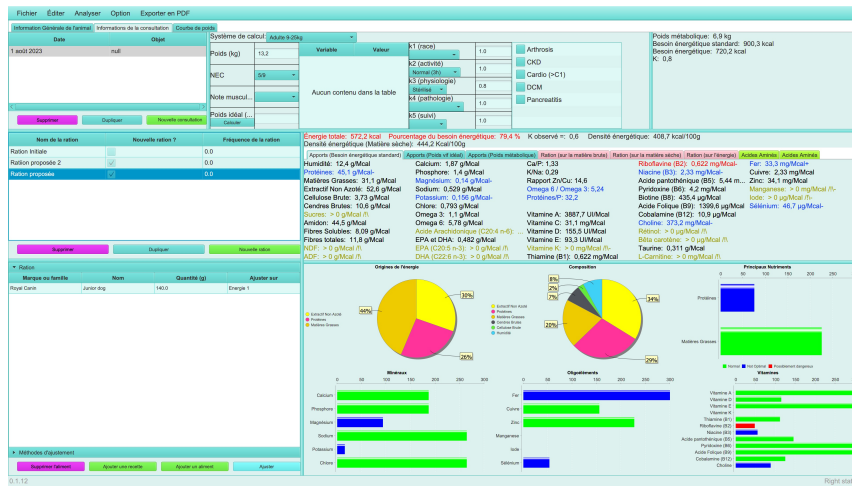
Afin de contourner les éventuelles contraintes liées aux droits d'auteur, qui pourraient menacer l'intégrité du projet, la participation des étudiants ou de toute autre personne à

l'élaboration de VetNutri 1 était restreinte. Ces individus ne pouvaient contribuer que de manière très partielle, à moins de créer leur propre version du logiciel, celui-ci étant open-source. Cette limitation freinait le développement de nouvelles fonctionnalités, telles que l'intégration de nouvelles espèces. Pour remédier à ce problème, VetNutri 2 ne contiendra plus les calculs et besoins nutritionnels spécifiques à chaque espèce en dur dans le code. Ces éléments seront plutôt stockés dans des bases de données modifiables et partageables sous forme de modules. Ainsi, un chercheur externe pourrait créer un module pour analyser et ajuster des rations destinées, par exemple, aux chevaux ou aux suricates, et partager ce module avec d'autres utilisateurs selon ses propres conditions et en étant crédité pour ce travail. Cette modification permettra également d'ajouter des besoins spécifiques liés à certaines affections. Cependant, cette nouvelle fonctionnalité augmente la complexité du logiciel, qui doit désormais interpréter les calculs provenant de modules tiers, tout en gérant leur importation et leur exportation. De plus, cette refonte a bénéficié de quelques ajouts mineurs en termes de temps de travail mais majeurs en ce qui concerne la diffusion, tels que l'intégration de la langue anglaise et, bien sûr, l'inclusion d'un module permettant l'analyse graphique des aliments directement au sein du logiciel (figure 2.3).

À garder	À corriger	À ajouter
Une ration analysé sur 1 écran	Installation compliqué (sur Mac)	Modularité
Pas de limite dans l'analyse	Compatibilité écrans (petit et grand)	Multilangue
Hors ligne	Design	Analyse graphique des aliments
	Code facile à maintenir	Retrocompatibilité avec VetNutri 1
		Prise en charge des besoins spéciaux lors d'affections

TABLE 2.1 – Principaux éléments du cahier des charges de VetNutri 2 en comparaison au 1

La première version alpha de VetNutri 2 est devenue opérationnelle en septembre 2022, suivie par la version bêta en janvier 2023. Pour faciliter son déploiement, une vidéo tutorielle a été réalisée (<https://www.youtube.com/watch?v=YRoQknNosx4>). Les premiers retours des étudiants sont très positifs. Quant à l'accueil professionnel, VetNutri semble se positionner comme un outil expert, principalement utilisé par les vétérinaires dont une part de leur activité est dédiée à la nutrition. Ces derniers saluent sa polyvalence dans la formulation de rations diverses comme avantage principal. Toutefois, sa complexité d'utilisation est souvent pointée du doigt comme inconvénient majeur. Le logiciel est actuellement intégré dans le curriculum clinique de l'École Nationale Vétérinaire de Maison-Alfort et sera prochainement utilisé à l'École Vétérinaire de Unilasalle (Rouen).



(a) Écran principal de VetNutri 2



(b) Analyse graphique des aliments dans VetNutri 2

FIGURE 2.3 – Capture d'écran de VetNutri 2

Cette nouvelle mouture valorise les compétences des vétérinaires en leur fournissant les outils nécessaires pour une analyse indépendante des aliments. Bien qu'ayant déjà prouvé son efficacité dans un contexte éducatif, le potentiel de VetNutri 2 est actuellement sous-exploité. Afin de le rendre plus visible sur la scène internationale et d'en encourager l'utilisation, il serait pertinent de le présenter dans des publications de recherche et des expertises, où il pourrait servir d'outil précieux.

2.4 Bibliographie

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- [2] National Research COUNCIL. *Nutrient Requirements of Dogs and Cats*. 2006. ISBN : 978-0-309-08628-8. DOI : 10.17226/10668. (Visité le 18/02/2019) (cf. pages 40, 42).

- [3] Veronika HAJEK, Yury ZABLOTSKI et Petra KÖLLE. “Computer-Aided Ration Calculation (Diet Check Munich©) versus Blood Profile in Raw Fed Privately Owned Dogs”. In : *Journal of Animal Physiology and Animal Nutrition* 106.2 (2022), pages 345-354. ISSN : 1439-0396. DOI : 10.1111/jpn.13601. (Visité le 31/07/2023) (cf. page 41).
- [4] ANSES. *Table de Composition Nutritionnelle Des Aliments Ciguil 2017*. Base de Données. Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail, 2017 (cf. page 42).
- [5] *USDA National Nutrient Database for Standard Reference Legacy Release, April 2018*. Rapport technique. United States Department of Agriculture Agricultural Research Service, 2018 (cf. page 42).

Chapitre 3

Expertise et recherche en nutrition

Outre la création d'outils dédiés à l'enseignement et à la pratique de la nutrition, la société et la profession vétérinaire attendent également une expertise et une recherche indépendantes. Le défi réside dans le fait que VetAgro Sup ne dispose pas d'une équipe de recherche spécialisée dans la nutrition des carnivores domestiques. Par conséquent, même s'il semble difficile de mener une recherche thématique intense dans ce domaine, il est néanmoins crucial de garantir un minimum d'ouverture vers la recherche et la méthodologie scientifique sur cette thématique. En effet, bien que ces travaux puissent sembler minimes en termes d'importance scientifique, ils jouent un rôle précieux à la fois dans l'enseignement dispensé aux étudiants et dans le rayonnement de l'établissement.

3.1 Ouvrir à la recherche en nutrition

3.1.1 Thèses d'exercice vétérinaire

La fondation de cette ouverture à la recherche et à l'expertise en nutrition repose sur l'encadrement d'étudiants en thèse vétérinaire et lors des stages de fin d'étude en ingénierie. Depuis ma prise de fonction, vingt-cinq thèses vétérinaires ont été sous ma supervision, dont cinq le sont toujours (tableau 9.3). Il a été ardu au début de structurer cette activité d'encadrement en sélectionnant des axes spécifiques pour les sujets de thèse. Ainsi, durant les deux premières années, un grand nombre de sujets bibliographiques ont été explorés. Toutefois, pour chaque thèse, en complément de la revue bibliographique, il était requis de réaliser une synthèse sous forme de guide pratique destiné à la profession vétérinaire. L'impact sur celle-ci est toutefois souvent mineur. L'une des thèses bibliographiques qui répond le mieux aux attentes professionnelles est certainement celle réalisée par Mme Hauwiller en 2021. Ce travail vise à effectuer une analyse critique de quatre ouvrages destinés au grand public, portant sur la nutrition et souvent en contradiction avec les conseils des vétérinaires lors des consultations. Dans son manuscrit, chaque notion évoquée dans ces livres est scrutée à la lumière de la littérature scientifique, offrant ainsi des

éléments de réponse fiables à la profession.

Nom	Année	Titre
Agathe Champetier	2018	Nutrition Et Cancer Chez Les Carnivores Domestiques : Création D'Un Guide Sur La Prise En Charge Nutritionnelle
Anne-Cécile Cael	2018	Réalisation D'Un Guide De Prise En Charge Du Surpoids Et De L'Obésité Chez Le Chien Et Le Chat À Destination Du Vétérinaire Et Du Propriétaire
Camille Traroni	2019	Evolutions Récentes De La Prise En Charge Du Chat Diabétique
Alyzée Galy	2019	Rédaction D'Un Guide À L'Attention Du Propriétaire Pour La Réalisation Pratique D'Une Ration MénagÈre Pour Un Chien
Charlotte Garot	2019	Nouvelles Tendances Nutritionnelles Chez Le Chat : Élaboration D'Un Guide Sur Les Différents Types De Rations
Alma Taleux	2019	Influence De La Ration Alimentaire À L'Entraînement Et En Course Sur Le Score Corporel, Les Performances Sportives Et La Prévalence D'Affections Chez Des Chiens De Traîneau : Exemple Lors De La Grande Odyssée
Marine Riffart	2020	Comparaison Des SystÈmes De Rationnement Français (INRA) Et Américain (NRC) Chez Le Cheval Et Le Poney
Ernelle Thiery	2020	Comparaison Des Aliments Industriels Physiologiques Complètes Pour Chiens Et Chats En Fonction Des Informations Fournies Par Leur Étiquetage, De Leur Prix Et De Leur Positionnement Mercatique
Corentin Treich	2020	Positionnement De La Structure Vétérinaire Libérale Vis-À-Vis De La Vente D'Aliments Pour Animaux De Compagnie
Tristan Michel	2021	Etude De L'Impact Du Type De Ration Sur Les Performances De Reproduction En Élevage Canin. Exemple Du Berger Australien
Gamou Gueye	2021	Démarche D'Utilisation Des Compléments Alimentaires Chez Le Cheval : Rédaction D'Un Guide Pratique
Léa Chevalier	2021	Perception De La Qualité Et Confiance Des Vétérinaires Praticiens Dans Les Aliments Industriels Pour Chien Et Chat : Une Étude Qualitative
Emilie Hauwiller	2021	Lecture Critique D'ouvrages De Vulgarisation Sur L'alimentation Commerciale Non Thérapeutique Des Chiens Et Des Chats De Compagnie
Simon Goddet	2022	Elaboration De Documents Supports Accompagnant La Prise En Charge Et Le Suivi Des Animaux De Compagnie En Nutrition
Solenne Dutreuil	2022	Mise En Place D'Un Système Permettant La Prescription D'Aliment Basée Sur Les Preuves En Cas De Diabète Sucré, D'Insuffisance Rénale Ou D'Arthrose Chez Le Chien
Quentin Mainfroy	2022	L'alimentation Sans Céréale Et Ses Conséquences Chez Le Chien Et Le Chat
Caroline Chambon	2023	Étude De L'Espérance De Vie Des Chiens Et Des Chats En France Entre 2003 Et 2020, Et Influence Des Conditions Socio-Économiques Des Propriétaires
Lucile Mange	2023	Étude De L'Évolution De La Médicalisation Des Chien Et Des Chats En France Entre 1997 Et 2020
Clelia Marra	2023	Aliments industriels physiologiques pour chiens et chats : analyse du crédit accordé aux informations fournies par le fabricant et à l'équilibre global de l'aliment, grâce à l'élaboration d'une grille d'évaluation de la créance
Marie Bouvier	(2024)	État Des Lieux Et Évolution De L'Alimentation Des Chiens Et Des Chats De 1997 À 2020
Laura Gioanni*	(2025)	État Des Lieux De La Vulgarisation Des Connaissances En Nutrition À Disposition Des Propriétaires De Carnivores Domestiques, Des Demandes Et Besoins De Ces Propriétaires Face À L'offre Grandissante Des Marques.
Maiwen Moreau	(2025)	Compréhension Des Facteurs De Risques Professionnels Et Personnels Motivant La Décision D'interrompre La Pratique Clinique Vétérinaire Chez Les Diplômés De Moins De 5 Ans. Étude Qualitative Au Sein Des Territoires Ruraux De La Région Auvergne Rhône-Alpes.
Vulcain Grosdenier	(2025)	Création d'un système d'analyse de ration à destination des primates du nouveau monde
Thomas Fernandez	(2025)	Création d'un système d'analyse de ration à destination des primates de l'ancien monde

TABLE 3.1 – Thèses vétérinaires encadrés ou en cours d'encadrement. Les noms d'étudiant en gras indiquent les thèses avec production originale et non uniquement bibliographiques. Les années entre parenthèse indique une thèse en cours (année prévue de fin). * Étudiante de l'ENVA

À partir de la fin de l'année 2019, une priorité a été accordée aux sujets de nature plus exploratoire. Ces derniers peuvent être catégorisés en trois axes principaux : les études de cas cliniques, la contribution au développement de systèmes d'analyse de rations, et les études à orientation sociale.

3.1.1.1 Les études de cas

Le but de ces thèses est d'élargir le corpus de connaissances par l'étude de situations dans lesquelles divers types d'aliments sont administrés. Ces travaux sont contextuellement spécifiques et dépendent des opportunités qui se présentent. Par exemple, la recherche menée par Mme Taleux en 2019 a été réalisée dans le cadre de sa participation à la Grande Odyssée, une course de chiens de traîneaux.

Un autre travail d'intérêt est celui de M. Michel (2021). Cette étude propose l'hypothèse selon laquelle le type d'alimentation (BARF^a) ou industrielle peut influencer les paramètres zootechniques d'élevages canins qui, *a priori*, ne présentent pas de pathologies spécifiques. L'alimentation BARF gagne en popularité parmi les éleveurs qui lui attribuent souvent de meilleurs résultats que les rations industrielles. Toutefois, en clinique, on observe de plus en plus fréquemment des problèmes de reproduction associés à ce régime, bien que cela ne soit pas corroboré par la littérature scientifique. L'objectif de cette étude est de vérifier, ou de réfuter, cette perception.

Pour minimiser les variations liées à la race, le Berger Australien a été choisi, en raison de l'intérêt personnel de l'étudiant pour cette race. Cinq élevages ont été recrutés pour chaque type d'alimentation. Malgré la variabilité des rations entre les élevages de chacun des groupes, seules les rations BARF présentaient des carences en vitamines et minéraux, selon une analyse effectuée avec VetNutri. Bien qu'aucune répercussion clinique n'ait été rapportée, et contrairement aux croyances des éleveurs du groupe BARF, la fécondité des élevages utilisant le BARF était significativement inférieure (médiane de 5,4 contre 7,5 chiots par chienne mise à la reproduction), ainsi que le gain moyen quotidien du poids (GMQ) durant le deuxième mois de vie (GMQ médian à 67g/jour contre 103g/jour). Bien que cette étude présente certaines limitations (taille de l'échantillon, hétérogénéité des groupes, méthodologie...), elle constitue la première recherche sur l'impact potentiel de l'alimentation BARF sur les paramètres zootechniques. Outre la portée scientifique de l'étude, la nature de son encadrement en fait un double succès. M. Michel, ayant eu un parcours académique difficile et des défis d'engagement, a produit un excellent travail qui fera l'objet d'une future publication dans une revue internationale.

a. Alimentation crue, généralement préparée à domicile à base de viande, d'abats, d'os et de légumes, sans compléments alimentaires

3.1.1.2 Développement d'un système d'analyse de ration

Le second axe de recherche est intrinsèquement lié au développement de VetNutri, surtout depuis l'introduction de la capacité à créer des modules pour enrichir ses fonctionnalités. Dans cette section, on distingue des recherches bibliographiques et des études ciblant la qualité des aliments commerciaux.

Les études bibliographiques de la thèse de Mme Riffart (2020) et de MM. Grosdenier et Fernandez (2025) visent à établir un système de besoins nutritionnels, pour le cheval dans le premier cas, et pour les primates dans le second. Bien que l'objectif ultime de créer un système d'analyse nutritionnelle soit similaire, les contextes diffèrent. Pour Mme Riffart, deux systèmes majeurs existent déjà ; son travail consiste donc à les comparer pour synthétiser un système dans VetNutri conforme aux connaissances actuelles.

Concernant les travaux de MM. Grosdenier et Fernandez, il n'existe actuellement pas de système d'analyse nutritionnelle pour les espèces concernées. Leur tâche initiale consiste à compiler les différentes publications décrivant les rations des espèces en question, pour ensuite, avec de l'aide, analyser ces données et proposer des recommandations nutritionnelles spécifiques. Ces thèses font écho à la thèse universitaire développée dans la section 3.1.2. Ces travaux offrent aux étudiants une occasion d'approfondir leurs connaissances sur l'alimentation d'une ou plusieurs espèces, tout en mesurant les incertitudes existantes. À leur sortie, ils contribueront en tant que co-auteurs aux modules VetNutri.

En ce qui concerne les études sur la qualité des aliments industriels, elles cherchent à développer des outils novateurs pour estimer la qualité des aliments pour lesquels peu d'informations sont publiquement disponibles. Souvent, les vétérinaires se fient à la réputation d'une marque ; l'objectif ici est de quantifier cette confiance en utilisant différentes méthodologies. Mme Thiery a analysé les composants analytiques et les étiquettes de plus de 2000 aliments physiologiques pour chiens et chats, aboutissant à un outil pour estimer l'origine du phosphore. Son travail a été présenté lors du congrès de l'ESVCN de 2020^b, poster dans la section 3.3.2. Une autre thèse, celle de Mme Marra (2023), examine les pratiques des fabricants, notamment en ce qui concerne leur gestion de la qualité et leur recherche et développement, en se basant sur un questionnaire publié en 2010 par l'American Animal Hospital Association¹ mais jamais mis en œuvre. Enfin, la thèse de Mme Dutreuil (2022) évalue les preuves concernant différents aliments à objectif nutritionnel spécifique sur le marché, tout en proposant un système de notation. Ces travaux permettent aux étudiants de mettre en pratique une analyse critique des aliments industriels et répondent aux attentes des vétérinaires et de la société, thèmes explorés dans les thèses axées sur les études sociales.

b. European Society of Veterinary and Comparative Nutrition

3.1.1.3 Etude de la société

Afin de saisir en profondeur les complexités associées à la nutrition animale dans le contexte sociétal contemporain, des étudiants ont été intentionnellement guidé vers ces thématiques. Cette démarche pédagogique vise à leur fournir un cadre analytique robuste pour évaluer les besoins et les attentes de leurs futurs clients et confrères, tout en contribuant activement à l'avancement scientifique dans le domaine de la nutrition animale. Parmi les contributions notables dans ce domaine, il convient de mentionner les travaux de M. Treich (2020) et de Mme Chevalier (2023). Utilisant des questionnaires comme méthodologie principale, ces étudiants ont respectivement examiné les stratégies commerciales associées à la vente d'aliments pour animaux au sein des structures vétérinaires et l'évaluation qualitative de ces aliments par la communauté vétérinaire. Les conclusions dérivées de l'étude menée par Mme Chevalier ont par ailleurs inspiré la formulation de la thèse de Mme Marra, précédemment discutée.

Le lien que les propriétaires d'animaux entretiennent avec la nutrition de leurs compagnons a fait l'objet d'une investigation plus récente, spécifiquement dans la thèse de Mme Gioanni (affiliée à l'ENVA). Son travail, basé sur des entretiens qualitatifs, aspire à dresser un inventaire et une analyse des sources d'information sur la nutrition utilisée par les propriétaires et à identifier les lacunes de connaissances qui subsistent dans ce domaine.

En complément, trois études quantitatives en cours, menées par Mmes Chambon, Mange, et Bouvier (prévues pour 2023 et 2024), adoptent une approche multidimensionnelle sur la relation humain-animal en mettant l'accent, à divers degrés, sur la nutrition. Ces recherches exploitent les données empiriques recueillies par les études Kantar/Facco entre 1997 et 2020. Ces enquêtes, menées biannuellement auprès d'un échantillon représentatif de la population française, cherchent à cartographier les divers aspects de la possession d'animaux, allant de l'âge et la race à la santé, ainsi que les habitudes de consommation y afférentes. Grâce à un financement collaboratif de 20 000 €, en partenariat avec plusieurs fabricants d'aliments pour animaux et la FACCO, l'ensemble des données depuis 1997 a été numérisé. Cela représente un corpus de données impressionnant, couvrant 184 000 foyers et 126 000 chiens et chats, analysé à travers une centaine de variables et réparti sur six bases de données distinctes. Ces données ont déjà permis de générer des connaissances nouvelles, telles que l'évolution de l'espérance de vie des chats (section 3.3.3), l'émergence du phénomène des "pet parents" (vue précédemment à la figure 1.1), et les dynamiques changeantes concernant les points de vente d'aliments en fonction de l'âge des animaux. Étant donné l'ampleur de la base de données, il est anticipé que plusieurs articles scientifiques émergeront de ces thèses pour exploiter pleinement cette richesse d'informations.

Pour conclure, mon engagement dans des études centrées sur la profession vétérinaire m'a conduit à co-diriger, en collaboration avec le Dr Pierre Bruyère, les travaux de Mme Moreau. Ces travaux portent spécifiquement sur les motivations et les défis rencontrés par

les jeunes vétérinaires qui s'installent en milieu rural, mais qui se retirent par la suite de la pratique. La compréhension des facteurs incitant ces professionnels à quitter la pratique est cruciale, non seulement pour le bien-être de la profession vétérinaire, mais également pour la santé publique, étant donné la nécessité de maintenir un réseau vétérinaire efficace à l'échelle nationale.

3.1.2 Thèse universitaire en nutrition animale

Il arrive parfois que les encadrements de thèse ou de mémoire de fin d'études mènent à des carrières en recherche. C'est le cas pour Mme Flore Viillard, étudiante en cursus d'ingénieur à VetAgro Sup, qui souhaitait travailler en nutrition animale au sein de parcs zoologiques. J'ai eu l'honneur de servir comme son référent pédagogique lors de son stage de fin d'études au Parc Zoologique de Mulhouse en 2021. Ce stage visait à étudier la transition alimentaire chez cinq espèces de primates et à évaluer son impact sur leur comportement et leur bien-être.

Suite à cette expérience, Mme Viillard a manifesté son intérêt pour poursuivre une thèse en ce domaine. Mes conseils initiaux ont porté sur la recherche d'un directeur de thèse expérimenté en France ou en Europe, tout en l'aidant à valoriser les travaux menés à Mulhouse. Cette valorisation a abouti à la présentation d'un poster au congrès de la Société Française de Primatologie (référéncé en section 3.3.5.1), qui lui a valu le Prix Poster de Master de la société. Un article issu de cette étude est actuellement en raccepté pour le *Journal of Zoo and Aquarium Research* (article en section 3.3.5).

Une seconde étude portant sur la gestion alimentaire des ruminants dans les parcs zoologiques a été présentée au congrès de l'ESVCN en 2022 (poster en section 3.3.4). Après une année de recherche infructueuse d'un directeur de thèse, j'ai proposé d'encadrer Mme Viillard dans le cadre d'une thèse CIFRE avec un parc zoologique. Un financement a été obtenu auprès du Parc Animalier d'Auvergne pour étudier les impacts physiologiques, éthologiques et environnementaux des transitions alimentaires sur la faune captive.

La question des attentes sociétales relatives à cette recherche s'est également posée. Les travaux menés à Mulhouse ont mis en évidence l'importance de l'alimentation sur le bien-être des animaux en captivité, soulignant par ailleurs le retard des parcs zoologiques français en matière de nutrition. Les bénéfices sociétaux des parcs zoologiques sont de plus en plus étudiés². Ils offrent une connexion à la nature pour une population majoritairement citadine, avec des effets positifs sur le bien-être et la santé humaine^{3,4}. De plus, ils contribuent à sensibiliser le public aux enjeux environnementaux⁵.

Ces recherches fournissent également une opportunité personnelle de tester mes compétences en encadrement. Elles me permettent de diversifier mon expertise en m'éloignant des thématiques habituelles de mon unité de recherche, tout en me rapprochant de mes sujets d'enseignement.

3.2 Expertises

Les activités d’expertise illustrent la contribution renouvelée de VetAgro Sup au débat public en matière de nutrition animale. À titre d’exemple, j’ai été auditionné en 2020 par Monsieur le député Loïc Dombreval dans le cadre de sa mission sur le bien-être animal. Cette audition a permis d’aborder les enjeux liés à l’alimentation des animaux de compagnie, notamment les lacunes en matière d’information du consommateur, et de proposer des pistes d’amélioration.

À compter de janvier 2022, l’ANSES m’a sollicité en tant qu’expert extérieur. J’ai d’abord agi en tant que rapporteur de l’expertise collégiale concernant une demande de modification du règlement européen 354/2020 afin d’y inclure un nouvel objectif nutritionnel particulier^c (l’avis émis à la suite de ce rapport est disponible à cette adresse : <https://www.anses.fr/fr/system/files/ALAN2021SA0194.pdf>). Cette expérience a été l’occasion de collaborer en collégialité avec d’autres spécialistes (trois, dont un autre nutritionniste) pour parvenir à un avis consensuel avec le CES ALAN^d. En septembre 2022, l’ANSES m’a nommé au sein de ce même CES ALAN. Les travaux du comité offrent l’opportunité d’échanger avec d’autres experts en alimentation animale (principalement des animaux de rente), de travailler sur d’autres aspects de la nutrition animale (évaluation des additifs, etc.) et de contribuer à la vie d’une institution nationale. De plus, l’indépendance, évoquée dans le chapitre précédent, trouve ici sa pleine réalisation, les liens d’intérêt étant vérifiés pour chaque dossier traité. Le maintien de cette indépendance est primordial dans un contexte où les experts en alimentation des animaux de compagnie sont rares.

Enfin, au début de l’année 2023, j’ai été invité par le think tank “Club Démeter”, spécialisé dans le domaine agricole et agroalimentaire. Mon intervention et la table ronde subséquente ont abordé l’importance et les enjeux de l’alimentation des carnivores domestiques au sein de la société française et de son industrie agroalimentaire.

En somme, ces diverses activités d’expertise et de collaboration interdisciplinaire incarnent non seulement l’évolution de la stratégie de VetAgro Sup en matière de nutrition animale, mais aussi la reconnaissance de notre institution comme un acteur clé dans ce domaine au niveau national. Elles témoignent de l’importance croissante de l’alimentation des animaux de compagnie dans les débats publics et les politiques de bien-être animal. De plus, elles illustrent l’indépendance qui est au cœur de ma démarche, des valeurs qui sont d’autant plus cruciales dans un contexte où les experts en alimentation des animaux de compagnie sont rares.

c. Indication d’utilisation d’un aliment dans un contexte particulier, souvent pathologique.

d. Comité d’Experts Spécialisés en Alimentation Animale

3.3 Publication

3.3.1 Poster :

You said “gastro-intestinal” food? Approach of petfood diversity during a student-conducted consultation.

Ce poster présente l’application synergique de VetNutri et des diagrammes alimentaires, mettant en lumière leur pertinence pédagogique ainsi que leur utilité dans la sélection d’aliments commerciaux pour animaux. Il démontre notamment les biais induits par les dénominations commerciales des aliments. À titre d’exemple, un animal atteint de lymphangiectasie exsudative avec perte de protéines s’est vu prescrire un aliment étiqueté comme “gastro-intestinal”. Or, cet aliment s’avérait être le plus riche en matières grasses disponibles sur le marché, une caractéristique contre-indiquée dans ce cas clinique spécifique. L’emploi des diagrammes alimentaires encourage les vétérinaires à examiner attentivement les nutriments clés avant de prescrire un aliment, augmentant ainsi la probabilité d’une prescription adéquate.

Ce travail a été présenté lors du congrès de l’ESVCN^e en 2019 à Turin, en Italie.

e. European Society of Veterinary and Comparative Nutrition

You said “gastro-intestinal” food ? Approach of petfood diversity during a student-conducted consultation.

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Introduction:

The choice of a petfood adapted to a clinical case is often difficult to perform by veterinary students. Indeed, between marketing, labelling, clinical data and the wide choice among available products, it is easy to be lost and to base their prescriptions on cognitive bias instead of scientific data. This presentation reports a case of protein-losing enteropathy with intestinal lymphangiectasia and its dietary management. Beyond the clinical aspect the authors want to discuss educational aspects.

Case History:

9 years old **Neutered male**

Polish lowland sheepdog

June 2018: Consultation at its usual veterinary clinic

- Diarrhoea with a loss of 4 kg since September 2017
- Diagnosis of protein-losing enteropathy with intestinal lymphangiectasia
- Prescription :
 - Prednisolone (1mg/kg BW)
 - Specific-purpose kibbles for gastrointestinal disorders (n°4 in figure 1)

December 2018: Consultation at the Nutrition Department of VetAgro Sup

- Weights 20.6kg (BCS 3/9)
- Diarrhoea diminished only slightly since June (Stool frequency: 4-5/day, consistency score: 4/5)
- A 2mg/kg BW dose of prednisolone has been tested but not tolerated by the dog.

Consultation: The consultation was performed by a student in the 4th year of the curriculum. As a part of case history was available before the consultation, a briefing based on literature was done before the consultation [1]. The aim of this briefing was to give students the necessary knowledge to perform the clinical reflexion, and thus to allow them to conduct the consultation as far as possible. One student took the history and led the physical examination under supervision. To help the student in the case assessment, he had a **nutrition software** and **diagrams of available food characteristics** (tools are presented in this congress [2]). Figure 1 presents some of these diagrams, the given food being number 4. According to this diagram and the briefing presenting the interest of **fat reduction** in management of protein-losing enteropathy with intestinal lymphangiectasia, the student easily assessed that this food was not the most appropriate for this case. With help of other diagrams, **he proposed to change food** for the number 9 (Hill’s i/d low fat). Then, with the software (figure 2), he estimated the MER at 1257 Kcal ME/d ($MER=130 \times BW^{0.75}/d$) and made sure that needs were covered- (main nutrient intake calculated in software are resumed in table 1. **Three days after** the food change, **diarrhoea stopped**. One month later, prednisolone dose was decreased to 0.5 mg/kg BW and the calorie intake increased to 1450 Kcal ME to recover its optimal weight.

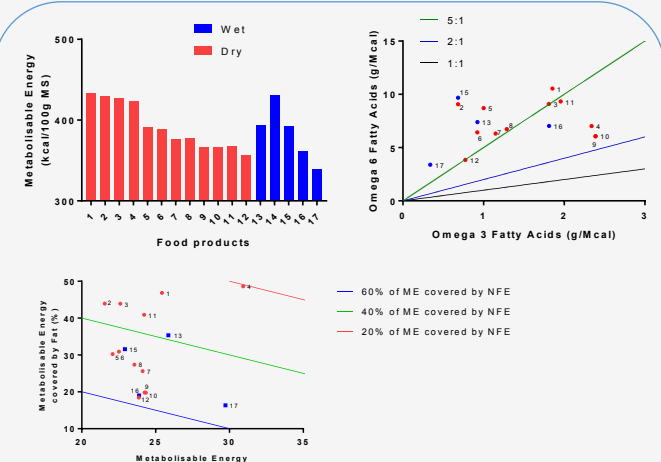


Figure 1: samples of the diagrams used to assess petfoods. Here specific-purpose petfoods for gastrointestinal disorders

Diet (petfood diagram number)	Initial (n°4)	Prescribed (n°9)
Calorie intake (kcal of ME)	1 357	1 272
Protein intake (g/kg BW ^{0.75})	12.3	8.5
Fat intake (g/kg BW ^{0.75})	7.9	2.8

Table 1: Calorie, protein and fat intakes for initial and prescribed diet, extracted from VetNutri Software.

Conclusion: More than clinical success, we report an approach that may popularize clinical nutrition reflexion among veterinarians by making it more achievable with the help of two complementary tools:

Diagrams

- Rapid overview of products
- No marketing bias

Software

- Rapid and Precise analysis of needs and intakes

References: [1] Okanishi H et al. The Clinical Efficacy of Dietary Fat Restriction in Treatment of Dogs with Intestinal Lymphangiectasia. J Vet Intern Med. 2014. [2]. Alves de Oliveira L et al. Reflexion and proposition of a tool for teaching small animal nutrition in veterinary cursus.

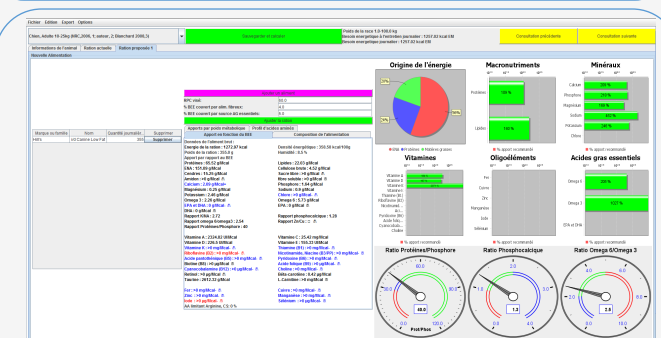


Figure 2: VetNutri software interface

3.3.2 Poster :

Labelled analytical constituent of commercial dog foods according to the main point of sale. Are there any clues as to the origin of phosphorus

Ce poster résume une portion des travaux de la thèse d'exercice d'Ernelle Thiery. Basé sur une base de données comprenant les informations relatives à 409 aliments différents, ce travail propose une méthode pour estimer la proportion de phosphore non organique dans les croquettes pour animaux. L'objectif est d'ajouter à la panoplie d'estimateurs de qualité existants un indicateur facilement calculable et pertinent pour la santé animale. En effet, le phosphore inorganique est souvent utilisé comme facteur d'appétence ou conservateur. Toutefois, sa haute digestibilité peut entraîner des pics de phosphatémie, associés à une toxicité rénale potentielle. Bien que cet indicateur soit loin d'être infaillible, il offre, en complément d'une analyse critique globale des aliments, une méthode objective pour le choix d'une ration physiologiquement appropriée.

Ce travail a été présenté lors du congrès de l'ESVCN^f en 2021, qui s'est déroulé en ligne.

f. European Society of Veterinary and Comparative Nutrition

Labelled analytical constituents of commercial dog foods according to the main point of sale. Are there any clues as to the origin of phosphorus?

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Introduction:

In our subconscious, a gradation has taken place between pet food depending on where it is sold. However, are these foods objectively different and could these differences have health consequences? Here we study the analytical constituents present on dog food labels in order to provide an objective view of the market. Finally, we propose an indicator to estimate the presence of soluble salts of phosphorus.

Data:

Foodstuffs studied

- Dry adult maintenance dog food

Sources of data

- Preferably the manufacturer, if not online or physical store

Price data

- The lowest price found for the largest commercial package

Classification

- According to their main point of sale and if they are grain-free or not : veterinary, mass retailers, online/pet store and grain-free.

Calculations:

Main Working Assumption

- Calcium comes only from bone materials

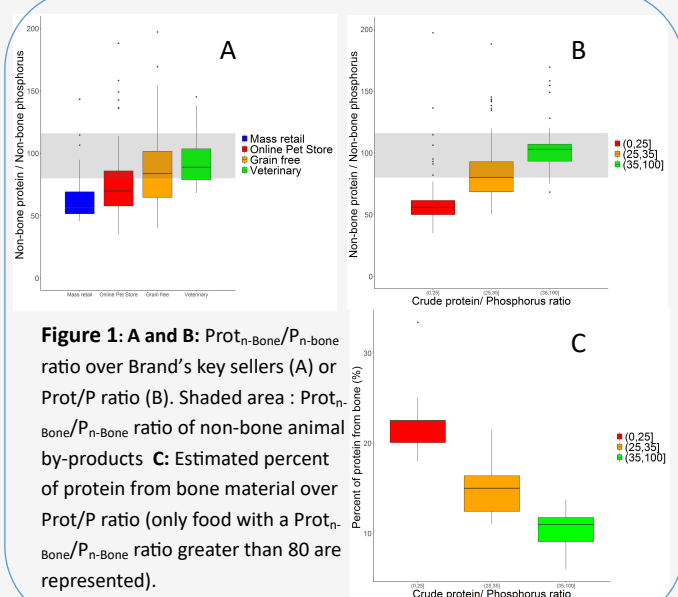
Energy	Soluble phosphorus	Proteins from bone materials
<ul style="list-style-type: none"> • NRC 2006¹ 	<ul style="list-style-type: none"> • Lineva et al.² • $P_{\text{bone}} = \text{Ca}/2$ • $P_{\text{n-bone}} = P - P_{\text{bone}}$ 	<ul style="list-style-type: none"> • Bone³: Prot/P ratio=6 • $\text{Prot}_{\text{bone}} = 6 * P_{\text{bone}}$ • $\text{Prot}_{\text{n-bone}} = \text{Prot} - \text{Prot}_{\text{bone}}$

Results/Discussion: A total of 409 dry commercial dog foods (320 with labelled phosphorus) were evaluated. The main results are presented in table 1. In this report, **grain free** products are **sold exclusively in online or pet shops**. All foods have **phosphorus levels higher than the minimum recommendation of FEDIAF (1.16g/Mcal)**⁴. The highest levels of calculated soluble phosphorus are reported in mass retail and online pet store product. In addition, the level of **non-bone phosphorus** in these products is **not correlated** with their level of **none-bone proteins** ($\text{Prot}_{\text{n-bone}}/P_{\text{n-bone}}$), which can be a source of organic phosphorus (fig 1A). In veterinary and grain free products, the $\text{Prot}_{\text{n-bone}}/P_{\text{n-bone}}$ ratio is closest to the non-bone animal by-products (80-116⁵). Thus, it can be assumed that the **phosphorus** content of the added **inorganic salt** would be higher in **entry-level products** where the ratio is well below the limits of 80. This highly soluble phosphorus could be harmful to the kidney health². To estimate the presence of this inorganic phosphorus salt the crude protein to phosphorus (Prot/P) ratio can be used (fig 1B). When this ratio is less than **25**, it appears that a **significant amount of phosphorus** has been added to the diet, above 35 it is less likely and section 25-35 is uncertain. To explore the **discrepancies** between the **two ratios**, we studied the percentage of **protein of bone origin** as a function of the Prot/P ratio for all foods with a $\text{Prot}_{\text{n-bone}}/P_{\text{n-bone}}$ ratio greater than 80 (Fig 1C). Thus a Prot/P ratio of less than 25 in the absence of added phosphorus could be a sign of a **high proportion of bone protein in the food (>20%)**.

It should be noted that the phosphorus levels provided on the label might be incorrect or underestimated. In addition, our working assumption: "Calcium comes only from bone material", while true in some cases, is not generalizable to all commercial dog foods. Therefore, the Prot/P ratio should only be used through a global analysis of the food.

Brand's key sellers (n _{phos} /n)	Protein (g/Mcal)	NFE (g/Mcal)	Fat (g/Mcal)	P (g/Mcal)	P _{n-bone} (g/Mcal) Calculated ²	Protein _{n-bone} vs P _{n-bone} ratio	Price (€/Mcal)
Mass retail (44/69)	68 ^A [62-71]	117 ^A [103-127]	35 ^A [32-40]	2.7 ^A [2.6-3.0]	0.94 ^A [0.8-1.13]	56 ^A [51-69]	0.79 ^A [0.59-0.95]
Online/Pet store (143/168)	66 ^A [61-71]	119 ^A [107-135]	35 ^A [30-39]	2.5 ^B [2.3-2.8]	0.80 ^B [0.66-0.97]	70 ^B [85-87]	1.0 ^B [0.78-1.17]
Grain free (99/138)	79 ^B [67-95]	93 ^B [65-110]	41 ^B [38-46]	2.7 ^A [2.4-3.1]	0.84 ^{AB} [0.67-1.1]	84 ^C [64-102]	1.35 ^C [1.2-1.54]
Veterinary (34/34)	72 ^B [65-84]	107 ^C [91-122]	41 ^B [33-44]	2.1 ^C [1.8-2.3]	0.73 ^C [0.51-0.79]	91 ^D [82-125]	1.19 ^D [0.96-1.43]

Table 1: Analytical constituents from different dog foods (median, the first and the third quartile). Different letters in the same column indicate a statistically significant difference (Kuskall Wallis).



Conclusion: This study highlights the differences between products depending on where they are sold, especially with regard to phosphorus. While more research is needed to understand the implication of added inorganic phosphorus on dog kidney health, its level needs to be communicated to the consumer.

References: 1. National Research Council. Nutrient Requirements of Dogs and Cats (2006). 2. Lineva et al. J Anim Physiol An N 103, 317–323 (2018). 3. Shirley et al. Poultry Science 80, 626–632 (2001). 4. FEDIAF. Nutritional Guidelines (2019). 5. ANSES. CIQUAL food database (2016).

3.3.3 Poster :

Life expectancy of French cats between 2003 and 2022

Ce poster (seul l'abstract est repris) synthétise une portion des travaux de la thèse d'exercice de Caroline Chambon. Utilisant les données des enquêtes FACCO/KANTAR, et plus particulièrement les déclarations concernant l'âge au décès des chats, cette étude calcule l'espérance de vie annuelle en fonction des conditions socio-économiques des propriétaires. Les résultats de cette recherche révèlent une augmentation significative de l'espérance de vie des chats au cours des deux dernières décennies. Ces données contredisent les rumeurs circulant sur Internet, selon lesquelles les aliments industriels auraient contribué à une diminution de l'espérance de vie des animaux de compagnie. De plus, l'étude souligne que le lieu de résidence, mais non le type d'habitation, est corrélé avec l'espérance de vie des chats en France.

Ce travail a été présenté lors du congrès de l'ESVIM^g en 2023 à Barcelone.

g. European Society of Veterinary Internal Medicine

Life expectancy of French cats between 2003 and 2022

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Over the last twenty years, the medicalization of cats has progressed and they are increasingly considered as members of the family with the care and attention that this implies. The aim of this study was to determine the evolution of the life expectancy of French cats over the last two decades and the influence of geographical and socio-economic criteria on it.

Cats' life expectancies were calculated from a life table constructed with the results of the question: "If your pet died in the last two years, how old was he/she when he/she died?". This question was part of the surveys carried out by a polling institute on a representative sample of the French population (10 to 15 thousand respondents per survey), approximately every 2 years (2003-2022). The questionnaire also retrieved information about the pet-owners' home location, the household composition and their socio-professional categories. Details on the pets living within the household (species, breed, age, feeding method...) were also collected. Results are showed \pm the standard deviation or with their 95 % confidence interval estimated by the bootstrap method (n=1000).

Mortality rate in the first eight years of life decreased from 7.4 % \pm 1.4 in 2003 to 3.2 % \pm 1.1 in 2022. This difference has contributed to an increase in life expectancy of 2.5 years in 19 years, going from 9.6 years [9.4-9.7] to 13.1 [12.8-13.5]. On this same period a significant increase in spaying and neutering has been observed: 60 % and 72 % respectively in 2003, compared to 85 % and 86 % in 2022. The increase in life expectancy was higher in bigger cities. In Paris area it reached 14.5 years [13.5-15.5] in 2022, compared to 12.6 years [11.8-13.3] in cities with less than 2,000 inhabitants. Interestingly, cats owned by retired people (2018 to 2022 data) had a significantly longer life expectancy (14.0 years [13.3-14.7]) compared to those owned by the rest of the population (11.9 [11.2-12.8]). However, the type of dwelling (house, garden, flat...) was not a factor with a statistically significant effect on feline life expectancy in this study.

Cat life expectancy has increased significantly over the last 19 years in France. However, life expectancy depends on the size and density of the urban area and the socio-professional category of the owners.

Acknowledgement: The authors gratefully acknowledge the support of FACCO and the companies Affinity Petcare, Hill's Pet Nutrition, Nestlé Purina, Royal Canin and Pro-Nutrition for the purchase of the database.

3.3.4 Poster :**Comparative survey of ruminant rations in zoological parks in France**

Ce poster résume une partie de l'enquête menée par Flore Viillard sur la prise en compte de l'alimentation des ruminants dans les zoos français. L'étude souligne un manque significatif d'attention portée à l'alimentation, en particulier pour les espèces folivores. En effet, seulement 40% des parcs fournissent des feuillages à ces animaux. De plus, il est constaté que les analyses alimentaires sont rarement effectuées. Ces résultats mettent en exergue la nécessité d'intégrer des nutritionnistes au sein des équipes des zoos pour assurer une alimentation adéquate et un bien-être optimal des animaux.

Ce travail a été présenté lors du congrès de l'ESVCN^h en 2022 à Zurich.

h. European Society of Veterinary and Comparative Nutrition



Comparative survey of ruminant rations in zoological parks in France



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Introduction:

During qualitative interviews prior to the survey, it was noted that the formulation of ruminant rations is often based on agronomic knowledge and advice from feed and pellet suppliers. In addition, the breeding objectives and number of species are also very different in these two types of animal structures. Thus, zoos must adapt rations to the food requirements and behaviour of each species, but also ensure the financial and logistical sustainability of the food they offer. Faced with these latter imperatives and the lack of resources to guide them, French zoos may underestimate the basics of feeding ruminants, especially folivores, and overlook the qualitative analysis of their diets.

Materials & Methods:



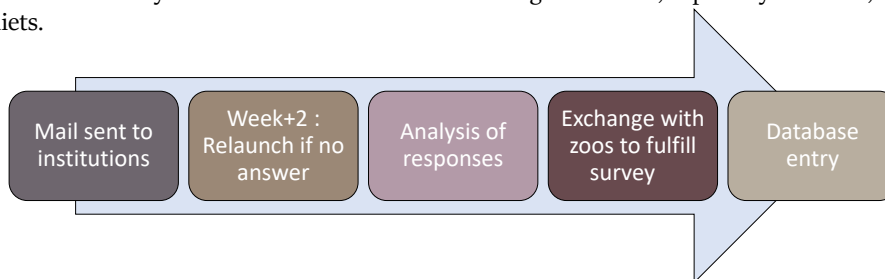
March to June 2021



97 surveys sent | 15 responses
(15,4%)



63 species | 135 herds



Statistical analysis: Kruskal-Wallis test and Dunn's post hoc test, statistical significance at p-value < 0,05

Results and discussion :

Table 1. Percentage of zoos using each of the feeds in their ruminants diets

	Browser	Grazer	Intermediate feeder
Hay (dried grass)	20%	100%	90%
Alfalfa (dried)	71%	25%	31%
Browse	40%	5%	20%
Pellets (starch or cellulose based)	95%	97%	97%
Fruits and vegetables	40%	65%	38%

Table 2. Percentage of zoos conducting laboratory analysis related to their ruminants diets.

	Yes	No
Water analysis	33%	67%
Forage analysis	20%	80%

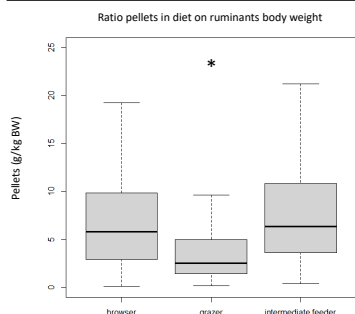


Figure 1. Ratio of the amount of pellets distributed to the live weight of the animals according to their feeding strategy in the study zoos.

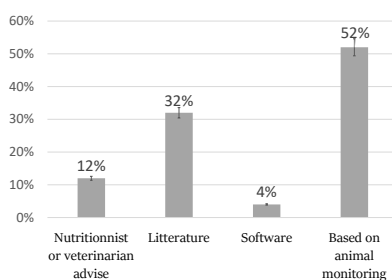


Figure 2. Method of formulating rations used in the study zoos.

- Hay is the most commonly used forage except for browsers where 71% of zoos provide alfalfa compared to 89% for European zoos. (Gussek et al, 2017) (table 1)
- 60 % of French zoos of the sample do not provide foliage to browsers (table 1) vs 4 to 20% in German zoos. (Hummel et al, 2006; Gussek et al, 2017)
- Browser, grazer and intermediate feeder do not receive the same proportion of pellets according to their body weight (figure 1). In comparison, grazers receive the least amount of pellets. This might be explained by better availability of vs browse and more accessible livestock guidelines.
- Pellets are an important part of the ruminant ration in zoos: the differences in diet between captive and wild animals, and zoo and farm animals, also impact on tooth abrasion. (Kaiser et al, 2009)
- Forage analysis (CP, NDF, ADF, energy, minerals) is not a common practice in the nutritional strategy of French zoos (Table 2) although it's recommended. (Ullrey & al, 1997, rev.2004)
- French zoos develop a large part of their rations based on field experience (61.5%) and/or word of mouth (15%), especially from their suppliers. (figure 2)
- The majority (80%) of the zoos in the study would like to receive a visit from a nutritionist, but this profession is limited in France, which may cause an imbalance compared to European publications where the profession is more developed.

Conclusion:

- It can be useful to evaluate and improve feeding management precisely and individually in French zoos and to accompany them in the progression of their method.
- French zoos seem to be moving away from the agronomic practices necessary to support their ration formulation and from the literature available for captive exotic ruminants through conferences, publications and experiences of European zoo.
- Several additional indicators have been calculated and are available on request.

Acknowledgments : The authors would like to thank French zoological parks for making data collecting possible.

Main references : Gussek et al. (2017) Journal of Zoo and Aquarium Research, 5(1), 62–70. <https://doi.org/10.19227/jzar.v5i1.152>; Hummel et al. (2006) EAZA, 29–61. Kaiser et al (2009) Mammalian Biology - Zeitschrift für Säugetierkunde 2009, 74(6):425-437; Ullrey, Duane E. (1997, revised by Jonni 2004) Hay Quality Evaluation in Nutrition Advisory Group Handbook

3.3.5 Article :

Multi-criteria study on fruit-free diets transition for Cebidae and Cercopithecidae.

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Research article

Multi-criteria study on a change to a fruit-free diet in Cebidae and Cercopithecidae

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Abstract

Nutrient intake of captive primates does not necessarily reflect that of their wild counterparts. Diets in captivity are often higher in non-structural carbohydrates and lower in fibre, resulting in health issues such as obesity, dental issues, diarrhoea and behavioural problems. The main objective of this study was to establish and monitor a change to a fruit-free diet in five species of primates (*Ateles fusciceps rufiventris*, *Cercopithecus hamlyni*, *Allochrocebus lhoesti*, *Cercopithecus roloway*, *Sapajus xanthosternos*). Nutrition and ethology was monitored, including an assessment of the nutritional composition of diets before, during and after diet change; monitoring of faeces consistency; observation of feeding choices; and the occurrence of aggressive behaviour and vocalisations by scan sampling. The initial diet included cultivated fruits and vegetables and some extras (cereals, animal and vegetable proteins) and was higher in non-structural carbohydrates, in particular sugar, than recommended in husbandry guidelines. After a diet change of four weeks during which fruits were gradually removed, a decrease of mean sugar content by more than half and an increase in fibre was achieved. Improved faeces consistency was observed for spider monkeys *A. f. rufiventris* and Hamlyn monkeys *C. hamlyni* (change in Bristol stool score: 6 to 4 and 7 to 3, respectively). An increase in time spent feeding was observed for capuchins *S. xanthosternos* and Hamlyn monkeys (1.5 to 2 times longer). These findings underline the beneficial effects of changing to fruit-free high-fibre diets in zoo-managed primates.

Introduction

As nutrition is one of the five pillars of animal welfare (Mellor et al. 2020), modern zoos aim to provide their animals with a high-quality diet in sufficient quantity. Over the past two decades, zoos have initiated diet changes, moving from feeding animals based on habit and empiricism to feeding based on objective nutritional needs and welfare criteria (Britt et al. 2015; Mellor et al. 2020; Plowman 2013). This transformation of feeding habits is particularly challenging for non-human primates in zoos. Primate diets in zoos are often composed of cultivated fruits. However, cultivated fruits generally have

higher sugar and lower fibre content than wild fruits (Milton 1999; Schwitzer et al. 2009). Negative effects of excessive carbohydrate consumption in captive non-human primates have been identified, e.g., diarrhoea (Cabana 2014), dental issues, low reproductive success, obesity (Streicher 2004) and aggressive behaviour (Britt et al. 2015; Cabana 2014; Plowman 2013).

Shifts to fruit-free diets (without cultivated fruit) in primates have been carried out since the early 2000s in several European zoos. This initiative was first introduced at Paignton Zoo, showing the benefits of removing fruits from the diet of a variety of primate species (*Colobus guereza*, *C. polykomos*, *Cercopithecus*

diana, *Papio hamadryas*, *Ateles hybridus* and *Macaca nigra*) (Plowman 2013). Food intake, nutrient composition, individual dental health, body condition, faeces quality and the overall cost of the feeding regimen were improved by the diet change (Plowman 2013). This thus served as a reference for other facilities seeking to change to a more naturalistic diet, and for the development of similar diets in other primate species. For instance, a change to fruit-free diets in lemurs at Paignton Zoo and Newquay Zoo led to a decrease in aggression and self-directed behaviour (Britt et al. 2015). Singapore Nature Reserve replaced eggs, rice, bread and fruit with sprouted legumes, whole grains, vegetables, forage and insects in most of their primates' diets (*Lemur catta*, *Pithecia pithecia*, *Pygathrix nemaeus*, *Trachypithecus auratus*, *Nasalis larvatus*) and also observed a decrease in aggressive behaviour (Sha 2014).

The present study describes the change to a fruit-free diet for five primate species at Mulhouse Zoo. Before the diet change, clinical problems such as obesity and behavioural problems such as gluttony and agonistic behaviour during meals were identified in several primate species. Based on previous studies, the dietary change was expected to correct these problems. However, to the authors' knowledge, the change to a fruit-free diet had not yet been described for some of the species involved in this study (*Sapajus xanthosternos*, *Cercopithecus hamlyni* and *Allochrocebus lhoesti*). The objective was to conduct a multi-criteria study on the effects of the change to a fruit-free diet in five species of frugivorous primates with different levels of folivory in their natural feeding behaviour. In particular, energy and nutrient intake, faeces consistency, body condition scores, time spent eating and behaviours associated with feeding were evaluated before, during and after the diet change. This inclusive study design with nutritional, behavioural and health criteria allows comprehensive understanding of the effects and limitations of these dietary changes.

Materials and methods

Subjects and housing

One group each of spider monkeys *Ateles fusciceps rufiventris*, Hamlyn monkeys *Cercopithecus hamlyni*, L'Hoest monkeys *Allochrocebus lhoesti* and Roloway monkeys *Cercopithecus roloway*, and two groups of yellow-breasted capuchins *Sapajus xanthosternos* (Capuchin 1 and 2) were included in the study. The size, structure and main health concerns of each group are summarised in Table 1.

All subjects were housed in the same building. Each group had 24 hr access to an indoor and an outdoor enclosure at all times. All enclosures were visible to the public. The study was conducted between April and June 2021.

Study design

Data were recorded during three distinct phases. During the first period (I, Initial), the primates were fed the initial diet that included cultivated fruits. During the last period (F, Final), they received the target fruit-free rations. Period C (Diet change) was the change period between the two rations and lasted for 4 weeks.

In each period, behavioural monitoring during feeding was carried out five times for each primate group and body condition scores (BCS) were estimated four times per individual. For 15 days per period, the offered and leftover food items were weighed for each group, and faeces consistency was monitored with 15 faecal samples per group.

Diet composition

Until the beginning of the study, the primate groups were fed a mixed diet of vegetables (about 30% of the diet as fed), fruits (about 60% of the diet as fed) and some extras such as dog pellets, cereals, cheese, chicken breast, nuts, eggs, flaked maize and insects. After the 4-week diet change, a fruit-free diet was provided to the primates. The fruit-free diet consisted mainly of vegetables, insects, pellets, and various nuts accounted for an average of 33.4% of the daily energy intake (Table 2). Due to their more folivorous nature, additional leaves were also included on a daily basis for Hamlyn monkeys and on alternate days for Colombian black spider monkeys.

To facilitate the work of zookeepers and due to seasonal variation in vegetable supply, vegetables were categorised into four groups: green leaves, starches, high sugar content and medium sugar content, based on the criteria detailed in Table 3. Criteria were decided internally using composition tables from the French Agency for Food, Environmental and Occupational Health and Safety (ANSES 2017).

Diet was analysed using Zootrition software. Table 4 compares the initial and fruit-free diets. Non-structural carbohydrates (NSC) were obtained by deduction (%NSC=100–%(protein–lipid–neutral detergent fibre (NDF)–ash)) and represent carbohydrates that are not considered as fibre.

Bananas, chicken breast, flaked maize and dog pellets were removed on the first day of the diet change; then, the rest of the fruits were gradually removed and the quantity of vegetables

Table 1. Composition of the primate groups submitted to the diet change

Species	Group size and structure	Main concerns
Capuchin 1	1.2 adult pair and one immature animal	Occasional aggression
Capuchin 2	1.2 adult pair and one immature animal	Frequent aggression, hyperphagia
L'Hoest	1.4 family group including two immature females	Frequent aggression
Roloway	1.2 mother and her two offspring	Frequent aggression, overweight female
Hamlyn	1.2 including one immature male	Frequent loose faeces and diarrhoea
Spider monkey	1.2 two adults and one geriatric female	Overweight females

Table 2. Diet ingredients used at Mulhouse Zoo (given as percentage fed) for the original (O) and fruit-free (FF) diets

Species	Capuchin		L'Hoest		Roloway		Hamlyn		Spider monkey	
	O	FF	O	FF	O	FF	O	FF	O	FF
Vegetables	47	92	47	94	66	91	57	93	57	91
Fruits	38	0	47	0	22	0	30	0	36	0
Cereals	3	0	1	0	2	0	3	0	1	0
Eggs	1	0	0.5	0	1	0	1		0.5	0
Dog pellets	3	0	1	0	2	0	3	0	1	0
Primate pellets	1	1	1	1	2	1	2	1	0.7	1
Chicken breast	3	0	1	0	2	0	3	0	1	0
Nuts	3	6	1	4	2	7	3	5	1	7
Cheese	1	0	0.5	0	1	0	2	0	0.3	0
Insects	0	1	0	1	0	1	0	1	0	1
Leaves	0	0	0	0	0	0	0	Twice/week	0	Twice/week

increased in parallel. The amount and variety of fruits distributed at each meal decreased.

Daily food weighing and desiccation coefficient

Keepers followed a strict feeding plan indicating the amount of daily food to be administered to each of the six groups of primates and its distribution within six meals per day (given at 0830, 0930, 1100, 1400, 1530 and 1700). Daily food and leftovers were weighed using an S30-I-30000 scale (Mettler Toledo, France; B3C; maximum 30 kg; precision ± 2 g) during 15 days of each period of the study (initial diet (I), diet change (C) and fruit-free diet (F)). Prior to subsequent feeding sessions, all leftovers were removed and enclosures cleaned. As all leftovers lost water because of ambient humidity and temperature, weights were accordingly adjusted using a desiccation factor derived from the estimated moisture lost from similar sets of food placed in a room with the

same abiotic conditions as the enclosures. Food consumption was determined by subtracting the weight of leftovers after application of the desiccation factor from the total amount of food offered.

Body condition scoring

BCS were evaluated weekly for all study subjects over the entire study period. Scores were assigned according to the *Macaca mulatta* scale (Clingerman and Summers 2012). This scale ranges from 1 to 5. Primates were classified according to their BCS as overweight ($BCS \geq 4$), underweight ($BCS \leq 2.5$) or normal ($3 \leq BCS \leq 3.5$).

Faecal scoring

For 45 days, all faeces were photographed daily and identified by group. Scores for faeces consistency were attributed from these pictures according to the Bristol scale, ranging from 1 to 7

Table 3. Vegetable categories used at Mulhouse Zoo

Category	Criteria	Examples
Green leaves	Fibre/sugar ratio > 2; a lot of leaves	Salad, chicory, cabbage
High sugar content	% Sugar DM > 17	Aubergine, cucumber, tomato
Medium sugar content	% Sugar DM < 17	Broccoli, green bean
Starches	% Starch DM > 30; root vegetables	Potato, sweet potato

(Lewis and Heaton 1997). Scores between 3 and 4 are ideal for the species, whereas scores below 3 are too firm and scores over 4 too soft. Scores 6 and 7 were identified as diarrhoea.

Behavioural data collection

All individuals were observed using scan sampling for 20 minutes during the midday meal distribution, for a total of five observations per group and period. Meals were distributed in the indoor enclosure and animals were given free access to the outdoor enclosure during mealtimes. Scan sampling took place in the indoor enclosure. Every minute, food chosen by the observed individual was recorded. 'Other' was used if the individual was in the indoor enclosure but not eating and 'Not visible' if it was outside. All occurrence of aggressions and vocalisations were also counted.

Calculations and statistical analyses

Zootrition (E. Dierenfeld, version 2022.02.1) was used for diet analysis. RStudio (RStudio Team, version 2022.07.0) was used for statistical analysis. Kruskal-Wallis tests followed by post hoc Wilcoxon tests with Bonferroni correction were used to compare data among the three phases of the study for each species independently. Statistical significance was set at $P < 0.05$. In the text and tables, values given are medians with the first and third quartile values in parentheses, unless otherwise noted.

Results

Food intake

Following the introduction of the fruit-free diet, the quantity of food ingested in terms of dry matter increased significantly for all groups except for L'Hoest and Roloway monkeys (Figure 1).

Energy and nutrient intakes

For all groups except Roloway monkeys and Capuchin 2, energy intake during the midday and evening meals decreased when switching to the fruit-free diet. The proportion of consumed versus distributed energy decreased significantly for spider monkeys, Capuchin 1 and Hamlyn monkeys (Table 5). Although the total amount of energy consumed by the L'Hoest monkey group decreased, the proportion of distributed energy that was consumed increased (81.9% to 94%). The origin of energy consumed changed significantly for all groups of primates with an important decrease in digestible carbohydrate and an increase in fat and proteins.

Dietary preferences

To estimate the dietary preference of each group, the first food consumed by each individual during each observed meal was recorded (Figure 2).

Feeding duration

Feeding duration, measured during the first 20 minutes of the midday meal, increased significantly in capuchins, which is consistent with the increase in food intake (Figure 3). The largest increase in feeding time was observed for the L'Hoest monkeys, which more than doubled their median feeding duration. There was a significant increase in feeding duration between the initial and diet change phases for Hamlyn monkeys. However, during observations in the final phase, the Hamlyn monkeys did not eat at several mealtimes, creating a bias in the obtained results.

Body condition scoring

The BCS of 12 of the 20 individuals remained unchanged (Table 6). All changes that occurred were of a magnitude of 0.5 BCS

Table 4. Daily amounts (per group) and nutrient composition of the original (O) and fruit-free (FF) diets for all primate groups in the study based on the food sheets distributed to the animal teams

Species	Capuchin		L'Hoest		Roloway		Hamlyn		Spider monkey	
	O	FF	O	FF	O	FF	O	FF	O	FF
Amount as fed (g)	2088	2394	5307	4925	2817	2559	2325	2913	4602	5307
Dry matter (g)	894	510	1035	870	957	474	855	486	1764	1035
Metabolisable energy (MJ)	13.3	6.8	13.3	13.0	14.2	7.0	12.7	6.3	27.0	13.3
Energy density (MJ/g DM)	0.015	0.012	0.012	0.015	0.015	0.012	0.015	0.012	0.015	0.012
Protein (% DM)	12.2	22.4	20.6	20.6	13.4	21.4	13.6	22.4	11.7	20.6
Lipid (% DM)	4.9	7.6	8.9	6.6	4.9	7.3	5.3	7.7	5.4	8.9
NDF (% DM)	1.9	9.8	1.9	8.4	2.5	9.6	1.5	7.1	2.8	7.0
Ash (% DM)	3.6	6.9	3.2	6.1	3.4	7.5	3.1	6.2	3.7	6.1
Non-structural carbohydrate (% DM)	77.4	53.8	77.8	55.5	75.2	52.8	82.1	59.2	75.2	58.2

Table 5. Median energy and nutrient intake by group monitored during the change from the original diet (O) to a fruit-free diet (FF), with first and third quartile values given in parentheses. *, ** and *** denote statistically significant differences between the considered period and the initial period (I) with $P<0.05$, $P<0.01$ and $P<0.001$ respectively.

Species	Capuchin 1		Capuchin 2		L'Hoest		Roloway		Hamlyn		Spider monkey	
	O	FF	O	FF	O	FF	O	FF	O	FF	O	FF
ME consumed (% of offered)	95.2	78.7*	96.1	94.4	81.9	94.0*	90.0	90.4	95.2	83.1*	93.9	86.8*
Protein (% of consumed ME)	14.9	24.3***	15.0	24.5***	12.5	23.6***	17.3	23.3***	17.3	25.1***	15.4	24.7***
	[13.6-15.7]	[24.2-24.6]	[13.8-15.6]	[24.3-24.8]	[11.6-13.7]	[23.5-23.7]	[15.8-18.2]	[23.1-23.3]	[16.4-17.5]	[24.9-25.4]	[14.8-15.8]	[24.5-25.2]
Fat (% of consumed ME)	5.3	9.6***	5.2	8.7***	4.9	7.8***	5.2	7.5**	5.3	9.4***	5.1	8.24***
	[5.0-6.92]	[9.4-9.95]	[5.0-6.1]	[8.3-9.2]	[4.8-5.1]	[7.6-8.0]	[5.1-6.9]	[7.3-7.8]	[5.1-6.0]	[8.7-9.6]	[5.0-6.6]	[8.0-8.5]
Digestible carbohydrates (% of consumed ME)	79	66***	80	67***	82	68***	77	69***	77	65***	79	67***
	[77-81]	[65-66]	[78-81]	[66-67]	[80-83]	[68-69]	[76-79]	[69-69]	[76-78]	[65-66]	[77-80]	[66-67]

increments. Three overweight individuals ($BCS\geq 4$) were among the seven animals in which BCS decreased. A single animal increased its BCS.

Faecal scoring

For all groups in the study, mean faecal scores decreased after the diet change, indicating that faeces became firmer (Table 7).

Table 6. Mean BCS data (on a scale of 1–5) for Mulhouse Zoo primates during the study. F stands for Female, M for Male, A for Adult and J for Juvenile. The number for capuchins indicates the group

Individual	April 2021	June 2021
Capuchin 1 (M,A,1)	3.5	3.0
Capuchin 2 (F,A,1)	3.0	3.0
Capuchin 3 (F,J,1)	3.0	3.0
Capuchin 4 (M,A,2)	3.5	3.0
Capuchin 5 (F,A,2)	3.0	3.0
Capuchin 6 (F,J,2)	2.5	2.5
L'Hoest 1 (M,A)	3.0	3.0
L'Hoest 2 (F,A)	3.5	3.0
L'Hoest 3 (F,A)	3.0	3.0
L'Hoest 4 (F,J)	3.0	3.0
L'Hoest 5 (F,J)	3.0	2.5
Roloway 1 (M,A)	3.0	3.0
Roloway 2 (F,A)	3.5	3.5
Roloway 3 (F,A)	4.5	4.0
Hamlyn 1 (M,A)	2.5	3.0
Hamlyn 2 (F,A)	3.5	3.5
Hamlyn 3 (F,A)	3.5	3.5
Spider monkey 1 (F,A)	4.0	3.5
Spider monkey 2 (F,A)	4.0	3.5
Spider monkey 3 (M,A)	3.5	3.5

Vocalisations

Vocalisations during feeding times were one of the major concerns reported by the zookeepers. For all groups except Capuchin 2, there was a significant decrease in vocalisations during the 20 minutes following midday food distribution (Figure 4). Aggressive behaviour was observed too rarely during feeding at any of the feeding periods to allow a reasonable comparison.

Discussion

The five primate species studied here are known as frugivorous (Colyn and Rahm 1987; Curtin 2002; Dew 2005; Hansell et al. 2020; Kaplin and Moermond 2000). 'Fruits' in the common sense used for human food differ in many ways from the fruits that these primates can find in their natural environment. For the same amount of energy, wild fruits provide more protein, fat, fibre and vitamin C and less easily digestible carbohydrates (Schwitzer et al. 2009). The composition of easily digestible carbohydrates is also different: sugars of wild fruits are mainly composed of fructose and glucose while in cultivated fruits glucose is replaced by sucrose (reviewed in Milton 1999). Vegetables (e.g., aubergine, cucumber, tomato, broccoli, green beans) have a composition much closer to that of wild fruits in terms of macronutrients, vitamins and sugar (ANSES 2017).

During the initial observation period (I), the high attractiveness of the fruits was observed for all species (Figure 2). Even after a large decrease in fruit quantity in the proposed diet during the diet change period, fruits remained one of the first food items eaten by the primates. One hypothesis to explain this preference for cultivated fruits is their high sucrose content. Indeed, in humans, sucrose has a much higher sweetening power than glucose (which is more present in wild fruits) (MacDonald 1988 cited in Milton 1999). The second hypothesis is the higher energy density of fruits, especially because of their lower fibre content. Predominance of starch-rich vegetables as the first choice of food during meals after the diet change would confirm the second hypothesis. However, after the removal of fruits, food preferences seem to be much more diversified, with differences between primate species. Only vegetables with a medium sugar content remain almost absent from the first choices of the monkeys after the diet change. A longer observation of the food preferences within a fruit-free diet is needed to confirm this diversification.

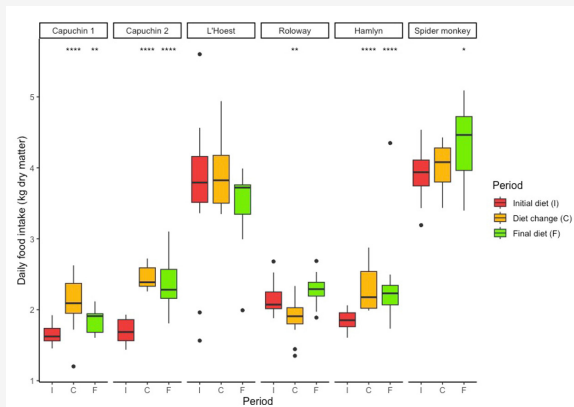


Figure 1. Daily food intake as dry matter by each group of primates on each diet. *, **, *** and **** denote statistically significant differences between the considered period and the initial period (I) with $P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.0001$, respectively

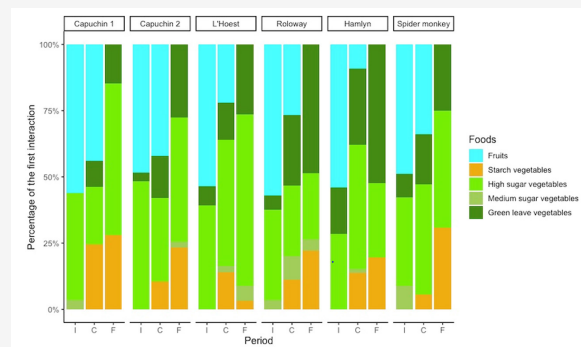


Figure 2. First food consumed per individual of each group following the distribution of the midday meal. I = initial period; C = diet change period; F = fruit-free diet

Even though aggression could not be reasonably scored based on aggressive interactions, according to the zookeepers, mealtimes had less conflict after implementation of the fruit-free diet. This observation is substantiated by the decrease in vocalisations in almost all groups and is consistent with observations of lemurs (Britt et al. 2015). Conflicts associated with a high sugar diet have been described in the literature, notably in human medicine, and an increasing amount of research on animal models seems to link sugar to drug-like effects that may explain the problematic behaviours associated with sugary foods (Avena et al. 2008; DiNicolantonio et al. 2018; Zorrilla and Koob 2019). The diet change did not appear to have a significant effect on the number of vocalisations in the Capuchin 2 group, whereas it did in the Capuchin 1 group despite similar housing conditions and group composition. Capuchin 2 is a social group with more tension and a smaller inside enclosure. Moreover, the individuals in Capuchin 2 spent more time eating indoors together after the diet change, whereas they ate outside during the first phase of the study. These

points suggest that although tensions remained in this group, the dietary change resulted in less conflict-associated access to food. Longer-term monitoring of the effect of diet change on Capuchin 2, as well as ethological observations in the outside enclosure, would be useful to determine the actual effect of removing fruits from the diet on the social behaviour of the animals.

Beyond a modification of sugar intake, the removal of fruits and their replacement by vegetables has other consequences on the ration that may have had repercussions on the animals. By increasing the amount of fibre and therefore decreasing the energy density of the ration, an increase in satiety leading to a decrease in the amount of energy ingested was expected. A decrease was observed in Capuchin 1, L'Hoest and spider monkey groups. In the Capuchin 1 and spider monkey groups, this was combined with a decrease in the proportion of energy consumed in relation to that distributed, confirming a higher satiety. In addition, the decrease in energy intake allowed a reduction in and normalisation of the BCS of the two overweight black spider

Table 7. Median (minimum–maximum) faecal scores (on a range from 1 (very firm) to 7 (liquid diarrhoea), Bristol scale) for primate groups in Mulhouse Zoo during the study

Period	Capuchin 1	Capuchin 2	L'Hoest	Roloway	Hamlyn	Spider monkey
April 2021	3.7 (3.3-4.2)	3.7 (3.2-4.2)	4.3 (3.7-4.8)	4.8 (4.0-5.7)	5.0 (4.4-5.2)	5.6 (5.0-6.1)
June 2021	3.3 (3.0-3.7)	3.0 (3.0-3.0)	3.7 (3.5-4.0)	4.6 (4.0-5.3)	3.9 (3.5-4.2)	4.2 (3.8-4.6)

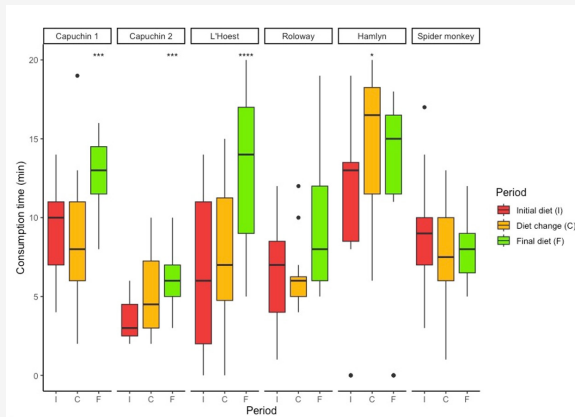


Figure 3. Duration of food consumption per individual of each group in the first 20 minutes following distribution of the midday meal, by period and diet. *, **, *** and **** denote statistically significant differences between the considered period and the initial period (I) with $P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.0001$, respectively

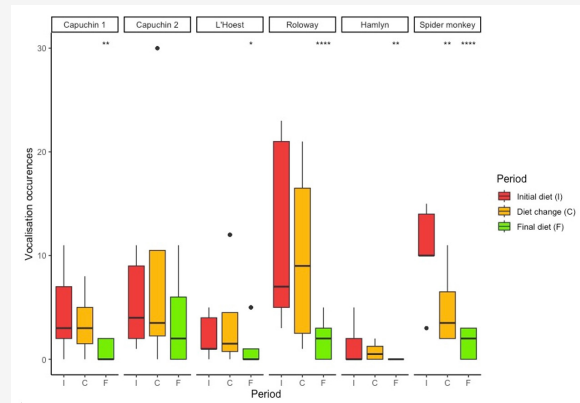


Figure 4. Number of vocalisations in the 20 minutes following the distribution of the midday meal. *, **, *** and **** denote statistically significant differences between the considered period and the initial period (I) with $P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.0001$, respectively.

monkeys. However, satiety does not seem to have been reached in L'Hoest monkeys. Indeed, although feeding time increased and vocalisations decreased, the proportion of energy consumed as a proportion of that offered increased. This lack of satiety might not be due to the fruit-free diet, but rather to a concomitant change in the amount distributed to this species. The study revealed that the amount of food originally distributed to the L'Hoest monkeys had been calculated on an erroneous basis that counted the 6-month-old female still nursing as an adult. This overestimation resulted in the wastage of almost 20% of the quantity distributed in the initial diet.

The fruit-free diet had positive effects on stool quality, especially in Hamlyn and spider monkeys. In these two species, stools became significantly firmer after dietary change. While faeces of spider monkeys were often soft or diarrhoeic during the initial observation phase, stools of the whole group became firmer with the diet change, and a faecal score of 4 was reached at the end of the study, compared with an ideal score of 3 (Lewis and Heaton 1997). Change to a fruit-free diet had a similar effect on faeces consistency in Hamlyn monkeys. Before the diet change, the male of the group was prone to frequent watery diarrhoea of unknown aetiology. Repeated bacteriological and parasitological testing always showed negative results and this phenomenon only responded to the short-term action of Smecta ND (diosmectite, Ipsen Consumer Healthcare, Boulogne Billancourt, France) before the diet change. However, as soon as fibre intake was increased, an effect was noticed on the stools of this group: faeces reached a firm and ideal consistency during the final phase. Softer stools were still observed occasionally, often associated with sugar-rich vegetables such as tomatoes or beetroot, or increased visitor density. Indeed, these episodes of softer faeces or diarrhoea may also be associated with the administration of unusual feeding items such as chips, candy or chocolate by the visitors despite prohibition signs placed in front of the enclosure. It should be noted that the lack of effect of the diet change on faeces consistency in the Roloway group can be explained by the fact that the male

Roloway monkey was on antibiotics (amoxicillin-clavulanic acid, 20 mg/kg q 12 h PO) during the study and diarrhoea is one of the main potential side effects of this treatment.

Before the diet change, there was some concern among the zookeepers at Mulhouse Zoo regarding acceptance of the new diet, with fruit being reported as the preferred ingredient, and regarding whether the primates would become more aggressive, even temporarily, during the gradual withdrawal of fruit. Doubts were also raised about whether expectations in terms of improved health and behaviour were too high. Despite negative staff perceptions of this diet change, the animals became slimmer and intra-group tension reduced. The change in rations has improved the acceptance of new vegetables by the primates. The staff noted that leafy vegetables were rarely sorted and new foods such as mushrooms that were assumed by the staff to be unpalatable are now welcomed by the animals. These results are consistent with those obtained in a survey of zookeepers who noted that monkeys were motivated by their diet whether the rations were with or without cultivated fruit (Hammerston et al. 2019). According to staff feedback, categorisation made the preparation of the ration easier, although preparation time was longer than for the original diet.

While this study provides significant results, there are many limitations that should be considered in future work. One of the main biases is the strong decrease in L'Hoest monkeys' feeding quantity at the same time as the dietary change. More generally, the study was conducted between April and July 2021 and does not consider the effects of seasonality such as perceived variation in food quality or variation in food intake due to weather and temperature. Additional parameters would be interesting to study. For instance, BCS is a subjective measure that should be studied over a long period of time, which may explain the lack of change found in this study. Ideally, a protocol of weighing animals prior to diet change should be implemented to allow more accurate monitoring (Plowman 2013). However, the animals involved in this study were not trained for weighing. Had blood

samples been taken from the primates before and after the dietary change, health status and blood triglyceride values could have been monitored. Finally, a multi-year study including animals from several zoos should be considered to better understand the advantages and limitations of fruit-free rations for primates.

Conclusion

The present study highlights findings regarding the switch to a fruit-free diet in five different species of primates based on various criteria: food intake, energy consumed, body condition score, faecal score, feeding duration and vocalisations. Diet change to a fruit-free diet had beneficial effects on the health and behaviour of the primates from Mulhouse Zoo included in the study. Its implementation allowed a significant decrease in the amount of available energy and non-structural carbohydrates administered to the animals, and an increase in the proportion of neutral detergent fibre delivered. Moreover, groups seemed to ingest less energy after the dietary change. These changes in diet composition and ingestion had clear positive impacts on the behaviour (fewer vocalisations) of the frugivorous species and on the faecal scores of the folivores.

Acknowledgements

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3.3.5.1 Poster :

Étude multicritère sur les régimes sans fruits pour les cébidés et les cercopithèques

Un poster a été réalisé sur cette même étude, il a été présenté au congrès de la société française de primatologie de 2022 et y a reçu le prix de poster de master.



ETUDE MULTICRITÈRE SUR LES RÉGIMES SANS FRUITS POUR LES CÉBIDÉS ET CERCOPITHÉCIDÉS



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Introduction

De nombreux problèmes de santé et de comportements observés chez les primates peuvent être liés à une teneur élevée en sucre dans la ration. Une transition alimentaire vers un régime sans fruit a été mise en place sur 5 espèces de primate du parc zoologique de Mulhouse en 2021. L'étude vise à évaluer l'acceptation du nouveau régime, la consistance des selles, l'état corporel des individus, le temps consacré à l'alimentation ainsi que le nombre de vocalises.

Matériels & Méthodes

Sujet : Atèle noir de Colombie (CBSM) (*Ateles fusciceps rufiventris*) (1.2), cercopithèque d'Hamlyn (*Cercopithecus hamlyni*) (1.2), cercopithèque de l'Hoest (*Allochrocebus lhoesti*) (1.4), cercopithèque de Roloway (*Cercopithecus roloway*) (1.2) and capucin à poitrine jaune (S1 et S2) (*Sapajus xanthosternos*) (1.2 et 1.2) au parc zoologique et botanique de Mulhouse.



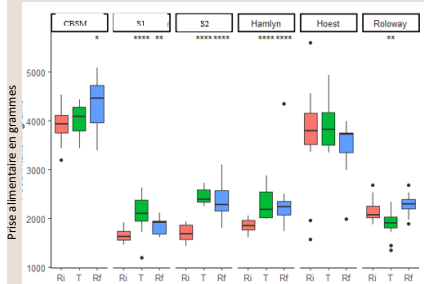
Régime initial (Avril 2021)

Transition (4 semaines, Juin 2021)

Régime final (Juillet 2021)

Résultats & discussions

Prise alimentaire



- Obtenue par pesée des rations et restes alimentaires avec application d'un coefficient de dessiccation
- Diminution de la prise alimentaire : satiété permise, sauf pour les Hoest
- Effet lors de la transition alimentaire pour les Roloway puis nouvelle augmentation : compétition pour nouveaux aliments ?

Score fécal



- Score de Bristol avec évaluation quotidienne
- Raffermissement des selles : atèles et Hamlyn
- Stabilité de la fermeté des selles : Hoest et capucins
- Diminution de la fermeté : Roloway (mâle sous traitement antibiotique)

Niveau d'énergie ingéré

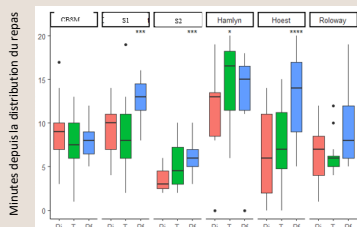
Espèce	<i>Ateles fusciceps rufiventris</i>		<i>Sapajus xanthosternos</i> S1		<i>Sapajus xanthosternos</i> S2		<i>Cercopithecus hamlyni</i>		<i>Allochrocebus lhoesti</i>		<i>Cercopithecus roloway</i>	
	Original	Fruit-free diet	Original	Fruit-free diet	Original	Fruit-free diet	Original	Fruit-free diet	Original	Fruit-free diet	Original	Fruit-free diet
Energie métabolisable distribuée (kcal)	2088	1986	949	1004	936	1040	962	950	2610	1799	1193	1177
Energie métabolisable consommée (kcal)	2021	1690	869	791	897	974	882	786	2122	1691	1024	1065
Energie métabolisable consommée (%)	93.9	86.8	95.2	78.7	96.1	94.4	95.2	83.1	81.9	94.0	90.0	90.4

- Analyses faites sur Zootrition® (Dierenfeld, 2021)
- Diminution de la proportion d'énergie consommée en Rf : limitation de la prise énergétique
- Au niveau des Hoest, la question de la satiété se pose à nouveau car la consommation d'énergie augmente.

Vocalise

- Scan sampling, 20 minutes d'observations après les repas, 5 répétitions par phase
- Diminution du nombre de vocalise après les repas : baisse de l'appétence, limitation de la compétition alimentaire
- Aucune interprétation éthologique a été effectuée sur ces vocalises.

Temps d'alimentation



- Scan sampling, 20 minutes d'observations après les repas, 5 répétitions par phase
- Augmentation du temps de repas pour les capucins (30% et 60% respectivement) et les Hoest (300%)
- Capucins S2 : on note que les individus mangent plus souvent en groupe lors de la période Rf.

Conclusion

- Transition alimentaire : les fruits doivent être retirés rapidement pour éviter tout conflit.
- Les primates à tendance majoritairement frugivore ont été des résultats hétérogènes : les groupes ont réagi de façon différente à la mise en place du régime.
- Les primates à tendance majoritairement folivore ont des résultats homogènes et ils ont réagi de façon significative à l'ensemble des critères étudiés.
- L'effet de l'individu ne doit pas être minimisé
- Une surveillance sur le long terme notamment des états corporels est nécessaire pour évaluer l'effet du changement de ration sur les individus.

Ri : période avec le régime initial
T : transition alimentaire
Rf : période avec le régime final (régime sans fruit)

Remerciements

Les auteurs tiennent à remercier les soigneurs animaliers du parc zoologique de Mulhouse d'avoir rendu possible la collecte de données.

Bibliographie : Britt et al (2015); Cabana (2014,2018); Caravaggi et al (2018); Clingerman and Summers (2012); Dierenfeld (1993); Gomis (2007); Goodchild and Schwitzer (2008); Lewis and Heaton (1997); NRC (2003); Nijboer et al (2000); Plowman (2013); Schwitzer (2001,2008); Streicher et al (2013); Turner and Harrenstien (1985)



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Deuxième partie

Réponses aux attentes sociétales dans le domaine de la gestion des populations de rongeurs

Chapitre 4

Enjeux, stratégies et mise en œuvre

Bien que la partie consacrée à la nutrition soit, en raison de son contexte spécifique, principalement animée de manière individuelle, la recherche sur les rongeurs, les risques sanitaires associés, et la gestion de leurs populations est, en revanche, effectuée en équipe. Dans ce cadre collaboratif, chaque membre apporte ses compétences spécifiques et contribue au projet en concertation avec les autres. Il devient dès lors ardu de déterminer qui a initié une idée en premier lieu ou qui a joué un rôle déterminant dans tel ou tel projet. Ce chapitre présente les thématiques abordées, leur articulation, ainsi que les productions associées aux travaux de recherche qui ont bénéficié de mon implication.

4.1 Contexte

Ce double positionnement entre la nutrition et la toxicologie peut paraître surprenant. Il m'est difficile de résumer en une seule phrase l'ensemble de mes activités. Bien que je sois principalement reconnu dans le domaine de la nutrition des carnivores domestiques, la majorité de mes contributions scientifiques, tant en termes de quantité que de qualité, se situe dans le domaine de la toxicologie.

Initialement, mon poste, ouvert en 2017, était destiné à être attribué à l'UMR CarMeN (INSERM, INRAe et Université de Lyon) avec un projet centré sur l'obésité. Toutefois, en l'absence de collègues de l'unité sur le campus de Marcy L'Étoile (à l'exception d'une en anesthésiologie), cette affectation m'aurait placé en Activité Scientifique Individuelle dans un laboratoire distant d'une heure en transport en commun de l'école. Cette situation était peu souhaitable pour un jeune enseignant-chercheur. De plus, ayant participé durant ma thèse à la mise aux normes des animaleries de l'unité 1233, rester à CarMeN aurait eu le double inconvénient de compliquer ma situation de recherche et d'handicaper une unité interne de VetAgro Sup.

Par conséquent, en 2018, contre l'avis de ma direction d'établissement, je suis retourné dans l'unité de mon doctorat, "Rongeurs Sauvages, Risques Sanitaires et Gestion des Po-

pulations” (USC/INRAe 1233). Au sein de cette unité, mes recherches ont porté sur les préoccupations sociétales liées à l’écotoxicité des rodenticides. J’ai étudié l’exposition sur des espèces non cibles et exploré des solutions pour réduire cette écotoxicité. Chacune de ces thématiques fait l’objet d’un co-encadrement doctoral, respectivement avec Mme Moriceau et M. Rached. J’ai également développé une dernière thématique sur l’application des principes des 3R^a dans le cadre de l’étude des rodenticides.

En réponse aux attentes sociétales, mes compétences ont également été mises au service de l’unité pour être à la pointe de la mise à disposition des données de recherche. Suite à la publication de la loi pour une république numérique de 2016, nous avons publié l’ensemble de nos articles sur les archives ouvertes HAL dès l’expiration de l’embargo de six mois. La loi ne nous permet pas de mettre en ligne les articles mis en forme par l’éditeur, sauf si l’article est publié dans un journal *open access*. Les mises en forme des *pre-prints* sont peu pratiques pour le lecteur. Par conséquent, pour faciliter l’accès, les articles ont été mis en forme en L^AT_EX.

4.1.1 Problématiques des rodenticides

Comme il sera précisé ultérieurement dans les articles constituant ce mémoire, les rongeurs cohabitent avec les humains partout où ces derniers se sont installés. Cette cohabitation engendre diverses difficultés, qu’elles soient agricoles, économiques, technologiques, sanitaires ou environnementales. Bien que les espèces de rongeurs varient, la volonté humaine de les éradiquer demeure constante, devenant même un enjeu politique dans certaines villes comme Paris.

Face à la problématique des rongeurs, la solution majoritairement employée est l’utilisation d’anticoagulants anti-vitamine K (AVK), qui induisent la mort des rongeurs par hémorragie. Cependant, cette approche chimique présente des risques tant pour l’utilisateur que pour l’environnement, notamment la faune non cible. La prise en compte de ces risques est cruciale pour l’utilisation raisonnée de ces molécules et leur acceptabilité sociétale. L’écotoxicité est devenue une préoccupation majeure, notamment en raison de l’émergence de résistances aux AVK dans les populations de rongeurs (voir chapitre d’introduction en section 5.1.1) . Cela a conduit au développement de nouvelles molécules anticoagulantes, plus efficaces mais également plus persistantes dans l’environnement.

Il est donc impératif de travailler à l’élaboration de solutions de gestion des populations moins écotoxiques mais tout aussi efficaces. L’utilisation de mesures moins efficaces pourrait faciliter la sélection d’animaux résistants, entraînant une course vers des molécules encore plus nocives. Aussi, il faut prendre en compte les retours de terrain faisant état de résistances pour étudier des manières de les contourner sans augmenter les doses de rodenticides.

a. Réduire, Remplacer et Raffiner. Principes fondamentaux de la recherche sur l’animal

Cette problématique s'étend également au bien-être des animaux de laboratoire. Pour développer de nouveaux rodenticides, des tests sont nécessaires, tant pour décrire leur pharmacocinétique que pour assurer leur efficacité. La réglementation exige l'application des principes des 3R^b tout en garantissant la fiabilité des tests. La population souhaite également une réduction de l'utilisation des animaux de laboratoire, surtout dans le cadre de procédures sévères.

En somme, la gestion des populations de rongeurs est un équilibre délicat entre trois composantes : le risque, l'efficacité et le bien-être animal. L'omission de l'une de ces composantes compromettrait son acceptabilité sociétale, avec le risque de se retrouver sans alternatives si de nouvelles méthodes de lutte ne sont pas développées en temps opportun.

4.2 Stratégies et mises en œuvre

Dans le contexte contemporain de la gestion des populations de rongeurs, trois enjeux majeurs émergent comme des piliers incontournables pour une approche socialement responsable : les risques sanitaires et environnementaux, l'efficacité des méthodes de gestion, et le bien-être animal dans le cadre des expérimentations. Ces enjeux, bien que distincts, sont intrinsèquement interconnectés et nécessitent une approche intégrée pour résoudre les défis complexes posés par la cohabitation entre les rongeurs et les populations humaines.

Ce travail de recherche vise à approfondir chacun de ces enjeux, à explorer leurs interconnexions et à présenter des stratégies de gestion qui tiennent compte de cette complexité. L'ambition est de contribuer à l'élaboration de solutions qui offrent un gain net dans la balance bénéfice-risque et qui répondent aux exigences éthiques et sociétales contemporaines.

4.2.1 Risques de l'emploi des rodenticides

Dans cette sous-section, l'analyse des risques se focalise sur l'exposition et la toxicité des AVK pour les organismes non ciblés, englobant l'humain, les espèces aviaires telles que le vautour, ainsi que les espèces domestiques. Les objectifs de ces études sont pluriels. D'une part, elles cherchent à déterminer et à quantifier l'impact réel des AVK sur ces diverses populations. D'autre part, elles visent à établir des données de référence longitudinales pour suivre l'évolution de cet impact au cours du temps.

Ces données revêtent une importance cruciale pour évaluer l'efficacité des politiques publiques et des innovations technologiques en matière de réduction de l'écotoxicité des AVK. L'acquisition de données objectives constitue un prérequis incontournable pour une analyse bénéfice-risque rigoureuse de l'utilisation de ces molécules. Cette nécessité est

b. Réduire, Remplacer et Raffiner

accentuée par le fait que ces substances sont inscrites sur la liste des molécules candidate a substitution par l'Union européenne¹.

4.2.1.1 Études de cas d'exposition et d'intoxication

L'un des dangers les plus fréquemment cités associés aux AVKs concerne la faune non cible. En raison de leur utilisation généralisée et de leur persistance environnementale, les AVKs peuvent affecter ces animaux de deux manières : soit directement par l'ingestion d'appâts (exposition primaire), soit indirectement par la consommation de rongeurs contaminés (exposition secondaire). Au fil des années, des mesures ont été mises en place pour limiter l'exposition primaire, notamment l'obligation d'utiliser des boîtes d'appâtage sécurisées. Toutefois, il est impératif de quantifier objectivement le risque pour différents types d'animaux, allant des animaux de compagnie aux animaux sauvages et aux animaux de rente, car les enjeux varient considérablement.

J'ai contribué à cette thématique par la rédaction d'un chapitre dans le livre "Poisoning" publié en 2017 (section 5.2.1.1)². Ce chapitre compile des données issues de la littérature ainsi que des analyses provenant de centres anti-poison humains et vétérinaires, offrant une vue d'ensemble des expositions aux AVKs. Il a été observé une diminution des cas chez les humains et des appels concernant les animaux domestiques. Cette baisse est attribuée à l'entrée en vigueur de réglementations imposant l'utilisation de boîtes d'appâtage sécurisées. Un autre point notable est la surreprésentation des chiens dans les appels (environ 80% des appels pour AVK), suivis des chats (environ 10%) et enfin des animaux de rente. Cependant, ces données sont principalement basées sur les appels et ne permettent pas de distinguer les suspicions d'exposition des expositions réelles ou des cas d'intoxication.

L'exposition réelle des animaux de compagnie a été étudiée plus en détail dans l'article de Mahjoub et al. 2022 (repris en section 5.2.1.2)³. Pour estimer cette exposition, des échantillons de selles ont été collectés auprès de chiens (n=304) et de chats (n=289) en bonne santé par des vétérinaires en France et en Belgique. Les taux d'exposition aux AVKs étaient respectivement de 2,6% pour les chiens et de 4,5% pour les chats. Ces taux sont nettement inférieurs à ceux observés chez les renards sauvages, où les prévalences d'exposition varient de 23%⁴ à 50%⁵. Cette différence significative peut s'expliquer par la réglementation en vigueur et par une moindre exposition secondaire chez les animaux domestiques.

L'impact de l'exposition des animaux domestique aux AVKs prend une dimension particulièrement critique lorsqu'il concerne les animaux de rente, dont les produits sont destinés à la consommation humaine. Dans ce contexte, l'article de Moriceau et al. 2020 (section 5.2.1.3)⁶ présente une étude de cas d'un élevage laitier où les brebis (n=23) ont ingéré un sac de 20 kg d'appât en grain contenant de la chlorophacinone à une concentration

de 50 mg/kg. Bien que cette exposition ne représente pas un risque élevé d'intoxication pour les ovins, en raison de la dégradation ruminale des AVKs⁷, elle soulève des questions cruciales concernant la présence potentielle de chlorophacinone dans le lait et les risques associés pour les consommateurs. L'évolution de la concentration de l'AVK dans le lait a été suivie quotidiennement du deuxième au septième jour post-exposition. Sur la base de ces mesures, une analyse de risque pour le consommateur a été effectuée, suggérant un risque relativement faible. Un autre résultat d'intérêt concerne la relation linéaire observée entre la quantité de chlorophacinone présente dans le lait et celle dans le plasma des brebis. En outre, bien que cette exposition n'ait pas entraîné de signes cliniques manifestes, elle a été suffisante pour provoquer une augmentation significative du temps de prothrombine chez les brebis, trois jours après l'exposition.

Enfin, les AVKs représentent également une préoccupation majeure pour la préservation de certaines espèces, notamment les oiseaux de proie. Ces derniers sont principalement exposés aux AVKs de manière secondaire, via leur alimentation. Toutefois, ils peuvent également être exposés de manière primaire en raison d'actes malveillants. À cet égard, un suivi rigoureux est indispensable pour formuler des propositions visant à minimiser ces risques. Cela est illustré dans l'article de Moriceau et al. 2022⁸ (section 5.2.1.4). Cette publication rapporte les résultats d'autopsies et d'analyses de résidus d'anticoagulants sur 156 rapaces retrouvés morts entre 2008 et 2019 en France, et plus particulièrement dans le Sud-Est. Outre la prévalence significative de l'exposition de ces oiseaux aux AVKs, qui est plus élevée chez les rapaces prédateurs que chez les charognards, l'article soulève la question complexe de l'attribution de la cause de la mort aux AVKs, une tâche souvent difficile à accomplir sur des cadavres. En effet, parmi toutes ces expositions, seules deux ont été suspectées d'être des intoxications aux AVKs. D'autres éléments suggèrent que l'exposition aux AVKs est généralement non mortelle dans la plupart des cas : **i/** la majorité des échantillons sont exposés à plusieurs AVKs (64%), ce qui semble indiquer une exposition multiple ; **ii/** d'autres contaminants tels que le plomb ou des insecticides (comme le carbofuran) ont été détectés. Cependant, même si les AVKs ne sont pas directement responsables de la mort en provoquant une hémorragie, pourraient-ils néanmoins jouer un rôle secondaire en affaiblissant l'animal à faible dose, comme le suggèrent certaines publications⁹ ?

Cette première partie soulève de nombreuses questions et met en évidence les lacunes d'une approche basée uniquement sur la détection des AVKs. Il est donc impératif de mieux caractériser les outils existants et leurs limites, et d'initier le développement de nouvelles méthodologies pour suivre plus efficacement l'impact des AVKs sur la faune non cible.

4.2.1.2 Différencier exposition et intoxication

Afin de différencier une simple exposition aux AVKs, sans conséquences cliniques, d'une intoxication potentiellement mortelle, nous avons entrepris une analyse exhaustive de la littérature. Cette démarche est détaillée dans l'article de Rached et al. 2020¹⁰ (voir section 5.2.2.1). À l'heure actuelle, la méthode standard pour diagnostiquer une intoxication aux AVKs repose sur la quantification des résidus de ces rodenticides dans le foie ou le plasma des animaux affectés. Bien que des valeurs seuils aient été proposées¹¹, ces dernières sont largement empiriques et ne tiennent pas compte des variations pharmacologiques inter- et intra-espèces. Il est donc crucial de découvrir des biomarqueurs plus spécifiques. Notre revue fournit un aperçu complet des paramètres toxicodynamiques et toxicocinétiques des AVKs dans différentes espèces animales, et évalue divers biomarqueurs en termes de leurs avantages et inconvénients. En effet, certains biomarqueurs ne sont pas applicables à tous les états physiologiques de l'animal. Nous introduisons également de nouveaux biomarqueurs potentiels et discutons de leur pertinence.

En complément de cette revue, des études originales ont été menées, notamment celle publiée par Rached et al. 2023¹² (voir section 5.2.2.2). Cette recherche évalue les concentrations de bromadiolone, un AVK spécifique, dans trois matrices biologiques (foie, plasma et selles) chez des rats mâles et femelles sur une période de 30 jours. Nos résultats indiquent que le plasma n'est pas un médium fiable pour cette évaluation, en raison du risque de faux négatifs. En revanche, l'étude de la diastéréoisomérisation de la bromadiolone dans les fèces permet de distinguer une exposition récente d'une exposition plus ancienne. Toutefois, ces conclusions appellent à une vérification plus large, notamment en ce qui concerne d'autres AVKs et espèces animales. Des travaux supplémentaires sur les biomarqueurs, notamment en relation avec la vitamine K époxyde hépatique, sont en cours de publication.

4.2.1.3 Étude sur le risque reprotoxique

Si l'exposition des animaux aux AVKs représente une problématique majeure, celle des humains mérite également d'être évaluée. L'adoption massive de ces molécules est due, non seulement à leur efficacité, mais aussi à leur sûreté d'emploi, garantie par l'existence d'un antidote efficace, sûr et disponible. Néanmoins, la question de l'exposition à de très faibles doses demeure. Ces doses, bien que non suffisantes pour entraîner une hémorragie, pourraient avoir d'autres effets, notamment sur un fœtus. En effet, les AVKs sont classés comme molécules toxiques pour la reproduction, une classification basée sur des études concernant la Warfarine (ou coumaphène)^c et sur des retours de son utilisation en médecine humaine^{13,14}. En application de la méthode de prédiction par *read-across*, prévue par la réglementation REACH, cette conclusion a été étendue aux autres AVKs par

c. AVK de première génération, à demi-vie courte, aujourd'hui abandonné en tant que rodenticide mais toujours utilisé en médecine humaine

l'Union Européenne, notamment ceux de seconde génération. En conséquence, les appâts contenant plus de $30\mu\text{g}/\text{kg}$ ne sont plus utilisables par les non-professionnels depuis 2018. Bien que cette limite n'ait pas de conséquence majeure sur l'efficacité pour la majorité des molécules, elle pourrait entraîner une inefficacité sur les rongeurs résistants aux AVKs et ainsi faciliter la sélection de ces sous-populations. Il semble donc nécessaire de confirmer ou d'infirmer la pertinence de l'utilisation du *read-across* entre les différentes générations d'AVKs, notamment en raison de leur pharmacologie distincte.

Pour explorer ce point, nous avons réalisé une étude pharmacocinétique comparative entre différents AVKs (deux de première génération, à savoir la Warfarine et le coumatétralyl, et un de seconde génération, la bromadiolone), en portant une attention particulière à leur distribution. De plus, nous avons mis en place une procédure pour évaluer l'exposition des fœtus *in utero* et, enfin, nous avons évalué l'exposition des nouveau-nés via le lait maternel. Ces expériences ont été réalisées sur des rates en gestation ou en lactation, selon le cas. Les résultats sont rapportés dans Chetot et al. 2020¹⁵ (repris en section 5.2.3.1). Cette étude montre que l'exposition des fœtus ou des nouveau-nés est nettement inférieure avec une molécule de seconde génération qu'avec les deux autres. Ce point explique les résultats obtenus dans un autre article de l'unité, qui met en évidence l'absence d'effet tératogène avec la bromadiolone, contrairement à la Warfarine¹⁶. Cette faible exposition semble s'expliquer par une captation quasi-totale de la bromadiolone lors du premier passage hépatique, réduisant ainsi fortement sa circulation. Cependant, il convient de rester prudent avant d'étendre ces conclusions à d'autres molécules et espèces.

4.2.2 Vers de nouveaux outils de gestion des populations

L'étude de nouvelles solutions vise à évaluer des moyens innovants de contrôle des populations de rongeurs qui répondent aux préoccupations soulevées dans l'axe précédent, sans compromettre leur efficacité par rapport aux méthodes existantes. Ces moyens peuvent être une évolution de méthodes existantes ou une amélioration d'une technique déjà en place. Mes recherches se sont concentrées sur deux opportunités pour augmenter l'éco-compatibilité des AVKs : la vitamine K dans l'alimentation et l'étude des stéréoisomères des AVKs.

4.2.2.1 Alimentation et exploitation du coût biologique

Alors que les AVKs inhibent le recyclage de la vitamine K, cette dernière peut également être obtenue par l'alimentation. Notre travail met en évidence l'importance de cette source alimentaire de vitamine K dans la résistance aux anticoagulants et dans la compensation du coût biologique induit par mutation du gène *vkorc1*.

La première illustration de ce phénomène se trouve dans Debaux et al. 2019¹⁷ (repris en section 5.3.1.1). Cette étude fait suite à des recherches antérieures montrant que les

mutations de *vkorc1*, qui confèrent aux rongeurs une résistance aux AVKs, ont un coût biologique : une diminution du recyclage de la vitamine K. Ce coût a été mis en évidence *in vitro*¹⁸ et ses conséquences physiologiques ont été démontrées *in vivo*, notamment par l'apparition de calcifications vasculaires chez les individus résistants nourris avec un régime carencé en vitamine K¹⁹. Notre étude complète ces données en analysant l'évolution du pool tissulaire de vitamine K (phylloquinone et ménaquinone 4) dans divers organes tels que le foie, les testicules, les reins et les poumons, suite à une alimentation carencée en vitamine K. D'autres biomarqueurs liés à l'activité de la vitamine K, tels que l'ostéocalcine non carboxylée (ucOC) et le temps de prothrombine, ainsi que les propriétés enzymatiques des microsomes de chaque tissu, sont également étudiés. Cette publication permet de mieux comprendre l'impact des mutations sur le pool de vitamine K disponible et souligne que, pour un même pool de vitamine K, les biomarqueurs des lignées résistantes se dégradent contrairement à la lignée sauvage. Ceci ouvre la voie à de nouvelles stratégies de lutte contre les rongeurs résistants en tentant de limiter leur accès à la vitamine K alimentaire.

L'importance de ce type de stratégie est d'autant plus cruciale que nous avons identifié un mécanisme de résistance qui ne repose pas sur une mutation du gène *vkorc1*, mais sur un apport alimentaire en vitamine K suffisant pour contrer l'action des AVKs. Cette hypothèse est avancée dans Abi Khalil et al. 2021²⁰ (section 5.3.1.2). Cette recherche tente d'expliquer la résistance des campagnols terrestres (*Arvicola amphibius*) aux AVKs, précédemment observée²¹. Elle démontre que cette résistance ne peut être attribuée à une enzyme VKORC1 résistante, mais révèle que les campagnols ont des concentrations hépatiques de vitamine K1 six fois supérieures à celles observées chez le rat. De plus, ces concentrations varient en fonction de la période de l'année, avec des pics en juillet et novembre et des creux en août et février. Ainsi, la prise en compte de l'apport alimentaire en vitamine K apparaît essentielle pour garantir l'efficacité des AVKs et réduire les quantités utilisées.

4.2.2.2 Révolution des anticoagulants par les stéréoisomères

Il est bien établi depuis plusieurs années que la warfarine présente des demi-vies différentes pour chacun de ses stéréoisomères, une variabilité expliquée en partie par des voies métaboliques distinctes. Récemment, l'USC 1233, et notamment Etienne Benoit et Virginie Lattard, ont suggéré que cette propriété est probablement partagée par les autres AVKs, et pourrait constituer une solution pour réduire la rémanence des anticoagulants antivitaminé K²². Les anticoagulants de seconde génération possédant deux carbones asymétriques, quatre stéréoisomères sont obtenus pour chaque molécule. Toutefois, leur séparation n'est pas toujours aisée. La première étape consiste donc à étudier les diastéréoisomères, plus facilement séparables par chromatographie non chirale. L'opportunité d'étudier ensuite les stéréoisomères individuels sera évaluée.

L'étude pharmacocinétique et d'efficacité des diastéréoisomères des AVKs de seconde génération est décrite dans Damin-Pernik et al. 2017²³ (section 5.3.2.1). Cette recherche montre que les diastéréoisomères présentent des pharmacocinétiques différentes, l'une des formes étant plus rémanente que l'autre, mais avec une capacité équivalente à inhiber VKORC1. Ainsi, en substituant un AVK commercial par son diastéréoisomère le moins rémanent, on réduit les résidus environnementaux à la mort de l'animal. Seule la diféthialone semble montrer une différence moins significative de rémanence entre ses diastéréoisomères.

Cette particularité de la diféthialone nous a incités à étudier les propriétés de chacun de ses stéréoisomères dans deux articles distincts. Le premier, axé sur le rat mâle, est Lefebvre et al. 2020²⁴ (section 5.3.2.2), et le second, portant sur les rats et les souris des deux sexes, est Rached et al. 2022²⁵ (section 5.3.2.3). Bien que les pharmacocinétiques hépatiques des diastéréoisomères de la diféthialone soient similaires, celles de ses stéréoisomères varient considérablement, avec des demi-vies allant du simple au décuple et des AUC variant de plus de vingt fois. Plus important encore, le second article soulève la question des différences inter- et intra-spécifiques. Les stéréoisomères les moins rémanents ne sont pas les mêmes selon les espèces et les sexes, ce qui complique l'extrapolation des résultats d'une espèce à une autre, voire d'un sexe à l'autre.

Cette conclusion ouvre un débat sur l'expérimentation animale. Étant donné le grand nombre de combinaisons possibles (stéréoisomère ; espèce ; sexe), il existe un risque significatif de devoir utiliser un grand nombre d'animaux. Comment, dès lors, appliquer le principe des 3 R dans de telles conditions ?

4.2.3 Amélioration de l'expérimentation animale

Il est effectivement délicat d'aborder la question du bien-être animal dans le contexte de l'étude d'un rodenticide. Bien que les rongeurs considérés comme nuisibles ne soient pas actuellement protégés par des mesures spécifiques, ceux présents dans les animaleries sont soumis à la réglementation européenne. Cette dernière exige d'éviter toute souffrance inutile en affinant les méthodes de recherche et les conditions d'hébergement. Elle préconise également de réduire le nombre d'animaux utilisés dans les expérimentations et de les remplacer par des méthodes alternatives lorsque cela est possible. Ces trois exigences forment le principe des 3R (Raffiner, Réduire, Remplacer). Toutefois, l'application de ces principes ne doit pas compromettre la qualité de la recherche ou des tests d'homologation. Les efforts dans ce domaine visent à trouver et à valider un équilibre entre ces différentes considérations.

Au-delà de l'aspect purement réglementaire et de l'enjeu pour l'acceptabilité sociétale, cet axe de recherche constitue également un impératif éthique pour les chercheurs impliqués dans les études *in vivo*.

4.2.3.1 Mise en place des points limites

Le point le plus urgent à aborder concerne la détermination des points limites dans le cadre des tests d'efficacité. Notre unité réalise des tests d'homologation pour les rodenticides conformément aux réglementations européenne et américaine. Ces tests visent à valider l'efficacité des appâts dans un environnement expérimental et sont considérés comme réussis si une mortalité de 90% est atteinte (avec 10 individus, 5 de chaque sexe). La mise en place de points limites, destinés à soustraire l'animal à une souffrance inutile, généralement par euthanasie, pose un dilemme dans ce contexte. En effet, un point limite non prédictif de l'effet de l'anticoagulant pourrait fausser les résultats du test.

Récemment, les directives européennes ont encouragé l'établissement de tels points limites sans les spécifier, ce qui expose les industriels finançant les tests au risque de rejet des points limites choisis lors de l'évaluation du dossier. Pour concilier les exigences réglementaires et éthiques, il est impératif de mener une étude pour valider ces points limites. Afin de minimiser l'utilisation d'animaux, cette étude a été réalisée en utilisant des données de tests d'homologation antérieurs. L'objectif est de déterminer des points limites qui soient presque certainement prédictifs de la mortalité de l'animal. Nous avons utilisé une ancienne procédure sans points limites mais avec un suivi clinique précis des animaux. Plusieurs hypothèses de points limites ont été testées pour ne retenir que ceux qui permettaient d'obtenir le plus grand nombre d'animaux les atteignant sans faux positifs. Cette étude a été présentée sous forme de poster²⁶ lors des congrès de l'EVPMC^d et de l'AFSTAL^e.

Il est à noter que ces données sont difficiles à valoriser dans des articles largement accessibles. En effet, compte tenu des débats actuels sur l'expérimentation animale et l'utilisation des rodenticides, l'impact d'un tel article sur le débat public reste incertain.

4.2.3.2 Amélioration du suivi des expérimentations

En dehors des tests d'homologation précédemment évoqués, les recherches sur la pharmacocinétique des anticoagulants peuvent entraîner des effets secondaires non recherchés sur les animaux. Lorsque ces effets surviennent, ils conduisent à l'atteinte d'un point limite et à l'exclusion de l'animal de l'étude. Pour minimiser ces occurrences, il est possible de recourir à l'antidote, la vitamine K. Toutefois, cette dernière peut légèrement modifier la pharmacocinétique des AVKs, en particulier si elle est administrée dans les heures suivant la prise de l'anticoagulant. Afin de prévenir l'apparition de signes cliniques tout en limitant l'utilisation de la vitamine K, il est essentiel de connaître l'évolution des biomarqueurs suite à l'administration des AVKs. Cela permet à l'expérimentateur de décider de l'utilisation ou non de la vitamine K.

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Comme précédemment, d'autres expérimentations seront valorisées pour réaliser cette étude. L'objectif est de comparer des méthodes permettant de suivre la pharmacodynamique des AVKs. Au-delà de son intérêt pour le suivi des expérimentations, cette recherche contribue également à l'identification de biomarqueurs, comme défini précédemment. Elle a abouti à deux publications : Lefebvre et al. 2017²⁷ et Jourdi et al. 2018²⁸ (respectivement en section 5.4.2.1 et 5.4.2.2).

4.2.3.3 Vers de nouvelles approches

De nombreux progrès restent à accomplir pour réduire l'utilisation des animaux de laboratoire, et notamment pour comprendre l'impact des AVKs sur des espèces où les possibilités d'expérimentation sont limitées. Une des solutions envisagées consiste à utiliser des modèles de toxicocinétique et de toxicodynamique. Ces modèles pourraient permettre une meilleure appréhension de ces espèces, de réduire le nombre de tests nécessaires en éliminant a priori les tests infructueux grâce à des simulations, et de faciliter les analyses bénéfice-risque pour chaque utilisation d'AVK. Cette nouvelle voie d'étude, ainsi que la stratégie associée, sont présentées en section 6.1.2.

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Chapitre 5

Publications

Dans ce chapitre, les publications introduites au chapitre précédent sont reprises et organisées en fonction de leurs thématiques.

5.1 Introduction de la problématique

5.1.1 Chapitre :

Comparative Biology of the Resistance to Vitamin K Antagonists : An Overview of the Resistance Mechanisms

Comparative Biology of the Resistance to Vitamin K Antagonists: An Overview of the Resistance Mechanisms

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Virginie Lattard

Additional information is available at the end of the chapter

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Abstract

Vitamin K antagonists (VKA) are used in human medicine as well as for the management of rodent populations. In both cases, we have to deal with inter-individual resistances. Many mechanisms of resistances are common in humans and rodents. Moreover, with the large use of vitamin K antagonist rodenticides, the resistant phenotype is overrepresented in some rodent populations. Consequently, some resistance mechanisms with a low prevalence in the human population have a higher prevalence in rodent population; thus, they can be more studied in rodents. The aim of this chapter is to cross knowledge coming from human medicine and rodent research in order to better understand each resistance mechanism. After an overview of the essential knowledge for the understanding of the VKA action, this chapter presents the different methods of VKA resistance studying and then it assesses the current knowledge on VKA resistance in humans and rodents.

Keywords: vitamin K antagonists, warfarin, comparative biology, pharmacogenomics

1. Introduction

Vitamin K antagonists (VKA) are inhibitors of the regeneration cycle of vitamin K. The diminution of the available and usable vitamin K in the body induced by VKA leads indirectly to a hypocoagulable status of the blood. The use of VKA in humans and rodents pursues this status. Nevertheless, the final aim is different for each. In the human case, VKA anticoagu-

lants are used to prevent venous and arterial thrombotic event. In spite of the increasing use of new oral anticoagulants, VKA and, more precisely, warfarin are the most commonly prescribed anticoagulants [1]. Considering rodents, VKA are used in pest population management. In this case, death resulting from hemorrhages is pursued. VKA are currently the most used and one of the best rodenticides for two reasons. First, the delay between VKA administration and death is of several days, which avoids association between bait and death by the other rodents. Second, conversely to other rodenticides, VKA have an antidote: the vitamin K, which increases their safety for the human population and nontarget species.

In humans, VKA anticoagulants have a narrow therapeutic range [2]. Under- and overdoses can have serious consequences by the lack of efficacy or adverse event. Warfarin has been ranked number 9 among primary suspect drugs having serious outcomes in the United States during the beginning of the 2000's decade [3]. Indeed, the VKA dose has to be modulated, reflecting the genotype of patients more than other common drugs. Thus, it is necessary to identify and characterize each gene and mutation which may influence the VKA dose. In this task, the VKA research on pest management can be helpful. Indeed, management of rodent population has to deal with VKA resistances as well as in human medicine. Many mechanisms of resistances are common in humans and rodents. Moreover, with the large use of vitamin K antagonist rodenticides, the resistant phenotype is over-represented in some rodent populations. Consequently, some resistance mechanisms with a low prevalence in the human population have a higher prevalence in rodent population; thus, they can be more studied in rodents.

After a rapid presentation of the basis of vitamin K and VKA mechanisms, this chapter presents the different methods to assess VKA resistance mechanisms. Then, an assessment of the VKA-resistant pathways described in humans and in different rodent species is performed.

2. Basis of vitamin K metabolism

2.1. Vitamin K

The name "vitamin K" gathers a great number of molecules. All vitamin K are based on a naphthoquinone core and are sorted in three classes, numbered from 1 to 3. The substitution on the carbon 3 of the core determinates the class of the vitamin K. Vitamin K1 is composed of only one molecule, the phyloquinone, where the carbon 3 is substituted by a phytyl moiety. It was the first vitamin K described in 1935 by Dam [4], and chemically identified and synthesized by Doisy [5]. Vitamin K2 regroups the menaquinones. The substitute is a chain of prenyl, and the number of prenyls is indicated in the name. For example, the menaquinone 4 side chain is composed of 4 prenyl. Finally, vitamin K3 or menadione is only constituted by the naphthoquinone core (**Figure 1**).

Phylloquinone is synthesized by plants [6]. Menaquinones are synthesized from phylloquinone. The number of prenyls of the synthesized menaquinone depends on the bacteria, fungi, and animals which synthesize them. Mammals are only able to synthesize menaquinone 4 with

the help of the *UBIAD1* gene [7, 8]. Consequently, other menaquinone forms come mainly from fermented alimentation or gut microbial synthesis [9, 10].

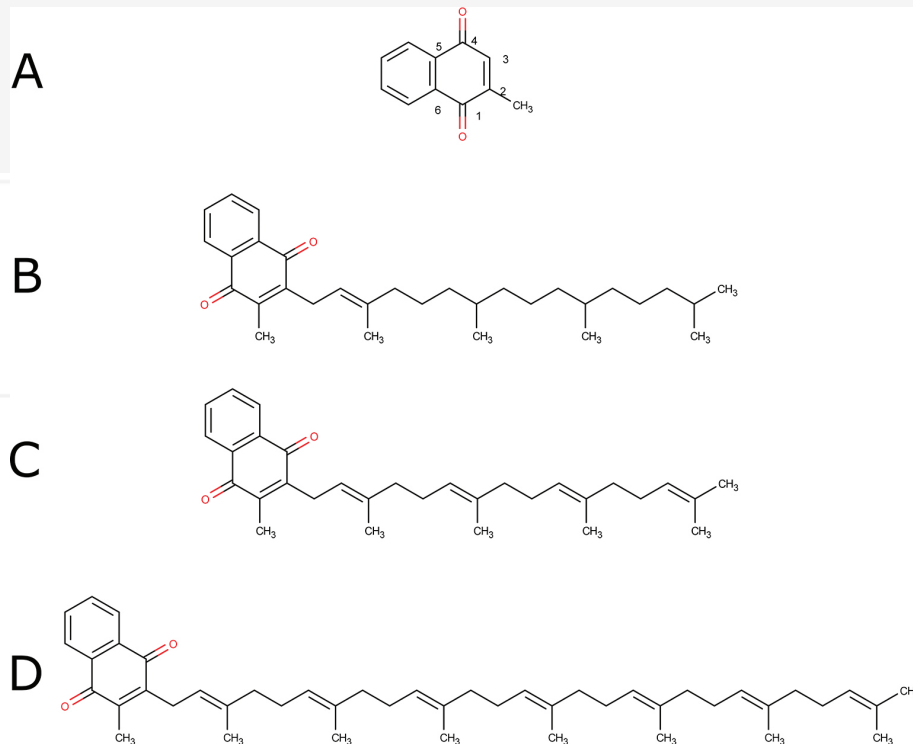


Figure 1. Examples of vitamin K: (A) menadione; (B) phyloquinone; (C) menaquinone 4; (D) menaquinone 7.

As many other fat-soluble vitamins, vitamin K's absorption increases with fatty intake [11]. The absorption of vitamin K occurs in the gut, nevertheless its mechanism has been unclear during many years [12]. Recently, a study suggested that the cholesterol transporter, the Niemann-Pick C1-like 1 protein, would be responsible for the vitamin K's absorption [13].

2.2. Vitamin K roles

The name of the vitamin K comes from the German word "koagulation." Indeed, when vitamin K was discovered, its deficiency involved bleeding [4]. Nevertheless, it is only in the 1970s that we began to understand the vitamin K's mechanism of action. Vitamin K is a cofactor of a post-transcriptional gamma-carboxylation which activates vitamin-K dependent proteins (VKDP) [14]. Four clotting factors of the coagulation cascade are the VKDPs, factors II, VII, IX, and X, which explain the bleeding issues observed in case of deficiency. Proteins C, S, and Z are also VKDPs involved in coagulation, but they have an antithrombotic effect. Nevertheless, the main effect of VKA is anticoagulation, even though the rapid decrease of these antithrombotic molecules can lead to a transient hypercoagulable state at the beginning or at the end of treatment with possible adverse events [15, 16].

The second great role of vitamin K is bone regulation with two VKDPs: osteocalcin and matrix GLA protein [17, 18]. This last protein is also involved in the protection against tissue calcification [19]. Vitamin K is involved in many other biological functions which are reviewed in Refs. [20, 21].

2.3. Vitamin K regeneration cycle

2.3.1. Vitamin K cycle

In spite of the low vitamin K level in food, vitamin K deficiency is rare [22]. Indeed, vitamin K is recycled by cells. The cycle is composed of two great steps: the use of vitamin K hydroquinone by the GGCX enzyme to activate VKDPs and the regeneration of the vitamin K hydroquinone from the epoxide form by VKORC1 (Figure 2).

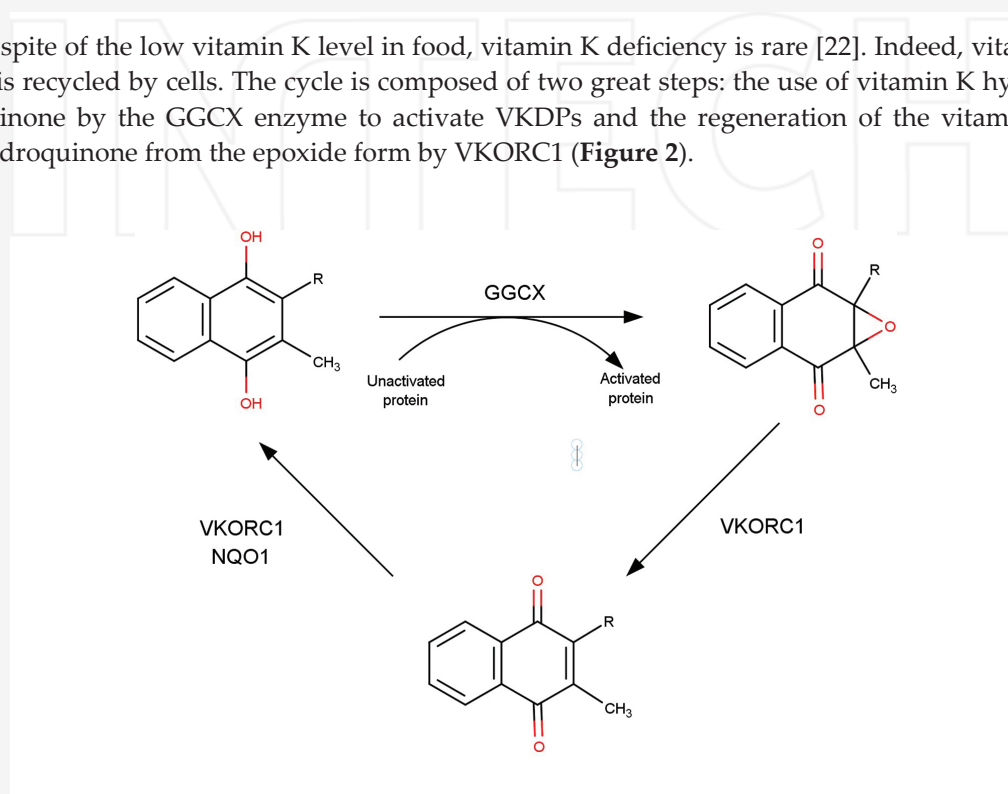


Figure 2. Vitamin K regeneration cycle.

In order to chelate calcium and to be active, the glutamate residues (Glu) of VKDPs have to be carboxylated to carboxyglutamic acid (Gla). This reaction is mediated by gamma-glutamyl carboxylase (GGCX). GGCX recognizes the VKDPs with the help of their propeptide [23, 24]. Then, GGCX removes the gamma-hydrogen of Glu residues and adds CO₂; the oxidation of vitamin K hydroquinone (KH₂) to vitamin K epoxide (K > 0) provides the required energy [25]. This reaction is performed in the endoplasmic reticulum [26].

2.3.2. VKORC1 structure

The *VKORC1* gene was located in 1969 on the chromosome 1 of rat by discovering a link between the coat color heritage and the resistance to warfarin [27]. Then it was located on the chromosome 7 of mice in 1976 [28] and on the human chromosome 16 in 2002 [29]. The first

characterization of the *VKORC1* activity was performed in 1974; nevertheless, at this time the responsible protein of this activity was unknown [30]. The identification of the *VKORC1* coding gene has only been performed in 2004 by two teams: one used short interference RNA functional screening [31] and the other used interspecies genetic linkage analysis [32]. Once identified and sequenced, the *VKORC1*'s structure and function study begun.

A complete review of the current knowledge on the *VKORC1* structure has been recently done [33]. *VKORC1* is a membrane protein of the endoplasmic reticulum. The activity of *VKORC1* seems to be due to CXXC patterns. *VKORC1* presents two CXXC patterns, with cysteines positioned at Cys43, Cys51, Cys132, and Cys135 in human *VKORC1*. These cysteines are widely conserved through species, which might indicate that they have a key role in *VKOR* activity [34]. Cys132 and Cys135 are located in a transmembrane domain. They seem to be essential for the *VKORC1* activity and might lead the nucleophilic attack supposed by a biochemical model of the *VKOR* reaction [34, 35]. Mutation of one of them to serine abolishes the enzymatic *VKOR* activity [36]. To reduce vitamin K, *VKORC1* needs to be itself reduced by a physiological partner. This partner and its mechanism of action are currently unknown. Schulman et al. proposed that the partner might reduce the loop cysteines (Cys43 and Cys51). Then the loop cysteines would transfer the reducing power to Cys132 and Cys135 of the active site [37]. However, other studies have reported that the mutation of one of these cysteines to serine has no consequence on the *VKORC1* activity [38–40]. Moreover, the conformation of *VKORC1* is still under debate between a topology with three transmembrane domains or four [41]. This last point is determining for the comprehension of the possible role of the loop cysteines. Indeed, they are either in the cytosol for the three transmembrane model or in the endoplasmic reticulum's lumen for the other model.

VKORC1 presents a strong homology between rodents and humans, allowing to compare the mutation between them. Mammals and bacterial *VKORs* are homologs, but their conformation and reaction seem different [42].

Mammals have another enzyme able to reduce the vitamin K epoxide, the *VKORC1L1*. It is mainly expressed in the extrahepatic tissues [43–45] and has a great similarity with *VKORC1* [46]. Its inhibition by VKA is lower than its homolog [43]. Nevertheless, due to its low hepatic expression, its influence on the anticoagulant resistance is negligible. However, it might explain that the other vitamin K functions are not significantly impacted by VKA treatments.

2.4. Vitamin K antagonists

First VKA has been discovered in 1941 and then isolated in spoiled sweet clover by Hueber and Link [47]. Sweet clover (*Melilotus officinalis*) contains coumarin. In a poorly preserved silage or hay, this coumarin can be changed to dicoumarol by *Penicillium* species commonly present in soil [48, 49]. The dicoumarol contained in spoiled forage causes a hemorrhagic condition called sweet clover disease. Some years after this discovery, Coumadin, a synthetic VKA also called warfarin, was synthesized [50]. This last molecule is still the most commonly prescribed anticoagulant. Concerning rodents, many new VKA have been developed to reply to resistance emergence.

Three VKA families are used: the 4-hydroxycoumarin derivatives, the 4-hydroxy-thiocoumarin derivatives, and the indane-1,3-dione derivatives (**Figure 3**). All derivatives are used against rodents. Conversely, in human medicine, only the 4-hydroxycoumarin derivatives (for instance, warfarin) and the indane-1,3-dione derivatives (for instance, fluindione) are used. Moreover, in order to deal with rodent resistances, second generation of 4-hydroxycoumarin derivatives and of 4-hydroxy-thiocoumarin derivatives have been designed with complex radicals (**Figure 3C and D**).

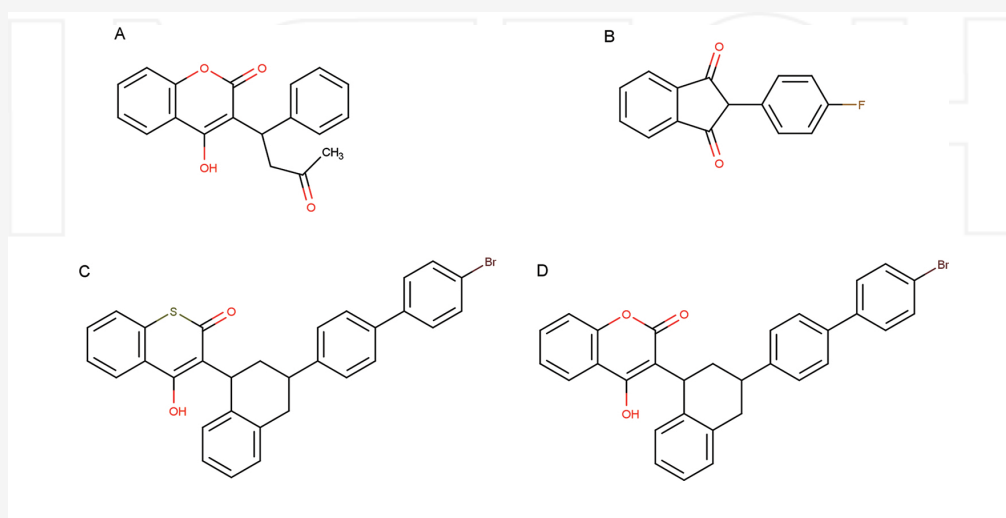


Figure 3. Examples of some VKA: (A) warfarin; (B) fluindione; (C) difethialone; (D) brodifacoum.

2.4.1. Mechanism of VKA action

Vitamin K antagonists stop the vitamin K recycling by performing noncompetitive inhibition of the VKORC1 enzyme [51, 52]. Nevertheless, the binding of VKA with VKORC1 enzyme is still a gray area. The reversibility of the binding is presently unknown [52–54], as well as the binding site. First, the binding site has been located at the level of the TYA motif (residues 138–140 in human VKORC1) close to the CXXC active site. Indeed, the mutation of the 139 tyrosine of this motif is associated with warfarin resistance in humans and rodents [55, 56], and by analogy the dicoumarol's binding site on NQO1 is also a TYA motif [57]. However, these mutations are moderately susceptible to second generation VKAs in rats [55, 58], which can suggest that other amino acids might be involved in this binding. Recently, Czogalla et al. have proposed a model involving three binding interfaces between warfarin and human VKORC1 [59].

2.4.2. VKA elimination pathway

The VKA treatments are established on the long term. Consequently, their elimination is a key factor which determines their liver concentration and finally their efficiency. The elimination pathway seems to depend on the molecule and on its enantiomeric form. Indeed, enantiomers of warfarin are eliminated differently. The (S)-enantiomer is metabolized exclusively by the

hepatic cytochrome P450 isoform 2C9 (CYP2C9), while (R)-enantiomer is metabolized by isoforms CYP1A2, CYP2C19, CYP3A, and hepatic ketoreductase [60, 61]. Although the (R)-enantiomer has a longer half-life, it is less efficient, and the modulation of its elimination has no significant impact on the coagulation [62–64]. However, the activity of CYP2C9 is critical in the determination of the warfarin dose. Indeed, CYP2C9 activity is influenced by many drug interactions [65–68], and polymorphism of CYP2C9 can also modulate the sensitivity to warfarin.

3. Assess the resistances

Since the discovery of rats that are resistant to warfarin in 1960 by Boyle [69], the assessment and the study of resistance mechanisms have become a key issue for the rodent population management and in human medicine. Many methods have been developed to study these resistance mechanisms. Their purpose is to isolate the possible origins of the resistance in a standardized model and to evaluate if the induced resistance factor is of the same order as that one observed *in vivo*. The resistance factor is the factor by which the dose or concentration of VKA required for a susceptible population must be multiplied to achieve the same effect within a resistant population. We present below the main methods, and for each we pinpoint its advantages and limits.

3.1. *In vivo* animal studies

In vivo studies were commonly used in rodent VKA research in order to evaluate the efficiency of an anticoagulant or the consequences of *VKORC1* mutations. The first resistance case in rodents was assessed by Boyle. He fed suspected resistant with containing VKA food and control rats during 5 days. Then, the death rate between the two groups was compared [69]. This kind of survival challenge is efficient to qualify a resistance state and remains the gold standard to test commercial rodenticides. Nevertheless, these tests are long and have many shortcomings concerning animal welfare and the repeatability. Moreover, it is difficult with this test to compare two VKAs and to assess the dose–response law.

Later, new methods based on blood clotting test have been developed [70–72]. They were standardized by the Rodenticide Resistance Action Committee in order to obtain a discriminating dose for each VKA [73]. These doses of anticoagulants, called effective doses 50 (ED50), were determined to quantify the susceptibility of rats to anticoagulants. ED50 is the dose leading to 50% of animals tested with a fivefold increase of the international normalized ratio 24 h after the administration of anticoagulants. This technique is more precise and rapid. Nevertheless, this method gives few information on the mechanism of resistance.

The origins of resistances in wild strain can be multifactorial. For example, they can involve *VKORC1*-linked resistance and cytochrome-linked resistance. It is possible to refine *in vivo* methods by introgressing the studied wild mutation in laboratory strain. With this method, it is possible to isolate the resistant mutation in a susceptible genetic background [55].

If the animal *in vivo* studies are still the gold standard to the understanding of the complex effect of VKAs and of resistant mutations, they are expensive and laborious in the preparation and care of the strain and in the realization of experiments. Moreover, it may be interesting to study more precisely each mechanism by *in vitro* methods [74].

3.2. *In vitro* enzymatic study

The VKOR activity can be reproduced *in vitro*. These methods are based on the kinetics of vitamin K quinone production by an enzyme system from vitamin K > O. To perform this reaction, the substrate (vitamin K > O) is beforehand synthesized according to the method described by Tishler et al. [75]. The evaluated enzyme can come from animal tissues or heterologous expression culture (cell, bacteria, yeast, etc.). Finally, dithiothreitol (DTT) is commonly used to transfer the reduction power to the enzyme. The studied VKOR-specific activity and the Michaelis constant are calculated [76]. It is also possible to add VKA to study its inhibition efficiency. In this case, an inhibition constant can be obtained for each couple of enzymatic system and VKA. Methods to perform these experiments are described in Refs. [43, 58, 76].

This simple experiment pattern allows us to assess the efficiency of large origins of enzymatic system. As the VKOR proteins are located in the membrane of the endoplasmic reticulum, they are present in the microsomal fraction of tissues. The commonly used microsomal preparation methods are described in Ref. [76]. Microsomes are prepared from other cell components by differential centrifugation. Microsomal enzymatic activity evaluates the enzymatic efficiency of whole microsomal VKOR activity and not only of the VKORC1 enzyme activity. Indeed, Hammed et al. have pinpointed that a modeling with two enzymes (VKORC1L1 and VKORC1) is necessary to explain the inhibition of the testis microsomal activity by warfarin [43]. Moreover, as for *in vivo* experiment, the strains with interesting mutations have to be selected and preserved.

To isolate the activity of one enzyme or to perform enzymatic activity with human-like enzyme or mutated enzyme, it is possible to perform a heterologous expression in yeast (*Pichia pastoris*) of VKOR enzymes and then extract the yeast's microsomes. The method is described in Ref. [58]. Yeast has neither endogenous VKORC1 nor VKORC1L1. Thus, this method allows the study of the influence of VKORC1 and/or VKORC1L1 mutation on their respective specific activities and VKA resistance without interaction. The yeast model has been validated for some mutations as Y139F for which the resistance rate of rat's liver microsomes is the same as that of yeast microsomes. Moreover, it is also possible to test some hypothetic mutations. The yeast expression method is more focused on the enzyme activity than tissue microsomes and opens many experimental opportunities. However, the studied activity is still the microsome activity. With the previous methods, enzymes are in their microsomal environment. In order to study more precisely VKORC1-linked resistances, it would be interesting to purify VKORC1.

The purification of VKORC1 was for many years a big business [77, 78]. Indeed, this enzyme loses its activity during the solubilization. It was only in 2006 than Chu et al. have purified the recombinant human VKORC1 produced from a baculovirus in insect cells which was still active after purification [79]. In this experiment, microsomes were washed from non-VKORC1

proteins. The artificial membrane inclusion of VKORC1 after heterologous expression in *Escherichia coli*, its extraction, and its purification have recently been described [80]. Briefly, the *hVKORC1* gene has been expressed in *E. coli* BL21(DE3) in a KSI-hVKORC1-His₆ construct [81]. The ketosteroid isomerase (KSI) part promotes the expression of the protein in insoluble inclusion bodies. KSI part was linked with hVKORC1 by a formic acid cleavage site. Finally, the construct was hexahistidine-tagged. After the expression, proteins were preserved under denaturing conditions and purified with a HisTrap Ni-NTA column. Then the KSI part is removed with formic acid. The refolding of hVKORC1 was performed with pulsed renaturation and "artificial chaperone." Then the proteins were incorporated in a lipid layer to form a liposome. Jaeneke reported that the obtained hVKORC1 liposome had only 10% of the specific activity of the enzyme obtained with HEK293 cell [80]. These new purification methods are not currently used in the resistance study and need improvement. Nevertheless, they open the way for a better understanding of the VKORC1 topology and the influence of mutations on VKOR activity.

The enzymology studies are the basis of our current understanding of VKORC1 mutations and interaction with VKA. It is currently the technique with the best reproducibility and more reliability. Nevertheless, some issues are still pending with these methods. The major issue comes from the DTT which gives the reducing power to VKORC1 in experiments. DTT is a powerful reductor which might bypass some dysfunctional mechanisms of mutated enzyme by directly activating the active site [37]. Moreover, high concentration of DTT (>1 mM) add a background level. To limit it, Krettler et al. proposed the use of tris(3-hydroxypropyl)phosphine instead of DTT [82]. Nevertheless, there is not enough knowledge currently on this new methods and its VKORC1 interaction to change the method. Moreover, tris(3-hydroxypropyl)phosphine is less efficient in VKORC1 enzyme reduction than DTT at usual concentration.

Another issue might be that these methods study only the impact of VKA and resistant VKORC1 enzyme on the vitamin K regeneration cycle and not on the vitamin K-dependent protein gamma carboxylation. The required lipid environment and detergents are different for the activity between GGCX and VKORC1. Thus, it is currently difficult to study the interaction between both enzymes in an *in vitro* enzymatic assay [83]. These discrepancies imply that enzymology results have to be analyzed in view of the *in vivo* results.

3.3. *In vitro* cell study

In order to evaluate the complete reaction from vitamin K reduction to gamma-carboxylation and to solve some enzymatic study issues, the functional study of the vitamin K cycle in mammalian cells has been recently developed by Tie et al. [83], which was then adapted by other teams [84, 85]. In these studies, the production of a complete carboxylated protein by cell culture was assessed. The cells were HEK 293 cells transfected with the studied *VKORC1* gene and a VKDP. Then, cells were cultured in an environment containing vitamin K epoxide and the studied VKA. All methods have followed the gamma-carboxylation of factor IX gla domain by its monoclonal antibodies recognition [86] or by measuring its activity (only for complete factor IX). Three different vitamin K-dependent proteins have been used: the simple factor IX protein (FIX) [59, 84, 87]; the chimeric protein FactIXGLA, which was protein C with its gla

domain replaced by the factor IX gla domain [40, 83]; the chimeric protein FIXgla-PRGP2, as previously the gla domain of the proline-rich gla protein was replaced by FIXgla domain [85]. Concerning the method using FIX [84, 87], the gamma-carboxylation was assessed by measuring the clotting activity of the concentrated culture medium mixed with factor IX-depleted plasma. For the FIXgla-PC method [40, 83], quantification is performed on the culture medium by an enzyme-linked immunosorbent assay (ELISA) with anti-FIXgla domain antibodies as coating antibodies. Although Jamil also used a chimeric protein transformed with a FIXgla domain for its quantification, in this case, it was the membrane protein, the proline-rich Gla protein 2, which was used. With this modification, carboxylated protein gla domains were presented at the surface of the cell, then recognized by monoclonal antibody conjugated to allophycocyanin and quantified by flow cytometer [85].

This new method is not standardized yet, and there is still a gray area in the interpretation of the obtained results. Indeed, some discrepancies exist between enzymatic assay and cell assay results [84, 88] and even between cell assay results [40, 59]. The origin of these discrepancies might be the interference of the cell's endogenous enzymes, in particular VKORC1L1, which is naturally more resistant than the wild VKORC1 enzyme [43]. In this way, standard cell assays have showed that all tested mutations were resistant [59, 84]. Tie et al. have dealt with this by knocking out endogenous *VKORC1* and *VKORC1L1* genes, using the transcription activator-like effector nuclease technology [40]. Thus, Tie et al. have confirmed the interaction of the endogenous enzyme in the cell assay, and they have showed that some mutations previously classed as resistant by Czogalla et al. [59] are in fact not resistant [40].

The second issue for the interpretation of cell assay is the lack of knowledge and control on it. This kind of assay is more complex than simple enzymatic activity; it involves many mechanisms that are currently uncontrolled or unknown: the level of the recombinant VKORC1 enzymes and the recombinant VKDP; the quantity and the efficiency of the physiological partner of VKOR enzymes, which are still unknown; the level of the endogenous GGCX protein; finally, the possible other mechanisms currently undescribed. All these elements determine if the studied enzymes are not bypassed and if the vitamin K reduction is the limiting reaction. These two conditions have to be fulfilled to validate completely the cell assay. However, some studies have pinpointed that the limiting reaction might be the VKORC1 enzyme reduction by its physiological partner and not the vitamin K reduction [89–91].

Although cell assays are recent and still have a gray area in the interpretation of their results, there is no doubt that they will become a key element of the study of VKA resistance mechanisms.

3.4. Pharmacogenomics study

The pharmacogenomics studies are the main source of *in vivo* information on the VKA resistance in humans. These studies assess the correlation between some genetic parameters and the warfarin dose, which allows to keep the patient's INR in the target zone. Among these parameters, the VKORC1 and the CYP2C9 polymorphisms are keys. But many other parameters, which might influence the VKA susceptibility, are unknown and cannot be evaluated by these studies. Pharmacogenomics studies assess the epidemiology of the mutations linked

to resistance and can help in the determination of the treatment's starting dose by taking in account of patient's genome. But, their contribution to the study of the resistance pathway is limited to the confirmation or not of the results obtained with other methods.

4. Resistance pathways

4.1. VKOR-linked resistances

4.1.1. VKORC1 human genotypes

The pharmacogenomic studies and the reports of warfarin treatment failures have pinpointed two kinds of polymorphisms linked to VKORC1 which influence the warfarin dose requirements. The first is linked to the polymorphism in the noncoding region and the second is linked to missense mutations.

The polymorphism in the noncoding region is the origin of the majority of VKA dose variations [92–95]. Indeed, the noncoding region influences the transcription level of VKORC1 [94]. Nevertheless, these variations are minor, few milligrams of increase or decrease. So, it is not really resistance to VKA, but rather a slight modulation of VKA sensibility.

Conversely, the VKORC1 coding mutations are rare, and some involve a real resistance to VKA. They are characterized by a dose to stabilize the anticoagulation, which is higher than the high-dose threshold defined by Watzka: phenprocoumon 3.0 mg/day, acenocoumarol 3.5 mg/day, warfarin (W) 7.1 mg/day, and fluindione 19.8 mg/day, for mean age patients [56]. Please note that the definition of warfarin resistance is not well-defined. Currently, there are more than 27 mutations that have been described in patients with high requirements of VKA dose. They are summarized in **Table 1**.

Mutation	Number of patients	Molecule	Dose (mg /day)	Stable anticoagulation	Ref.	Confirmation of resistance by <i>in vitro</i> method [†]
A26P	1	W	20	No	[106]	Yes [88]
		F	100	No		
A26T	1	W	6	No	[56]	No [88]
L27V	1	W	7	Yes	[107]	No [88]
		F	60	No		
H28Q	1	P	10	yes	[56]	No [88]
V29L	2	W	14	?	[32, 56]	
		P	26	Yes		
A34P	1	W	27	Yes	[108]	
D36Y	31	W	16.1 [3–36] [*]	~	[56, 96, 105, 106, 109]	No [88]
		F	45	No		

Mutation	Number of patients	Molecule	Dose (mg /day)	Stable anticoagulation	Ref.	Confirmation of resistance by <i>in vitro</i> method [†]
		A	7	No	–[112]	
		P	7 [6.9–7.1]	Yes		
D36G	1	W	20	Yes	[56]	No [88]
A41S	1	W	16	?	[94]	No [88]
V45A	1	W	45	No	[32]	No [88]
S52L	1	P	9	No	[113]	Yes [40]
S52W	1	P	10	Yes	[56]	No [40]
V54L	2	W	21 [10–32]	~	[105, 106]	Yes [88]
		F	60	No		
		A	8	No		
S56F	1	P	15	No	[56]	No [88]
R58G	1	W	34	?	[32]	No [88]
W59R	3	A	9.3 [8–12]	No	[113, 114]	Yes [40]
		P	9	No		
W59C	1	P	11	No	[56]	No [88]
W59L	1	P	15	No	[56]	Yes [40]
V66M	15	W	31.5 [20–42]**	Yes	[56, 103, 105,	No [40]
		P	9.5 [9–10]	~	106, 108]	
V66G	1	P	8	Yes	[56]	No [40]
H68Y	1	–	–	–	[115]	Yes [88]
G71A	1	P	6	No	[56]	No [40]
N77S	1	P	9	No	[56]	Yes [40]
N77Y	1	W	25	Yes	[56]	No [40]
I123N	1	P	21	No	[56]	Yes [88]
L128R	16	W	44	No	[32, 105,	Yes [40]
		F	80	No	106, 116]	
		A	13	No		
		P	30	No		
Y139H	1	P	9	No	[56]	Yes [88]

Adapted and completed with permission from Ref. [88].

* A case report, which includes a 2-year-old girl, is not included [117].

** A report of two cases was not used due to its imprecision on the doses [118].

† Results of the Oldenburg's team are not reported [59, 84].

? no data are present on the stabilization of the anticoagulation in study.

~ only some patients have a stabilization of their anticoagulation.

Table 1. Genetic variations in the coding sequence of human VKORC1 in patients requiring high dose of a vitamin K antagonist.

Conversely, another mutation has been described by Rost in two Libyan families, the R98W mutation. Homozygous patients with this mutation have a combined deficiency of vitamin-K-dependent clotting factor type 2 which causes bleeding [32]. This deficiency can be treated by a daily dose of vitamin K. Rost et al. have shown in enzymatic assays that R98W-mutated VKORC1, which was expressed in HEK cell, has a VKOR activity diminished of 90% [32].

Some mutations have a higher prevalence in some populations. Thus, the D36Y mutation is relatively well represented in some African populations [96]. The Ethiopian population and the Ashkenazi Jews population have a D36Y allele frequency of respectively 15% and 4% [97–99], while this mutation is absent in South African or in Chinese populations [100, 101]. Concerning the V66M mutation, it has been described in African and African-descent populations [102–104]. Finally, the L128R mutation has been described in different families [32, 105].

Nevertheless, the other reported mutations have been described only one time and sometimes on patient with unstabilized anticoagulation. These elements reduce the possibilities to determine a resistance factor for each. Moreover, the mutations have been often described as fortuitous events of pharmacogenomics studies [94]. Finally, the interactions with other mutations on cytochromes, GGCX, or noncoding part of VKORC1 genes can also modulate the warfarin dose and enhance or reduce the resistance. Thus, the assessment of these mutations only from case report entails a lot of bias.

In order to deal with the described bias, the *in vitro* results can be useful. The *in vitro* results from enzymology [88] and cell assays [40] have been reported in **Table 1**. Nevertheless, all mutations do not seem to be associated with resistance in *in vitro* assays. Only Oldenburg have found that all the VKORC1 coding mutations were resistant with a cell method assay; his results are not reported in the table [59, 84]. This discrepancy between assays might be due to the VKORC1L1 gene as afore-explained in the description of the cell assays. Moreover, enzymology assay pinpoints that some mutations drastically diminish the VKOR activity of the enzyme [88], whereas cell assays do not [40]. The study of the paralog mutations in rodents may help to understand the real impact of these mutations on the resistance.

4.1.2. VKORC1 rodent genotypes

Resistances have evolved differently in rodents according to their species. Indeed, the anticoagulant pressure is exerted differently on each. These differences depend on the behavior of the rodents, and more particularly the feeding behavior. Thus, some rodents feed preferentially on one food source (rat), while others feed on many sources (house mice). Thus, rats eat a lot of poisoned grains, whereas mice eat few poisoned baits and dilute them with other food sources.

Resistances have been largely described in house mice (*Mus musculus*) and in Norway rat (*Rattus norvegicus*). VKORC1-linked resistances are due to missense mutations. They have been detected whereupon rodenticide treatment fails. Due to the purpose of the VKA rodenticides, their doses used for management of rodents are important. Consequently, only huge resistances have been pinpointed and then isolated by introgressing the concerned genes in

laboratory strains. Thus, the noncoding mutations, which are involved in the modulation of VKA dose in humans, have not been described in rodents.

Concerning the Norway rat, it has been the first target of the VKA rodenticides and the first to develop resistances [69]. The first description occurred in Scotland, and rapidly the resistance spread all over Great Britain [119, 120]. Denmark has been the second country with warfarin-resistant rats [121]. Entrapments have pinpointed that 24.2% of Denmark's rats were resistant in the 1960s [122]. Since then, resistances have been brought to light from all around the world [123, 124].

Currently, 25 mutations have been described in Norway rats [125]. Nevertheless, only few mutations are widely present and linked with important resistances. The five main mutations in Europe are L120Q, L128Q, Y139C, Y139F, and Y139S [126]. These mutations have different frequencies depending on the geographical areas. Thus, Y139C is the main mutation in Germany and Denmark [126, 127], and Y139F in France [128]. Concerning Great Britain, important discrepancies on the frequencies of mutations have been pinpointed between counties [126, 129, 130]. The frequencies of rat mutations are disproportionate comparatively to human coding mutation frequency. Thus, Y139F mutation is detected in 21% of the French rats [128], but less than 1% of the world human population carries one coding mutation. Moreover, in some areas, the prevalence of the resistant rats is of 100% [131].

Concerning the house mice, their mutations have been described in 1961 in many countries [132]. Currently, more than 10 *VKORC1* mutations have been described [133]. Moreover, 80% of the trapped mice carried at least one *VKORC1* mutation in a study in Germany, Switzerland, and Azores [133]. Finally, some population of house mice have multiple mutations, Arg12Trp/Ala26Ser/Ala48Thr/Arg61Leu, which involve VKA resistance [133]. This mutation may come from an interspecific hybridization with *Mus spretus*. But, only little information is available on *Mus spretus*'s resistance to VKA [133].

4.1.3. Overall approach of human and rodent mutations

The presence of *VKORC1* mutations in humans with very low frequency (<1%) and the fast emergence of resistance in rodents indicate that the mutations were present in rodents well before the use of VKA as rodenticide. The large use of these molecules has selected the resistant mutations of *VKORC1* and increased their prevalence in rodent populations. Moreover, the use of excessive dose of VKA has selected the mutations leading to the most severe resistance, especially in *Rattus norvegicus* populations. Indeed, in Europe, while more than 20 mutations were published, only three mutations seem now widely spread. As the roof rat population is less exposed to VKA than brown rats, the prevalence of their *VKORC1* mutations might be close to human prevalence. The roof rat resistance linked to *VKORC1* mutation has not been widely explored. Recently, Goulois has characterized the Y25F mutation in roof rats [134].

Five amino acid positions of the *VKORC1* enzyme carry a described mutation in both humans and rodents. They are the positions A26, R58, W59, L128, and Y139. Nevertheless, only the mutations A26T, R58G, and W59R are identical. These three mutations are not common in humans, and the reported cases did not have a stable anticoagulation. The A26T mutation does

not seem to enhance the resistance in the rat [125]. These results were obtained with rat *VKORC1* cell expression, and they agree with the results obtained with human *VKORC1* [88]. Moreover, the frequency of this mutation is low in rats which might suggest that the resistance linked to this mutation is insufficient to be selected by the VKA rodenticide pressure. Concerning the W59R mutation, it diminishes considerably the VKOR activity of the enzyme in rats conversely to in humans [40, 125]. Finally, R58G mutation is described both in humans and mice. This mutation does not seem to involve resistance in mice, and as the W59R mutation in rat, it diminishes the VKOR activity [125]. Thus, the results obtained with *VKORC1* enzyme of rodents are in accordance with the *in vitro* results with human *VKORC1* gene or *VKORC1* enzyme.

The mutations at positions L128 and Y139 are the major mutations of the Norway rat. Moreover, the studies on the Y139F mutations have shown that the resistance factor of this mutation is conserved between *in vivo* and *in vitro* assays [55, 58]. In rats, these mutations involve resistance factors against warfarin, which are of 8 for the L128Q mutation and greater than 200 for the Y139 mutations [58]. Moreover, in rats, L128Q does not seem to diminish the VKOR activity while Y139F does it. Conversely, in humans, the L128R mutation conducts to a reduction of more than 90% of the VKOR activity in enzymatic assay, with the *VKORC1* enzyme produced in yeasts or HEK cells [32, 88]. But, this mutation does not modify the level of gamma-carboxylation in cell assay [40, 84]. Possible origins of these discrepancies are argued in Section 3.3. Consequently, it is currently difficult to conclude on the possible resistance linked with the L128R mutation in humans.

As aforementioned, some mutations decrease the efficiency of the VKOR activity. Matagrín has studied the origins of this loss of efficiency for the Y139 mutation [135]. He showed that Y139 mutations involve a diminution of the VKOR activity and the creation of inactive vitamin K metabolites (3-OH vitamin K) which are eliminated. This induces an increase of the food requirements of vitamin K in rodents carrying these mutations [136, 137]. In humans, the influence of coding mutations on nutritional requirement of vitamin K has not been well studied [22]. However, by analogy with rodent mutations, human mutations might be involved in cardiovascular diseases without VKA treatment [138]. Thus, the rodents carrying these mutations might be use to model and to better understand the possible consequences of these mutations on the human health.

4.2. Cytochrome-linked resistances

Cytochromes are essential elements in the elimination of xenobiotics. Thus, it would be expected that their polymorphism might result in an origin of resistances. But in human medicine, the cytochrome polymorphism is associated with an increase in the patient's VKA sensibility [93, 97]. Moreover, the polymorphism of CYP2C9 is a key element in the prediction of the VKA treatment dose [97]. Thus, no VKA resistance linked to cytochrome has been described in human medicine.

In rodents, expression profiles of cytochromes are different between sensitive and resistant rats carrying a Y139C mutation on *VKORC1* [139]. Nevertheless, it is difficult to identify the part of the resistance due to *VKORC1* mutation and the one due to the expression profiles of

cytochromes. Nevertheless, one example of warfarin resistance linked to cytochrome has been described without association with VKORC1 mutation in roof rats from Tokyo [140, 141]. This rat population overexpresses the cytochrome 3A2. Thus, the concentration of blood warfarin 1 h after the warfarin administration is eightfold lower in resistant rats. However, it is currently the only reported case of cytochrome-linked resistance.

5. Conclusion

The assessment and the comprehension of the resistance mechanisms are essential in the safety and the efficiency of VKA treatments and in the rodent population management. However, the complexity of the mechanisms and the interactions between them prevent the use of only one studying method. The understanding of the VKA actions and their interaction with individual variability can only be achieved by a multiscale approach which combines humans, rodents, and *in vitro* and *in vivo* knowledge.

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INTECH

5.2 Risques de l'emploi des rodenticides

5.2.1 Études de cas d'exposition et d'intoxication

5.2.1.1 Chapitre de livre :

**Poisoning by Anticoagulant Rodenticides in Humans and Animals :
Causes and Consequences**

Poisoning by Anticoagulant Rodenticides in Humans and Animals: Causes and Consequences

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Additional information is available at the end of the chapter

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Abstract

Anticoagulant rodenticides (ARs) are a keystone of the management of rodent populations in the world. The widespread use of these molecules raises questions on exposure and intoxication risks, which define the safety of these products. Exposures and intoxications can affect humans, domestic animals and wildlife. Consequences are different for each group, from the simple issue of intoxication in humans to public health concern if farm animals are exposed. After a rapid presentation of the mechanism of action and the use of anticoagulant rodenticides, this chapter assesses the prominence of poisoning by anticoagulant rodenticides in humans, domestic animals and wildlife.

Keywords: anticoagulant, rodent, poisoning

1. Introduction

The management of rodents around the world is a great concern, in many aspects. Rodents are ubiquitous and opportunistic animals, some such as the brown rats (*Rattus norvegicus*) and the black rats (*Rattus rattus*) are present at all continents except Antarctic [1]. These rodent populations are an ecological and an economic issue in the islands where they are not indigenous [2]. Agriculture is also affected by rodents; in France, for instance, water voles (*Arvicola terrestris*) devastate some lands [3]. Finally, rodents are a major nuisance in cities where their proximity to and interactions with human populations and infrastructure can cause impairments and become a hazard for the public health as they are reservoirs of many diseases. In

China, rodents destroyed the rice stock that would be sufficient to feed 200 millions of people [4] and the estimation of the cost induced by rodents' damage is about 19 billions of dollars [5]. Many similar cases have been recorded around the world [1, 6].

To deal with these concerns, rodent populations have to be controlled. One of the most used management methods is the chemical method based on the use of anticoagulant rodenticides (ARs). Anticoagulant rodenticides (ARs) have been used since the 1940s to control rodent populations. Warfarin was the first molecule used. But after its use for more than one decade, resistant strains of rodents to ARs have emerged [7]. To deal with resistance, this first generation of ARs has been supplemented by a second generation. ARs of the second generation are frequently named 'superwarfarins' or long-acting anticoagulant rodenticides. Indeed, these molecules are more potent than the first generation due to their longer half-life, which implies longer tissue-persistence and better efficacy.

Indeed, the consequence of the widespread use of ARs and more specifically the second generation of ARs, that are more efficient and more persistent, has been an increase of the exposure risks and the intoxication risks for non-target species such as pets, wildlife as well as humans. Nevertheless, anticoagulant rodenticides are renowned as a safe method to manage rodent populations. This safety is due to their mechanism of action as well as on the implementation of good practices in their use and by the respect of related regulations. Beyond this renown, it is important to monitor the impact of using ARs regarding the risk of untargeted species poisoning and to discuss on the remaining grey area in our knowledge on anticoagulant rodenticides.

Hence, after a rapid presentation of the mechanism of action and the use of anticoagulant rodenticides, this chapter assesses the importance of the exposure and the intoxication by anticoagulant rodenticides.

2. Anticoagulant rodenticides

The current anticoagulant rodenticide molecules belong to the family of vitamin K antagonist (VKA) molecules. The effects of VKAs have been observed in the 'sweet clover' poisoning of bovines, which results in a haemorrhagic disease and often the death of the animal [8–10]. Clover (*Melilotus officinalis*), used as fodder, contains coumarin a precursor of dicoumarol which is a VKA (**Figure 1E**). If clover fodders are not stored under proper conditions, fermentations may occur. These fermentations change clover coumarin in dicoumarol. Thus, clover fodder become toxic [10]. Dicoumarol was synthesised by Paul and Stahmann in 1941, opening the opportunity of use VKA as medicine and rodenticide [11]. Then other VKA molecules have been synthesised, including the famous warfarin (**Figure 1D**) and all other products that are more potent than dicoumarol [12].

The main molecules used in the rodent population management are presented in **Figure 1**. VKA molecules are derived from a coumarin (**Figure 1A**), thiocoumarin (**Figure 1B**) or 1,3-indandione (**Figure 1C**) core. The distinction of the second generation of AR from the first generation is the radical. In second generation, radical includes three benzene structures,

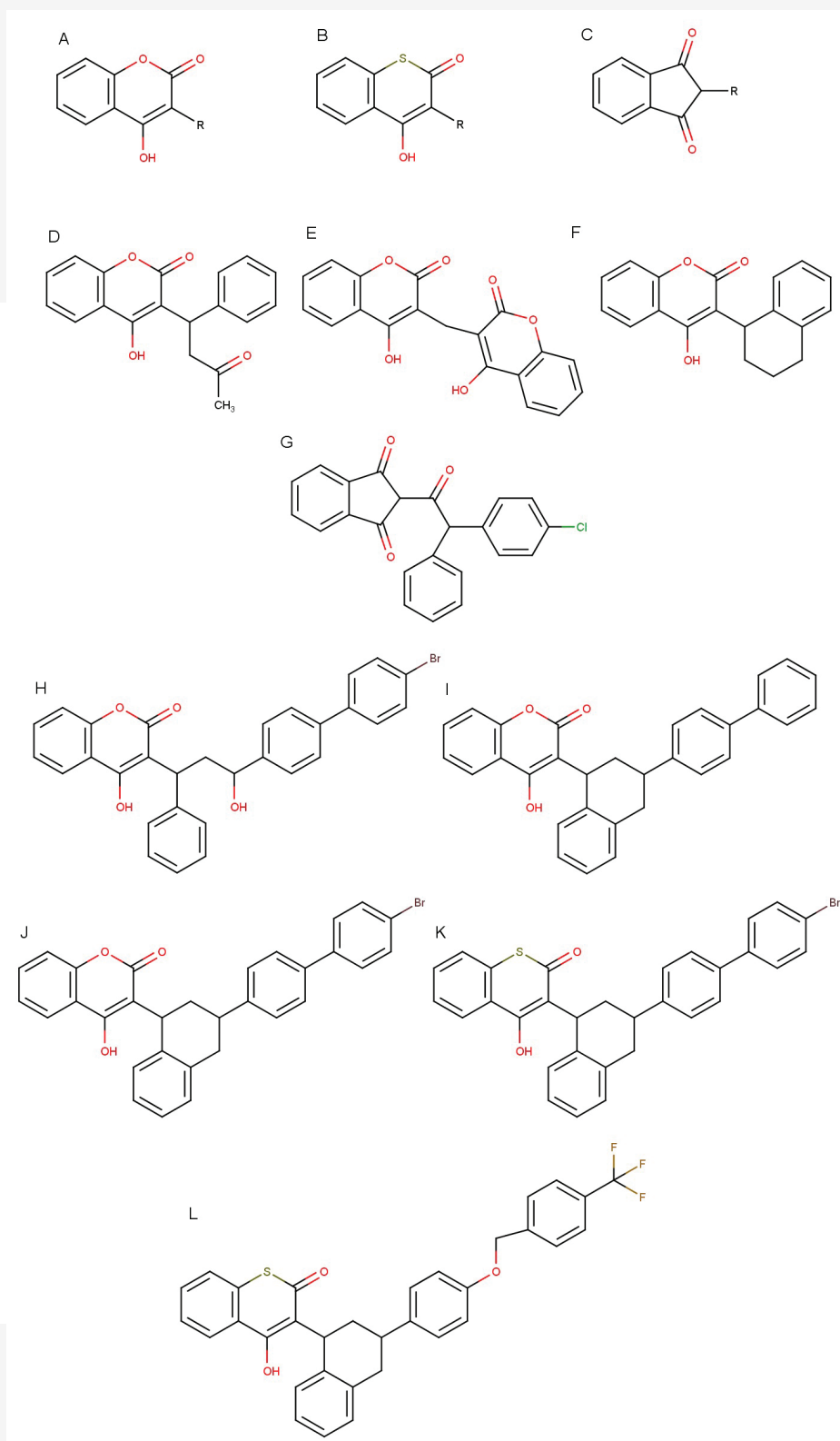


Figure 1. Chemical structure of: (A) coumarin core; (B) thiocoumarin core; (C) 1,3-indandione core; (D) warfarin; (E) dicoumarol; (F) coumatetralyl; (G) chlorophacinone; (H) bromadiolone; (I) difenacoum; (J) brodifacoum; (K) difethialone; and (L) flocoumafen.

which increase the fat solubility of the molecules and influence their pharmacokinetic properties. In order to understand the interest of VKAs and the key issue of their safety, it is important to present their mechanism of action and their pharmacokinetics.

2.1. Mechanism of action

Vitamin K antagonists (VKA) are non-competitive inhibitors of the vitamin K epoxide reductase enzyme (VKORC1) [13, 14]. This membrane enzyme of endoplasmic reticulum is responsible for the recycling of vitamin K. Vitamin K is a cofactor essential to many biotransformations of proteins and more specifically to obtain an active form of some clotting factors, the factors II, VII, IX and X. These factors, called vitamin K-dependent clotting factors, have to go through a post-translational gamma-carboxylation of their glutamate residues into gamma-carboxyglutamic acid to be able to chelate calcium and have their physiological activity [15, 16]. This reaction is done by gamma-glutamyl carboxylase (GGCX), which is another membrane enzyme of endoplasmic reticulum, and needs the oxidation of vitamin K hydroquinone to vitamin K epoxide to provide the required reducing power [17, 18]. Then VKORC1 recycles vitamin K epoxides to vitamin K hydroquinones (**Figure 2**) [19].

The amount of vitamin K provided by the majorities of food is not sufficient to offset the complete arrest of vitamin K cycle. Consequently, when VKORC1 is inhibited by VKA, a sufficient amount of vitamin K hydroquinone cannot be recycled from vitamin K epoxides to ensure the gamma-carboxylation of vitamin K-dependent proteins, and more especially the vitamin K-dependent clotting factors. Consequently, the blood concentrations of active vitamin K-dependent clotting factors decrease and lead to an increase of clotting times then, with time, to the death by haemorrhages.

2.2. Pharmacokinetics properties

Vitamin K antagonists are reputed to be highly and rapidly absorbed after *per os* administration. Then they are mainly stocked in liver. Their liver storage and their elimination are

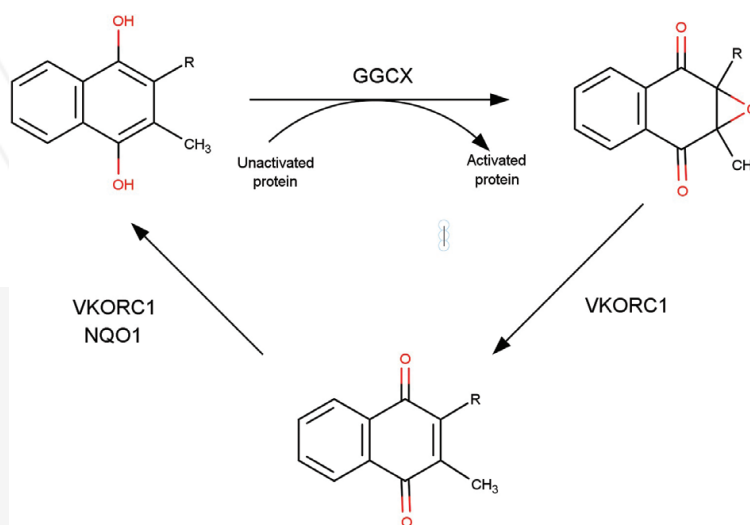


Figure 2. Vitamin K cycle.

key factors, which determine a part of their efficiency and their persistence. The elimination pathway depends on the molecule and on its enantiomeric form [20]. For example, enantiomers of warfarin are eliminated differently. The (S)-enantiomer is metabolised exclusively by the hepatic cytochrome P450 isoform 2C9 (CYP2C9) while (R)-enantiomer is metabolised by isoforms CYP1A2, CYP2C19, CYP3A and hepatic ketoreductase [21, 22]. Although the (R)-enantiomer has a longer half-life, it is less efficient and the modulation of its elimination does not have a significant impact on the coagulation [23–25]. There is a great discrepancy between tissue persistence of first generation and second generation of ARs. First-generation molecules have tissue persistence of few days while the second generation has tissue persistence of few weeks [26]. This point is a major concern for AR ecotoxicity.

Moreover, second-generation ARs (i.e. bromadiolone, difenacoum, brodifacoum, flocoumafen and difethialone) contain two asymmetric carbons systematically. Therefore, commercial second-generation ARs are a mixture of two diastereoisomeric forms (1R,3R)(1S,3S)-isomers and (1R,3S)(1S,3R)-isomers with different pharmacokinetic properties. For each second-generation AR, there is systematically one diastereoisomeric form with a shorter half-life than the other one (**Table 1**) [20, 27]. Proportion between stereoisomers in commercial baits is defined by regulatory documents. For example, bromadiolone must be a mixture of more than 70 of trans-isomers and fewer than 30% of cis-isomers. These differences in half-life between stereoisomers could be a fundamental point in the development of future more eco-friendly AR with modification of regulatory defined ratios.

2.3. Interest of anticoagulants in rodent population management

The first methods used to control rodent populations aim to kill them immediately. They were based on physical traps or on rapid killer molecules like strychnine. However, the neophobic behaviour of some rodents such as rats and their social organisation make these molecules ineffective. Indeed, the precocity of symptoms or death after a bait eating by congeners induces

Molecules	T _{1/2} (h)
Brodifacoum cis	120.8
Brodifacoum trans	68.7
Bromadiolone cis	26.9
Bromadiolone trans	75.6
Difenacoum cis	78.3
Difenacoum trans	24.2
Difethialone cis	71.6
Difethialone trans	52.9
Flocoumafene cis	76.7
Flocoumafene trans	177.4

Table 1. Half-lives of some anticoagulant rodenticide enantiomers.

bait aversion in the rodent population [28, 29]. Conversely, the time to onset of anticoagulant action is sufficient to avoid that rodents link their symptoms and death to bait eating [29].

Moreover, the delay and the mechanism of action of ARs are the keystone of their safety of use comparatively to other rodenticides. Indeed, the delay allows the possibility to implement a treatment after an exposure to ARs and the mechanism of action can be easily bypassed which offers an efficient and safe antidotes, the vitamin K.

Nevertheless, some issues exist with anticoagulant rodenticides, first the resistance of some population to some AR molecules. This issue has led to the creation of the second generation of ARs, which are more efficient against resistant strains [30]. However, this generation is more persistent which involves other issues. This persistence extends the duration of antidote treatment after exposure. Moreover, it entails a greater concentration of AR molecules in rodents after its death; thus, it might increase the risk of secondary poisoning of predators or scavenger animals. Consequently, to prevent poisoning of humans and animals, many actions have been implemented in the use of ARs.

2.4. Prevention of poisoning

In Europe, an anticoagulant rodenticide product can be registered either as a plant protection product or as a biocide. According to the kind of registration, restriction and modality of use are defined in order to prevent human and animal intoxication. Nevertheless, there are important differences on the modality and restriction among the European Member States. Here, we present some member state (MS) actions to prevent poisoning.

The majority of MS distinguishes the individual use of ARs and the professional use. Professional users are mainly the pest control operator, they have to be trained. In some countries like France or Italy, the sale of ARs is restricted for the individual user, thus, in France, individuals cannot buy more than 1.5 kg of AR bait. In other countries, like Germany, only trained professionals are allowed to use the second generation of ARs. Moreover, some molecules can be allowed as biocides and be forbidden as plant protection products.

The presentation of ARs is also regulated. Baits are presented as poisoned seed, paste or foam. Previously concentrated products like tracking powder and oil concentrate were used but they have been forbidden in many states. Thus, concentrations of the current AR baits are of the order of few dozen to few hundred milligrams of active product per kilogram of bait. The concentration depends on the efficiency of the active molecule. The main consequence of the use of products with low concentration is that it is difficult to reach the lethal dose at once for mammal heavier than rodents such as cats, dogs or humans. Nevertheless, the high half-life of some anticoagulants allows to reach this dose after a multi-exposure. The use of a bitter agent in bait is mandatory notably to avoid and limit exposure.

Finally, to avoid the exposure of untargeted species, in many states, baits have to be placed in secured bait stations. These stations have to be labelled to inform people on their content and on the action to perform in the case of exposure. Moreover, stations avoid the dispersion of baits which allows to control the consumption and they are waterproof, which prevent

water pollution. Nevertheless, some rodents are reluctant to enter in bait stations which might involve failing in pest control. In spite of all described elements to prevent exposures and intoxications of human and untargeted animals to ARs, many cases have been reported.

Therefore, some recent research makes effort to implement a third generation of ARs which is based on the stereochemistry concept, which would be efficient against resistant strains of rodents and be less persistent and thus less involved in secondary poisoning [20, 27].

3. Human exposures and intoxications

Intoxication with anticoagulant rodenticides is a major public health concern. The involvement of poison control centres is crucial in the record of poisoning cases in both rural and urban areas. Besides, emergency departments report rare cases of intoxication by suicide or homicide. Most of these poisonings occur following accidental exposure, especially ingestion in children. Bleeding severity is highly variable, depending on the rodenticide exposure and on the delay between the exposure and patient management. The diagnosis relies on simple coagulation tests. Emergency department physicians should be aware of anticoagulant poisonings since management differs according to the anticoagulant rodenticide including warfarin or long-acting superwarfarin types.

3.1. Epidemiology

The incidence of poisoning with anticoagulant rodenticides is difficult to assess, mostly based on national registries. In the literature, cases associated with bleeding are published as case reports or small series, probably corresponding to the most severe ones.

In the annual report based on the US National Poison Data System and published by the American Association of Poison Control Centers, data related to long-acting superwarfarin- or warfarin-type rodenticides intoxication are given separately. Over the last 5-year period (2011–2015), the cumulated number of exposures is 44,095 for long-acting superwarfarin-type and 1029 for warfarin-type drugs, with a single exposure in 97.3 and 95.6% of the cases, respectively [31–35]. Interestingly, the number of reported cases has slightly decreased since 2008 (**Figure 3**) [36–38]. The mean prevalence of exposure over the last 5 years is 3.4% for long-acting superwarfarin-type and 4.9% for warfarin-type drugs. The age distribution shows that children, especially those of less than 5 years old, are the most involved (**Figure 4**); only 9% of the reported cases are adults. Finally, clinical outcomes are reported (**Figure 5**). Remarkably, outcome is favourable in 93.6% of the cases, probably due to the limited ingested doses in relation to the bad taste of numerous rodenticides. The bitterness brought by the excipients in the currently marketed rodenticides considerably limits the ingested amounts, especially in young children. In cases associated with significant complications, severe bleedings are observed in less than 10% of cases, with fatal bleedings occurring in only eight patients among the 44,095 exposed patients during the last 5-year period in the USA. Overall, poisoning with rodenticides remains a rare cause of morbidities and fatalities [31–35].

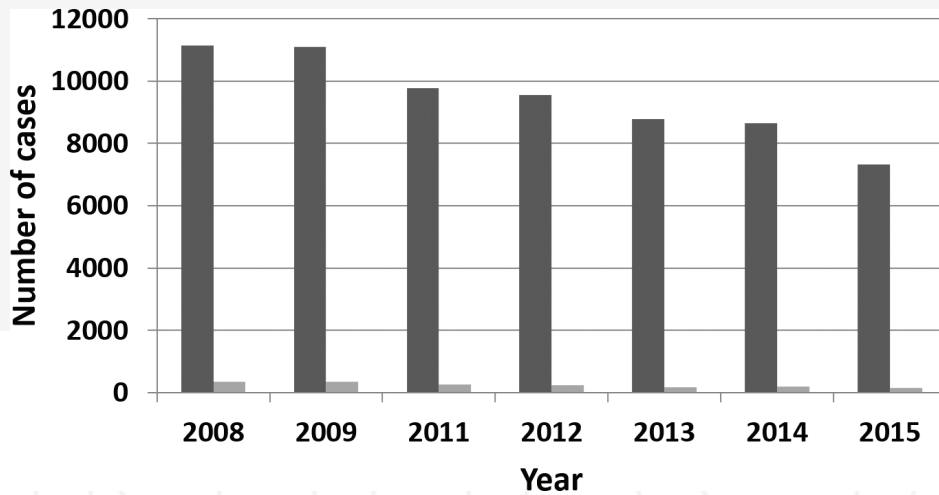


Figure 3. Number of poisonings with anticoagulant rodenticides reported by the American Association of Poison Control Centers from 2008 to 2015. Black bars: intoxications with long-acting anticoagulant-type rodenticides; grey bars: intoxications with warfarin-type rodenticides.

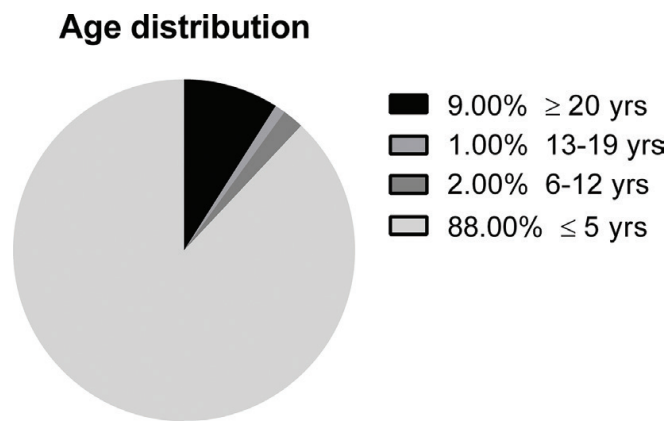


Figure 4. Distribution of the cases of poisoning with anticoagulant rodenticides reported by the American Association of Poison Control Centers in 2011–2015 according to the patient age.

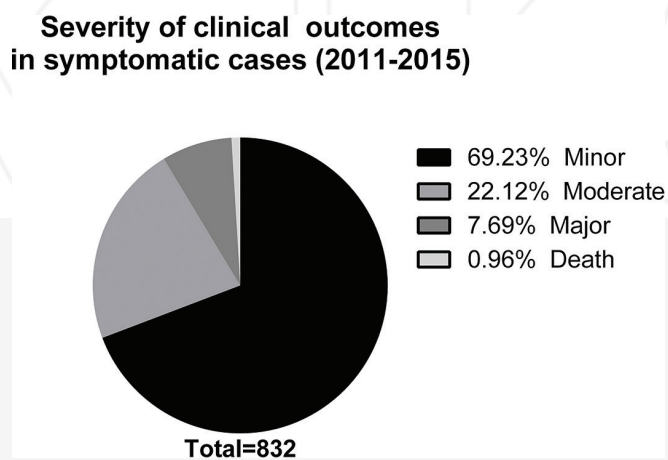


Figure 5. Distribution of the cases of poisoning with anticoagulant rodenticides reported by the American Association of Poison Control Centers in 2011–2015 according to the outcome.

3.2. Clinical outcomes and laboratory diagnosis

The threat in poisoning with rodenticides is the onset of severe bleeding. In humans like in rodents, anticoagulant rodenticides inhibit the enzyme vitamin K epoxide reductase complex (VKORC1) leading to the absence of vitamin K recycling, which is essential for the gamma-carboxylation of vitamin K-dependent proteins in the hepatocytes, especially clotting prothrombin, factors VII, IX and X. This leads to impaired functioning of gamma-carboxylated vitamin-K-dependent factors due to their inability to bind activated platelets. Given the half-lives of coagulation in humans, i.e. from 6 hours for FVII to ~60 hours for prothrombin, the onset of hypocoagulability and the risk of bleeding are delayed after the exposure to rodenticides. The risk of bleeding depends on the severity of the hypocoagulability state induced by rodenticides and on the duration of hypocoagulability. The spectrum of bleeding is wide: extended unexplained spontaneous ecchymosis, epistaxis, hematoma, bleeding from the gastro-intestinal or the genitourinary tract as well as intra-cerebral bleeding are reported [39–46].

The diagnosis of rodenticide intoxication has to be considered for any patient with prolonged prothrombin time (increased INR), prolonged activated partial prothrombin time; the vitamin K-dependent factor II, VII, X, IX coagulant activities are decreased while factor V coagulant activity and the fibrinogen level are normal [41, 43, 47, 48]. Liver dysfunction, cholestasis and severe starvation can be ruled out by normal liver enzymes and serum albumin concentration. Moderate to severe anaemia can be present, depending on the severity of bleeding. Special attention and high clinical suspicion are required in patients with apparent negative history of warfarin treatment. The diagnosis of rodenticide intoxication should be suspected when the international normalised ratio (INR) strongly fluctuates on vitamin K therapy, especially while high doses of vitamin K are required. The accessibility to anticoagulant rodenticides should be checked; monitoring of persons who deal with rodenticides in their home or workplace, especially those suffering from dementia or psychiatric disorders, is necessary [49]. The intoxication can be confirmed by the identification and measurement of the rodenticides in plasma by specific assays [42, 45, 50].

3.3. Principles of poisoning management

Acute life-threatening complications can be prevented with timely intervention. Immediate administration of high doses of phytomenadione (vitamin K1) and/or factor prothrombin complex concentrate (30 UI/kg FIX) can successfully reverse the anticoagulant effects of anticoagulant rodenticides. With tissue half-lives estimated at between 16 and 220 days, reversal of superwarfarin toxicity is a long-term issue. Therefore, long-term daily treatment for several weeks of phytomenadione is necessary. Treatment courses averaged 168 days. To avoid re-bleeding, close monitoring of INR is necessary. Adjunctive haemostatic therapy with recombinant factor VIIa and prothrombin complex concentrate has been used [50–54].

4. Overview on animal exposures and intoxications

To assess the importance of animal intoxications, it is important to discriminate two situations: the domestic animals and the wildlife. Concerning wildlife, the evaluations of exposures

and intoxications are often realised during focused scientific campaign and are often based on contamination studies or after an important mortality in wildlife. In domestic animal, besides the scientific campaign, there are, in some countries, animal specialised poison control centres, which can provide data on exposure, intoxications and linked symptoms.

In this part, it is important to take account of the differences between exposure and intoxication. Concerning exposure, it is the fact to take a dose of anticoagulants, it can be suspected by an owner who sees its animal eating baits, sometimes without knowing what the active substance is, or find in wildlife by pinpointing the presence of VKA in the sample. Intoxication is when the active substance induced clinical signs. This distinction is fundamental in the study of VKA toxicology. Indeed, to observe intoxication, the exposure dose and the delay of action has to be sufficient. This issue is discussed further concerning the wildlife exposure/intoxication studies.

4.1. Domestic animals

In France, two control poison centres are specialised in animals. The most important in terms of call number is the 'Centre National d' Informations Toxicologiques Vétérinaires (CNITV)' which responds to questions from owners or veterinarians on a 24-hour/7 day basis. We used this important database to assess the importance of VKA exposures and intoxications in domestic animals.

The data of the last 9 years have been analysed. During this period, the CNITV has received about 150,000 calls. Each month, 10.73% (CI 10.41-11.06) of solicitations are about VKA exposure or intoxication. Moreover, about whole VKA appeal is on domestic animals (99.2%). Appeals accrue from veterinary (69%) and owner (29%) mainly.

During the analysis of data, an important seasonality of the calls concerning VKAs has been pinpointed (**Figure 6**). Significant ($p < 0.05$) increases of the number of calls for VKA exposure are observed during the months of August, September and October followed by a significant decrease of appeal numbers from December to April, which is surprising. Indeed, based on our experience, the periods when people apply rodenticides in cities are at the beginning of winter (late November) and at the beginning of spring (March), when rodents are active and when the scarcity of food encourages rodents to eat baits. Conversely, during summer and the beginning of autumn, rodents can find many sources of food; consequently, they are less likely to eat baits, which increase the risk that baits are eaten by untargeted animals, notably dogs. However, summer is also the time when there is less human in cities and this element with a lenient weather encourages the presence of rodents outside where they are more visible. In response, cities and individuals might increase the number of baits, which is unfavourable for the rodent population management and rocket up the risk of pet exposures to VKA.

More generally, data pinpoint a trend reversal; before September 2013, the number of cases has significantly increased with a slope of 3.9% per annum ($p < 0.0001$) whereas after this date it has significantly decreased of 10.5% per annum ($p < 0.0001$). The increase trend has to be relativised as the total number of calls significantly increases during this period. However, after 2013, the total number of calls was stable ($p = 0.13$). Consequently, a significant decrease in the number of calls for VKAs after 2013 is confirmed. We hypothesise that the source of this diminution is the evolution of the regulation. Indeed, regulation has enforced the use of secured bait station since 2013, which seems to reduce the exposure of domestic species.

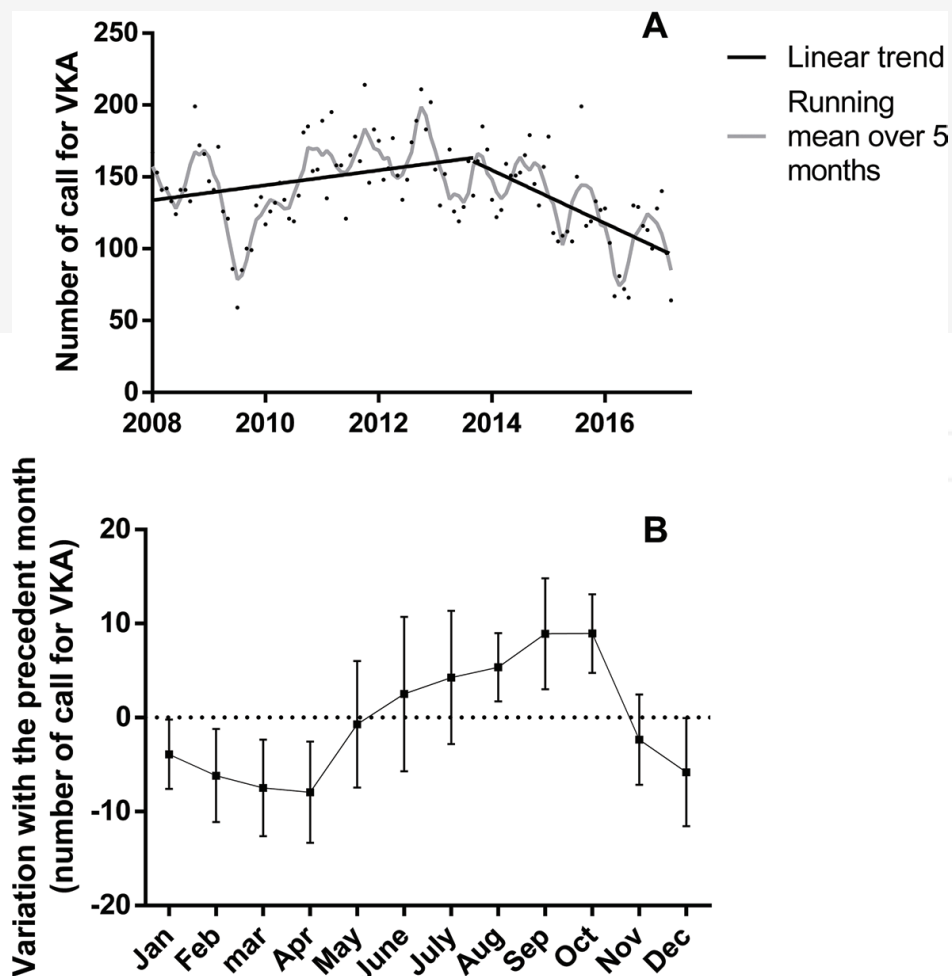


Figure 6. (A): Evolution of monthly calls for VKA exposure over time. Grey curve is the running means over 5 months. Dark lines are linear regression from January 2008 to September 2013 and from September 2013 to February 2017. (B) Variation of the number of calls for VKA exposure with the precedent month, values are represented as the mean of observed variations for the concerned month over the period 2008–2016 and its 95% confidence interval.

Pets and more specifically dogs are over represented (**Figure 7**). This might be explained by the lack of use of secured bait station by private individuals and by the behaviour of dogs. Poisoning is mainly accidental even if some malicious poisoning are reported (2.03%). The proportion of suspected malicious case concerning cats is significantly higher than for the general case. Indeed, cats and dogs represent, respectively, 19.14 and 63.64% of malicious reports. These uses of anticoagulant rodenticides with harmful intent against animals but also against humans have led to restrict the sale of rodenticides in some countries such as Italy [55].

Concerning molecules, in 22% of calls, the exact molecule is not identified. Nevertheless, exposures or intoxications with one of the six molecules authorised are reported, they are difenacoum, difethialone, brodifacoum, bromadiolone, chlorophacinone and coumatetralyl, which represent, respectively, 23, 18, 10, 9, 3 and 2% of the calls for AR. It is significant that the four main anticoagulants are second-generation ARs. This was predictable because first-generation ARs are less efficient on resistant strains of rodents consequently main ARs sold belong to the second generation.

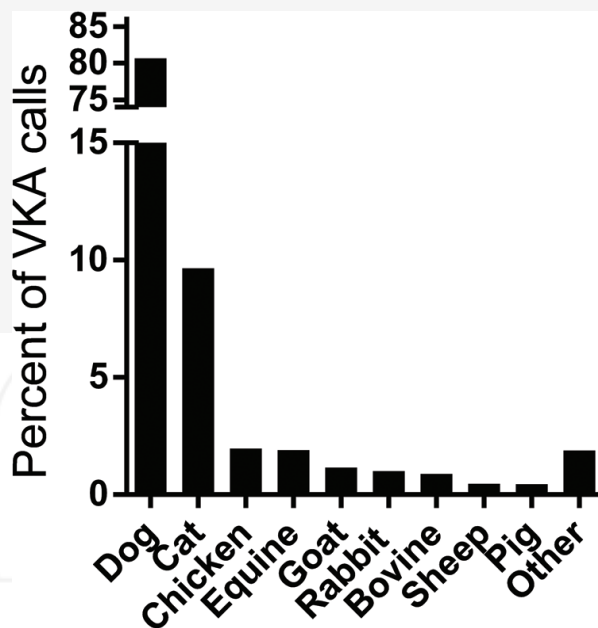


Figure 7. Percentage of species concerned by calls on VKA.

The consequences of an exposure without intoxication are completely different for pets and farm animals. Indeed, for pets when an exposure occurs, the aim is to prevent intoxication. In farm animals, more than intoxication prevention, the presence of anticoagulant molecules in products such as meat, eggs or milk has to be considered. Further, we discuss issues of ARs in pets then in farm animals.

In pets, depending on caller, the circumstances of appeal are different. Indeed, 90% of calls from individuals report exposure without intoxication, whereas the proportion of this circumstance drops significantly to 75% when it is a call from a veterinary ($p < 0.0001$). This can be easily understood, if an animal shows a symptom, the owner priority is to bring it to the veterinarian to be healed. Differences according to species are also pinpointed. In dogs, 81% of calls for AR exposure do not report symptoms versus 62% in cats ($p < 0.0001$). The source of this distinction may be the detection of the exposure, which is earlier in dogs. Moreover, cats may be more prone to be secondary exposed to ARs from intoxicated rodents and this kind of exposure is not visible by the owner. It should be noted that according to the low doses of bait and to the difference between the toxic doses for a rodent versus a cat, a large number of intoxicated rodents should be eaten to induce intoxication.

In dogs and cats, when an exposure is suspected and when it is possible, the best way to prevent intoxication is to induce vomiting in the first hour after the exposure. If medicines are not available, it is possible to use 10 volume hydrogen peroxide. Hydrogen peroxide solution can be given orally to animals at 1 mL for every 5 kg of weight. Be careful, salt must not be used to induce vomiting. Indeed, excess of salt can cause fatal hypernatremia [56].

Sometimes, even the exposure is uncertain or the absorption of VKA after vomiting is unknown, to confirm exposure, two means are currently tested by our team: the dosing of VKAs in plasma and their dosing in faeces. The difficulty of dosing in plasma is that for some VKAs, the presence in the plasma is temporary then VKAs are stored in liver. Therefore, dosing

in plasma is often associated with false-negatives. The dosing of VKA in faeces seems to be more reliable with an excretion that can be detected for several weeks.

As there is not any well-described toxicity dose, it is important to follow the possible effect of AR in order to prevent serious intoxication. Moreover, there is no correlation between the dose of VKA and the symptom severity [57]. Today, the gold standard to diagnose VKA intoxication is the prothrombin time [58]. It is advised realising a prothrombin time about 48 hours after the suspected exposure [59]. If the prothrombin time is elevated then a treatment has to be initiated. Other methods such as vitamin K clotting factor concentration measurement are explored to detect AR effects sooner. Factor VII seems to be a good candidate as its half-life after a VKA administration is the shorter [60].

If no treatment is given, symptoms may appear after 2–6 days [57, 58]. Symptoms are the classic signs of coagulopathy, which may be pinpointed by owner as bleeding, pale mucous membrane, haematomas, haematuria or haematemesis as well as their consequences on animal general condition as lameness, depression or lethargy [57, 58, 61]. Owing to intrathoracic bleeding, respiratory distress occurs frequently in anticoagulant intoxications [62–64].

Animals exposed with an elevation of prothrombin time after exposure or with AR-linked symptoms have to receive vitamin K₁ supplementation as long as the recycling mechanism is inhibited. Vitamin K₁ is given *per os* daily with a dose of 5 mg/kg of body weight. The duration of the VKA inhibition depends on the pharmacokinetics of AR molecule with huge differences between molecules. Thus, the duration of treatment after an exposure to the first-generation AR is estimated to 3 weeks versus 5–7 for the second generation. Nevertheless, there is a lack of studies to support these durations of treatment. Thus, a treatment can be initiated during at least 1 month then stopped during 48 hours. After 2 days of treatment discontinuation, the vitamin K regeneration mechanism can be assessed by measuring prothrombin time. If prothrombin time is elevated, treatment has to be replicated until a new assessment of regeneration mechanism else, the treatment can be arrested. Vitamin K₃ is inefficient to treat VKA intoxication. The treatment of symptomatic animals may require fresh plasma to reconstitute the pool of clotting factors urgently, moreover, in this case, it is recommended initiating the vitamin K treatment by an intravenous administration. If there is a proper compliance of the vitamin K treatment, the prognostic is excellent [57].

In farm animals, when an AR exposure occurs, the safety of the product has to be considered. Little information is available on the contamination of food following AR exposure. Nevertheless, many methods have been implemented to assess the residues in foodstuffs [65, 66]. Concerning meat, it has been shown that VKA molecules are present in muscle after exposure and that the cooking does not influence their activities [67]. Likewise, VKA molecules are also present in eggs after hen exposure, and are still detected in eggs 14 days after exposure [68]. Concerning the milk, it has been observed an excretion of VKA in human milk when a VKA is used as medication for the mother [69]. Consequently, it might be supposed that the same occurs in animals. Thus, when an animal is exposed, its litter should be separated of its mother and fed with relevant artificial milk. If separation is not possible or if diagnostic is late, litter should be supplemented with vitamin K₁. Concerning foodstuffs provided by an exposed animal, their management would be done in accordance with relevant authority.

4.2. Wildlife exposures and intoxication

Wildlife expositions or intoxications to ARs have been reported around the world for many mammals such as minks [70], bobcats [71], stoats and weasels [72], foxes [73, 74] and boars [67] and as well for many birds [75–77]. Exposition of fish was reported near an island where an eradication of rodent with brodifacoum was performed and the risk for human through the consumption appeared very low [78].

These intoxications may be primary when non-target species eat directly the bait. It is the case when baits are directly available without protection or when they are washed away and diluted in sea or river. In Spain, a study on water and soil samples revealed no imminent environmental risk in treated areas with chlorophacinone and brodifacoum [79]. However, the use of secured bait stations prevents this kind of exposition.

The secondary exposition occurs when a scavenger or a predator eats an exposed rodent. It is the most described exposition of wildlife to ARs and the most difficult to prevent. Many factors may influence the level of secondary exposition. First, due to the bait appetite, rodents can eat more AR than necessary to lead to their death, which might increase their concentration in AR. Moreover, if rodent is resistant to ARs, this phenomenon might be amplified. Indeed, a resistant rodent eats twice to fivefold more AR than susceptible rodent [1]. After the onset of symptoms in rodents, their behaviour evolves. They increase their activity during the day and stay longer in uncovered area, which enhances the risk to be hunted by predators [1]. The delayed action of ARs, inherent to its mechanism, allows rodents to eat several times the LD50 dose between the first bait intake and the death [1] and may as well increase the risk of secondary exposition. Pesticide usage has been correlated with non-target wildlife exposition [74, 75], and the intensity of treatment was related to incidence on local fox populations in France [80]. Finally, the diet is certainly going to influence secondary exposition and species like raptors, foxes and mustelids largely feeding on rodents when abundant are consequently the most at risk, as demonstrated for the red kite (*Milvus milvus*) [81]. The removal of visible rodent bodies helps to reduce the risk of secondary exposition [82] but is not always possible because of landscape limited access and in the case of aerial application [1]. Mitigation measures have been considered to protect predatory species but new approaches are still required [82].

Persistence and toxicity of the molecule are key factors. They depend on the used active ingredient [26, 83]. Historically, second-generation ARs had been designed to be more persistent and toxic on resistant strain. Thus, secondary poisonings of wildlife associated with the use of second generation are more often reported. But the development of new ARs recently proposed is based on the stereochemistry of second-generation ARs with reduced persistence but equivalent toxicity might greatly decrease the level of secondary exposition [20].

They are two types of consequences of the exposition of wildlife to ARs. First, if the species is eaten by human [67, 78], the consequences are comparable to those discussed for farm animals. Second, if the exposition is sufficiently important, it might lead to intoxication of the animals and to its death, which can be problematic mainly for endangered species. The rate of exposure of non-target species has often been evaluated, and summed liver concentrations above a limit of 0.2 mg/kg associated with clinical signs (i.e. macroscopic haemorrhages with no trauma) have been statistically characterised as representative of a high-risk toxic threshold

[84]. According to these criteria, hepatic concentrations above 0.2 mg/kg have been associated with mortalities in raptors and small mustelids from Denmark [85], in raptors and hedgehogs from Mediterranean region of Spain [86], in six raptor species from Canary Islands, Spain [77].

It is difficult to discriminate a simple exposition and intoxication in wildlife. Indeed, as well as in domestic animal, toxic doses are not well described for all species. Moreover, the majorities of exposition studies are performed on dead animal, and as the lesion induced by ARs is not specific, so it might be difficult to conclude to its implications. Less than 10% of exposed and dead birds have been confirmed to be intoxicated by ARs [1, 87]. Currently, there are no reports of a significant incidence of ARs on non-targeted species populations [82]. Nevertheless, impact of ARs on wildlife has to be more monitored in order to limit the impact of rodent population management. The probable future design of eco-friendly baits with new isomer ratio will change the need the way AR hepatic residues are monitored. The recently described multi-residue LC-MS/MS method [88] is an appropriate tool to start investigating second-generation AR diastereoisomer proportions in non-target wildlife and to evaluate their respective persistence in predators.

5. Conclusion

Anticoagulant rodenticides are a keystone of the rodent population management. Like other poisons, there is a risk of human or non-targeted species poisoning. The wide use of anticoagulant rodenticides near human living space and agriculture space involves an important exposure of humans and domestic animals. Nevertheless, since few years, many risk mitigation measures have been taken and the number of exposure in humans and domestic animals has decreased. Moreover, in contrast to the majority of chemical biocide, anticoagulant rodenticides have an effective antidote, the vitamin K. Consequently, anticoagulant poisoning is rarely fatal. However, the impact of anticoagulant rodenticides on wildlife is least well known and deserves more investigation.

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5.2.1.2 Article :

Asymptomatic Anticoagulant Rodenticide Exposure in Dogs and Cats—A French and Belgian Rural and Urban Areas Study



Asymptomatic Anticoagulant Rodenticide Exposure in Dogs and Cats—A French and Belgian Rural and Urban Areas Study

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Anticoagulant rodenticides (ARs) are important tools for controlling rodent pests, but they also pose a health threat to non-target species. ARs are one of the most common causes of pet poisoning. However, exposure of domestic animals to subclinical doses of ARs is poorly documented. To study the random exposure of dogs and cats to ARs, feces from animals showing no clinical signs of rodenticide poisoning were collected from a network of French and Belgian veterinarians. We analyzed fresh feces from 304 dogs and 289 cats by liquid chromatography-tandem mass spectrometry. This study showed a limited prevalence of AR exposure in dogs and cats of 2.6 and 4.5% respectively. In both species, access to the outdoors is a risk factor for ARs exposure. In contrast, the sex of the animals did not affect the ARs exposure status. The observation of the ratio of cis and trans isomers suggested primary exposure in dogs, but also in some cats. While primary exposure in dogs appears to be related to the use of ARs as plant protection products, primary exposure in cats may be malicious, as warfarin, an anticoagulant formerly used as a rodenticide and now used only in humans, was found in 4 of 13 exposed cats. Secondary exposure may also occur in cats. Our study showed reduced exposure in dogs and cats, compared to wildlife, which often has high exposure, especially in areas where rodent control is important.

Keywords: anticoagulant rodenticides, pets, dogs, cats, asymptomatic, non-target species, exposure

INTRODUCTION

Anticoagulant rodenticides (ARs) have been used worldwide for pest control since the mid-twentieth century (Fisher et al., 2019). Due to this intensive use, many animal species, whether or not targeted by these treatments, are regularly exposed to these compounds. ARs are classified into two generations: the first generation (FGARs) includes warfarin, diphacinone, coumatetralyl and chlorophacinone; these compounds have been used in the past for rodent control, but, due to increasing resistance mechanisms in the target species, they are now being replaced by the second-generation anticoagulants (SGARs) which include brodifacoum, bromadiolone, difenacoum, difethialone, and flocoumafen. SGARs have higher toxicity than FGARs and are much more persistent in tissues (Sánchez et al., 2012). Both generations of anticoagulants have the same mechanism of action, these pesticides break the vitamin K cycle in the liver by inhibiting the activity

of vitamin K epoxide reductase; ARs result in a progressive reduction in the pool of vitamin K required for the activation of coagulation factors II, VII, IX, and X (Suttie, 1985; Furie and Furie, 1988) leading to depletion and risk of bleeding.

The widespread use of these anticoagulant rodenticides also exposes non-target species that share the same treated area. This exposure can be observed in two different ways: either a primary or secondary exposure. Primary exposure results from direct ingestion of the poisoned bait whether it is a target or non-target species. Severe primary poisonings with ARs are widely documented and are among the most common poisoning in dogs (Lahmar et al., 2019; Bertero et al., 2020; Avolio et al., 2021; CNITV, 2022; Webmaster, 2022). In France the uses of ARs are divided into two categories, plant protection products (PPPs) and biocides (European Commission, 2022). PPPs are widely used in the field for the protection of crops against rodents while biocides are used to a limited extent in and around buildings. The mandatory use of bait boxes since 2013 has significantly reduced this primary exposure to biocidal products, but primary exposure to PPP products used in the field is possible for wildlife but also for domestic species that might consume these baits. This exposure remained largely possible until 2020, when such use was banned (Campagnol, 2020). Nevertheless, illegal uses are obviously still possible. The other type of ARs exposure is secondary exposure where the animal poisons itself by eating rodents that have been poisoned or chronically exposed to low doses of anticoagulants. This mode of poisoning has been widely studied in wild predators such as foxes, raptors or mustelids (Hosea, 2000; Sage et al., 2010; Elmeros et al., 2011; Nakayama et al., 2019). Such exposures are perfectly conceivable in domestic carnivores. In wildlife, it appears that animals exposed to ARs *via* poisoned rodents are frequently asymptomatic. Although hepatic residues of these pesticides persist for extremely long periods of time, low hepatic concentrations of ARs do not cause hemorrhagic syndromes but could have adverse effects, especially on the immune system (Vidal et al., 2009).

Veterinary professionals typically see asymptomatic animals with recent witnessed exposure to ARs or animals that are bleeding with suspected ingestion or known ingestion and delayed presentation. In domestic animals, especially in domestic carnivores the importance of asymptomatic exposure is totally unknown. In order to try to better understand this issue we propose to determine the prevalence of asymptomatic anticoagulant exposure in dogs and cats.

MATERIALS AND METHODS

Animal Sampling

To study the random exposure of dogs and cats to ARs, feces from animals showing no clinical signs of rodenticide poisoning (i.e., prostration and/or breathing difficulties and/or difficulty in mobilization and/or traces of bleeding) were collected from a network of veterinarians on animals that were consulted for any other reason not related to a suspicion of exposure to ARs (i.e., sterilization, vaccination, check-up, trauma, infection,

metabolic disease, etc). A fresh fecal sample from each individual was placed in a collection pot and accompanied by an information sheet on the animal when this information was known (age, sex, breed, location, reason for visit to rule out any suspect animals, etc). As the sampling of dogs and cats was done at different times and by different people such as students in training and practicing veterinarians, the sampling areas were different (Figure 1).

Anticoagulant Rodenticides Extraction

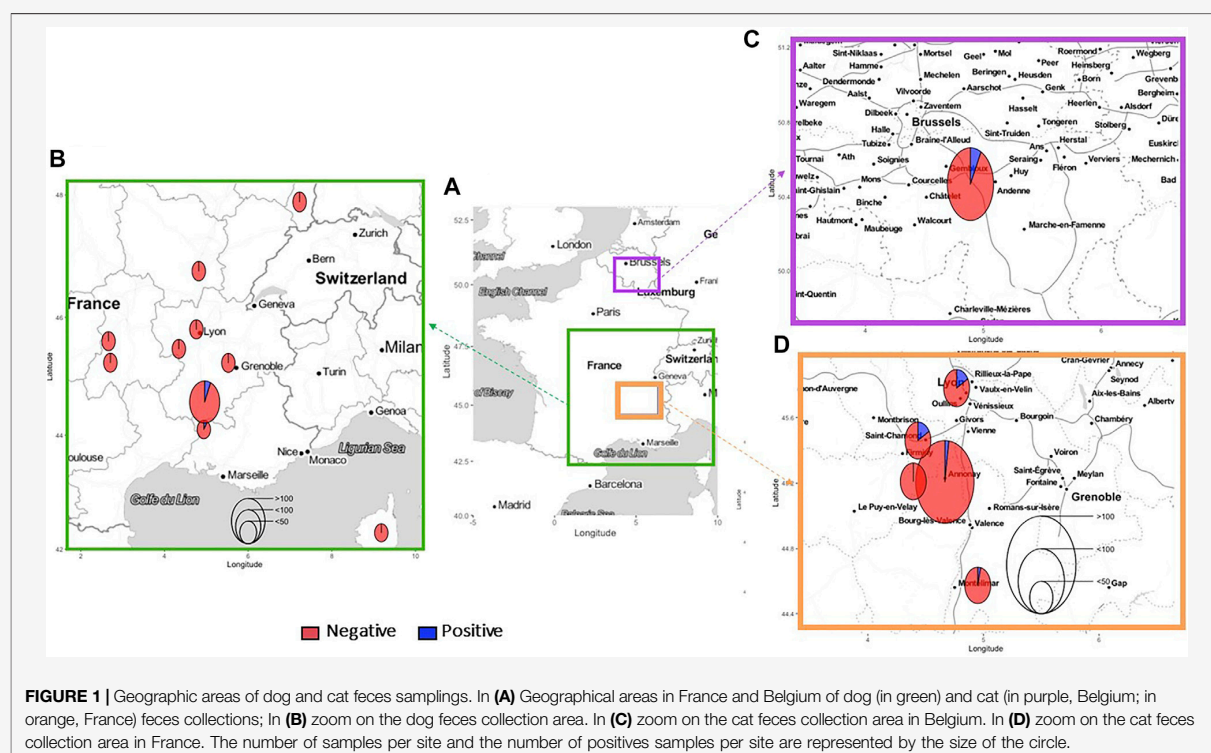
A weighing of 0.5 ± 0.05 g of dried feces was performed (i.e., a weight between 0.45 and 0.55 g) and placed in a 50 ml polypropylene tube of Falcon type. 10 ml of acetone was added and vortexed for 30 s. After resting for 1 hour, the tubes were shaken for 10 min by a PTR-60 rotator according to a sequence of cycles with orbital, reciprocal and vortex rotation. Centrifugation at 3000 rpm for 5 min was performed and 6 ml of supernatant was transferred to a test tube. The solvent was evaporated at 40°C under a nitrogen flow. The dry deposit at the bottom of the tube was taken up in 1 ml of acetonitrile, vortexed and added with 1 ml of hexane after 10 min of rest. The supernatant was then removed, and the rest was evaporated at 40°C under a light flow of nitrogen. At that stage, the dry extracts were taken up in 200 μ l of methanol. The supernatant was then placed in a vial with a syringe and a 0.2 μ m filter. The preparation was then injected (1 μ L) in LC-MS/MS for ARs titration according to (Fourel et al., 2018).

Anticoagulant Rodenticides Analysis

Anticoagulant rodenticides analysis was carried out following the method recently described by (Fourel et al., 2018). The ARs analysed in this study were three FGARs: warfarin, coumatetralyl, chlorophacinone and the five SGARs potentially used in European countries: bromadiolone, difenacoum, brodifacoum, flocoumafen, and difethialone. The chromatographic separation was achieved with a semi-porous Poroshell 120 StableBond C18 column (2.1*100 mm, 2.7 μ m) and MS/MS detection was carried out by a 6410 B Triple Quadrupole from Agilent Technologies (Palo Alto, CA, United States) equipped with ElectroSpray Ionization source in negative mode. Two fragment ions were recorded in dynamic Multiple Reaction Monitoring mode and a calibration curve was built up for each analyte. The limits of quantification varied between 1–2 ng/g and the recovery rates were above 70%.

Data Analysis

Because cats and dogs samplings were done at different periods and in different geographical areas, comparative analysis was not performed. As clinical and epidemiological data were not provided by the sampling veterinarians for all animals, the exposure risk analysis was performed by including only those animals for which epidemiological and clinical data were known. In order to qualify the living environment, three types of zones have been defined: urban zones: corresponding to municipalities with more than 2,000 inhabitants with grouped and collective housing, peri-urban zones: corresponding to municipalities with more than 2,000 inhabitants with individual housing close to an



urban center, and rural zones: corresponding to municipalities with less than 2,000 inhabitants.

RESULTS

Dog Sampling Description

Three hundred and four fecal samples were collected from dogs between 2016 and 2017 in southeastern France, in 16 different administrative departments, with 1–112 dogs per department (**Figure 1**, section B). The population descriptors are presented in **Table 1**. For some dogs, age ($n = 70$), sex and habitat ($n = 60$) and/or department of origin ($n = 43$), were not reported. The breed distribution of the sampled population was very heterogeneous, with a majority of hunting dogs (62.0%) in which 22.7% of the dogs were often used for hunting, followed by companion dogs (24.1%) and shepherds (13.9%). Regarding the geographical distribution of the samples, 58.4% were collected in rural areas, 9.4% in urban areas and 32.2% in peri-urban areas. Regarding the lifestyle of the dogs, the majority (53.2%) lived in a house and had regular access to a garden. A part of the population lived in kennels (28.3%) and 9.8% in apartments. Finally, 8.8% of the individuals lived primarily outdoors on farms.

Cat Sampling Description

A total of 289 fecal samples were collected in France (77.2%) and Belgium (22.8%) (**Figure 1**). In Belgium, the cats came from the city of Namur and were all stray cats. In France, the fecal samples

came from five administrative departments in the southeast of France, with 1–183 samples per department (**Figure 1**, section D). The European breed was the dominant breed at 99.3%. One hundred eighty eight cats were strays, 101 had a known owner. For 105 cats, the sex was not known. For the rest of the sample, the male/female sex ratio was 25.5%/75.5%, respectively. The median age of the population was young (2 years), although for 123 stray cats the age was not known. The age of these strays was estimated by the veterinarian to be between 1 and 3 years. The majority of the cats (63.7%) were from urban areas. 95 were from rural areas (32.9%) and 10 from suburban areas (3.5%). Only 6% of the cats lived strictly indoors.

Dogs' Exposure to Anticoagulant Rodenticides

Of the 304 dog fecal samples, only 8 contained ARs, a prevalence of 2.6%. Of these 8 samples, only one AR was detected; no multiple exposures were found (**Figure 2**). Only bromadiolone was detected. All dogs found positive were animals from the same department, Drome, in an area of 100 km by 100 km near the city of Montelimar, including five in an area of only 20 km by 40 km (**Figure 1** and **Figure 2**).

Of the 8 dogs exposed, five were purebred (a Griffon, a Jack Russel Terrier, a Shih Tzu, an English Setter and a Breton Spaniel) and 3 were of mixed breed, 2 from a cross with the beauceron and 1 from the labrador, representing 63% of the hunting dog breed. The average age of the exposed dogs was 7.4 years. Three dogs

TABLE 1 | Description of the dog and cat samples.

	Dog	Cat
Total number	304	289
Date of sampling	2016–2017	2019–2020
Age	(<i>n</i> = 238)	(<i>n</i> = 166)
Median	5.0	2.0
25–75% percentile	2.0–8.0	1.5–4.0
Min-Max	0.3–16	0.25–19.0
Sex Ratio		
Male	43.4%	16.3%
Female	36.8%	47.4%
Unknown	19.7%	36.3%
Department	(<i>n</i> = 261)	(<i>n</i> = 289)
Ain	1.5%	–
Allier	8.8%	–
Alpes Maritimes	0.8%	–
Ardèche	5%	63.3%
Corse	0.4%	–
Cantal	6.5%	–
Drôme	43%	9.3%
isère	2.7%	–
Haute Loire	2.3%	2.1%
Haute Loire	–	0.4%
Puy de Dôme	3.8%	–
Haut Rhin	0.4%	–
Rhône	14.6%	2.1%
Saone et Loire	5.7%	–
Vaucluse	4.6%	–
Belgium (Namur)	–	22.8%
Urbanization Level	(<i>n</i> = 245)	(<i>n</i> = 289)
Rural	58.4%	32.9%
Suburban	32.2%	3.5%
Urban	9.4%	63.7%
Lifestyle	(<i>n</i> = 245)	(<i>n</i> = 289)
Indoor	9.8%	5.9%
Outdoor	8.2%	65.7%
Indoor/Outdoor	82%	28.4%
AR Exposure	2.6% (i.e., 8/304)	4.5% (i.e., 13/289)
Exposed to 1 compound	2.6% (i.e., 8/304)	4.15% (i.e., 12/289)
Multi-exposed	0% (i.e., 0/304)	0.35% (i.e., 1/289)

were females, five were males. All exposed dogs had outdoors access, living in a house or a farm. Five lived in rural areas, two in suburban areas, and one in urban areas. Three dogs were used for hunting.

Median concentration of bromadiolone in feces was 13.41 ng/g (95% CI: 4.64–294.4) with a lower value of 4.64 and upper value of 264.4 ng/g (**Figure 4A**). Mean percentage of trans-isomers of bromadiolone in feces from exposed dogs was 80.5 ± 10.8% with a lower value of 61% and a higher value of 95% (**Figure 4C**).

Cats' Exposure to Anticoagulant Rodenticides

Of the 289 cat fecal samples, only 13 contained ARs, a prevalence of 4.5%. Of these 13 samples, 12 contained a single AR, one contained two different ARs. Among the samples exposed to a single AR, 7 contained ARs belonging to the FGARs (4 with

warfarin and 3 with coumatetralyl) and five contained SGARs (2 with brodifacoum, 2 with difethialone and 1 with bromadiolone) (**Figure 3** and **Figure 4B**). The multi-exposure sample with ARs contained brodifacoum and difethialone.

Eight cats were strays, five had a known owner. All cats had outdoors access, 9 lived exclusively outdoors. Eight cats lived in urban areas, 4 in rural areas, and 1 in suburban areas. Five cats lived in the Ardeche department, including the multi-exposed cat. The compounds detected in these five cats were variable (2 exposures to brodifacoum, 3 exposures to coumatetralyl, 1 exposure to difethialone). Five other exposed cats were stray cats from a parking lot in the city of Namur, Belgium (65 samples from this area). One cat exposed to brodifacoum and 4 cats, collected at the same time, were positive for warfarin. For the 3 remaining cases, two cats, one in the Rhône and one in the Loire, were positive for difethialone and one case was positive for bromadiolone in the Drome department.

Among the 13 AR-positive cats, 4 (2.3%) were neutered (*n* = 119) versus 9 (5.3%) were entire (*n* = 170).

The median concentration of FGARs in feces was 3.6 ng/g with extreme values for coumatetralyl of 2.0 and 13.8 ng/g (**Figure 4B**). The median SGARs concentration (i.e., brodifacoum, difethialone, and bromadiolone) was 22.6 ng/g with a lower value of 4.1 for difethialone and a higher value of 129.6 ng/g for bromadiolone. The mean percentage of cis-isomers of brodifacoum in the feces of exposed cats was 64 ± 36%, with a lower value of 28% and an upper value of 100% (**Figure 4C**). The mean percentage of cis-isomers of difethialone in the feces of exposed cats was 100 ± 0% (**Figure 4C**). The percentage of trans isomers of bromadiolone in the feces of the cat exposed to bromadiolone was 68%.

DISCUSSION

Asymptomatic exposure of wildlife to anticoagulants has been widely documented. However, exposure of domestic animals has been poorly studied. In addition, severe poisoning cases are very frequently reported in dogs, while very few cases are reported in cats (Walton and Otto, 2018). Besides, the subclinical exposure of these species is poorly documented, with no documented study in cats and only one study in dogs (Seljetun et al., 2020a). In this study, we evaluated asymptomatic exposure of cats and dogs to ARs without preconceived ideas. All samples were taken from apparently healthy animals with no clinical signs of anticoagulant exposure.

Since our objective is to assess the exposure to relatively minimal doses of ARs, the most sensitive biological sample is obviously the liver. However, the sampling is invasive and is not compatible with an exposure assessment study on a live animal. Plasma is very often used, but this biological fluid is not the most appropriate for an ARs exposure study. First of all, blood sampling is still invasive and requires a technical procedure. Overall, its use leads to many false negative results because the plasma circulation of FGARs is very transient and occurs at very low levels, much lower than hepatic concentrations (Vandenbroucke et al., 2008). For example, a recent study

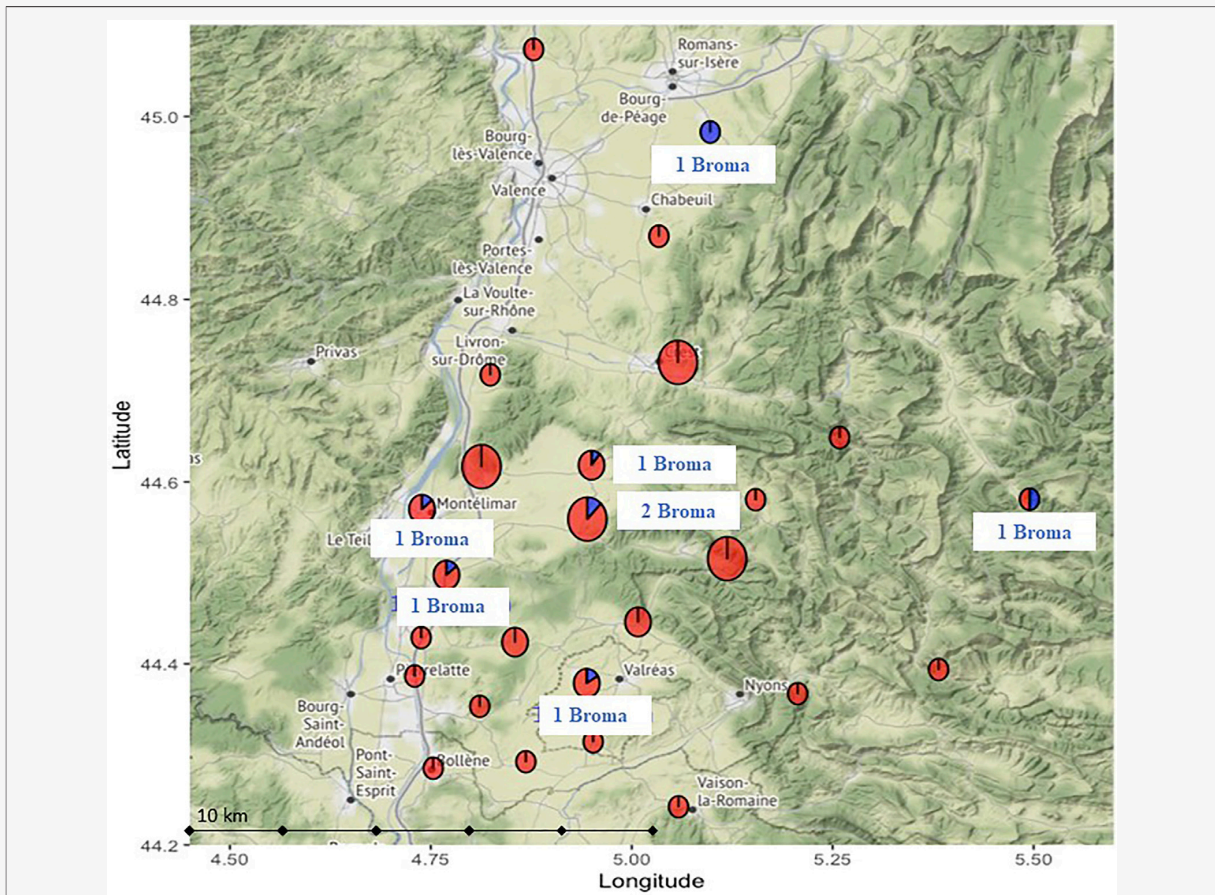


FIGURE 2 | Focus on the dog sample collection area ($n = 134$) near the French town of Montélimar in the Drôme department where the 8 exposed dogs were found, including five in a limited area of only 20 km by 40 km.

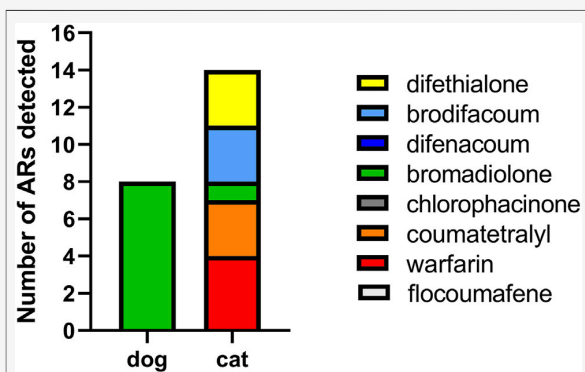
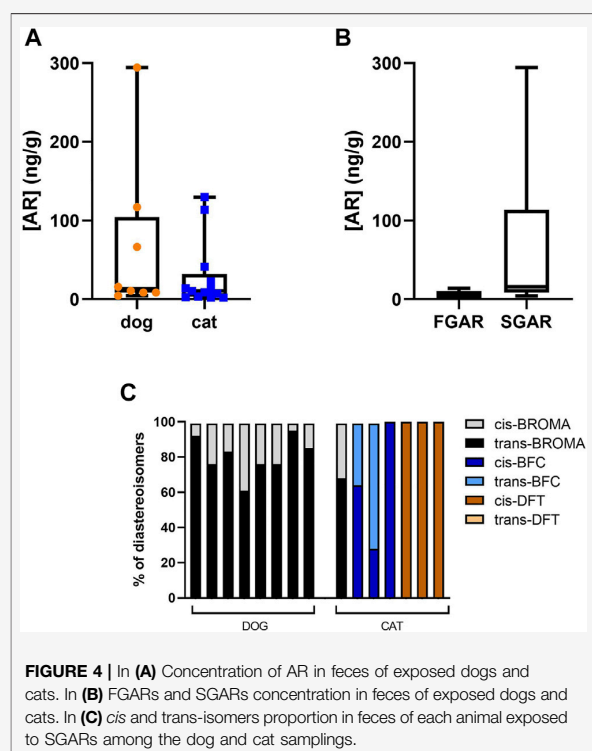


FIGURE 3 | Exposure to AR of cats and dogs. Occurrence of each AR compound in the exposed dogs ($n = 8$) and cats ($n = 13$) with one cat dually exposed to brodifacoum and difethialone.

shows that the distribution of bromadiolone in plasma at steady state is only 0.4% of the amount present in the body in rats and its presence becomes very quickly undetectable after the initial administration (Chetot et al., 2020). Recent reports have shown that fecal samples, although less sensitive than liver, may be an acceptable and non-invasive alternative for the detection of ARs in animals. Indeed, ARs would be subject to enterohepatic cycling resulting in a long half-life, but also excretion in feces (Watt et al., 2005). This biological matrix has been used to evaluate the pharmacokinetics of bromadiolone in foxes with fecal concentrations 30 to 50 times higher than those of plasma and detectable for more than 25 days post-administration (Sage et al., 2010). A comparative study on the detection of ARs between liver and feces showed that this matrix allowed a satisfactory detection of ARs on a sample of foxes, even if some false negatives are reported (Seljetun et al., 2020b).



In our study, the prevalence of exposure of dogs and cats to ARs were similar and rather low, 2.6 and 4.5%, respectively. This prevalence may be underestimated due to the biological matrix used. Nevertheless, in Norway, 110 healthy dogs were tested for ARs and showed that only one sample contained difenacoum (Seljetun et al., 2020a), whereas the prevalence in non-target wildlife such as foxes was 50% (Seljetun et al., 2019). Indeed, much higher levels of exposure have been reported in wild canids and felids, even when monitoring is performed on feces. Furthermore, a study conducted in France, using fox feces collected in an area where the ground vole is abundant, reported an exposure prevalence of 23% (Fourel et al., 2018). In bobcats in California, exposure has been reported to be close to 39% from plasma monitoring (Serieys et al., 2015). This difference in exposure between domestic and wild canines and felids may be partly due to the inclusion in our sample of animals without access to the outdoors. Nevertheless, after excluding them, the exposure prevalence (3.6% for domestic dogs and 4.8% for domestic cats) are still much lower than those reported for wildlife, considering that 9.8% of the dogs in our population lived in open spaces e.g., farms and 65% were stray cats. This low exposure suggests that the conditions of ARs use, especially the use of a bittering agent in bait (Hansen et al., 1993; Lei et al., 2015) and the use of ARs as a biocide in bait stations since 2013 (Lefebvre et al., 2017), allow to avoid exposure of domestic dogs and cats. Despite these precautions, there are unfortunately cases of poisoning in dogs when baits are improperly stored by their owners or when they are misused.

Some conditions leading to exposure of dogs and cats can be identified from our sampling. All animals exposed to ARs were animals with access to the outdoors. Cases of exposure have been found in both rural and urban areas. Unfortunately, the number of positive cases was not sufficient for statistical analysis. Neither gender nor age seems to be determining factors. Can the exposure be characterized as primary or secondary? Observation of the diastereomeric ratios of SGARs residues may provide an answer. Indeed, each SGARs is a mixture of pair of cis and trans diastereomers with different biological properties (Damin-Pernik et al., 2016; Lattard and Benoit, 2019). In fact, one diastereomer is systematically eliminated more rapidly than the other, at least in rats (Damin-Pernik et al., 2017). Baits contain known and regulated proportions of diastereomers. In Europe, SGARs contain more cis-isomers than trans-isomers, except for bromadiolone. The most abundant diastereomer is consistently the most persistent, so that the residues in the body of a rodent that has consumed a SGARs are composed almost exclusively of the most persistent diastereomer with a change in the proportions of diastereomers relative to the original active substance. An animal consuming a poisoned rodent is then exposed primarily or almost exclusively to the most persistent diastereomer, trans isomer for bromadiolone, cis isomer for all other SGARs. An animal consuming the bait is exposed to the diastereomeric pair, except for difethialone which is composed almost exclusively of cis isomer. The residues found in our SGARs-exposed dogs and cats are composed of both diastereomers, except for one cat exposed to brodifacoum and obviously the cats exposed to difethialone. These results suggest a primary exposure of all 8 dogs and at least 2 of the cats (the one exposed to bromadiolone and the one dually exposed). Nevertheless, the representativeness of the diastereomeric composition of the residues in the feces matrix compared to those in the liver matrix has never been studied. It is possible that the more persistent isomers remain in the liver, which would increase the proportion of less persistent isomers in the feces. Studies will be needed to address this specific point. While primary exposure is easily conceivable in dogs, it is more difficult to imagine in cats. The two cats could have been deliberately poisoned with homemade bait.

For the 8 dogs exposed to bromadiolone, they all came from the same department and from the same geographical area, which was quite small compared to the total sampling area (Figure 2). The samples were all taken between April and July 2017, a fairly short period of time. Outside of this area, no dogs were exposed to AR. It is quite surprising to encounter such a lack of positives outside this zone. Thus, the prevalence in this area reaches locally 6% (8/134 dogs sampled in the area). Bromadiolone could be used at the time of sampling either as a biocide to be used around and in buildings by professionals and amateurs, or as a phytosanitary product for the control of vole populations by farmers (subject to prior prefectural authorization, at low population density, with maximum quantities per hectare not to be exceeded). The use of bromadiolone as a PPP at that time and in that area could not be confirmed, but the exposure results suggest such use with or without authorization.

Concerning the exposed cats, we notice that more than 30% of the positive cats carry coumatetralyl which is a low remanence FGARs and therefore disappears rapidly from the body. This could suggest a regular predatory behavior in these felids. Cats, due to their exploratory instinct, explore a variety of environments which explains the different nature of the identified ARs and the multi-exposure. In addition, neutering status appears to have a slight influence, as entire cats have a positivity of 5.3 versus 3.3% in neutered ones. Sex hormones may influence the lifestyle of felines. It is reported that entire cats are more active, travel longer distances and control a larger territory than neutered cats. In addition, these felids still have an aggressive hunting instinct compared to dogs. Poisoned and less alert rodents can be easy prey (Crowley et al., 2019). It should be noted that in Belgium, some cats are positive for warfarin, an anticoagulant used as a human drug for the prevention of thromboembolic events. This ARs would no longer be used in modern rodent control. The 4 warfarin-positive cats are stray cats that were sampled in a limited urban area in Namur, Belgium. Several hypotheses could explain this observation. It is possible that old stocks of warfarin are still being used as a rat poison, or malicious poisoning or even contamination by wastewater containing residues of warfarin-based drugs consumed by the cats or their prey may be suspected (Gómez-Canela et al., 2014). The source of exposure could be also the consumption of human drugs, as described in cases of childhood poisoning (Brands et al., 2021), this remains unlikely in this case because they were all stray cats. This discovery leads to the questioning of the behavior of these felids that can expose themselves to other medications intended for humans.

Although subtoxic levels of ARs in animals do not cause hemorrhagic syndrome, these chemical residues could be not without consequences for the health of these animals. Reduction in body condition has been reported in raptors and in mustelids at sublethal concentrations (Elmeros et al., 2011; Martínez-Padilla et al., 2017). Some studies in barn owls have shown behavioral aberration after secondary exposure to ARs (Salim et al., 2014). Studies suspect that subtoxic levels of ARs might interfere with the immune response by disrupting leukocyte differentiation (Fraser et al., 2018), besides, other work indicates that ARs increase susceptibility to opportunistic infections in bobcats (Serieys et al., 2018). Nevertheless, experiments have shown that cats exposed to brodifacoum showed transient decreases in the production of certain cytokines, without significant impact on the immune response (Kopanke et al., 2018). Other felids such as lynx were found to be at high risk of exposure, with a record of fetal transfer of several ARs. In contrast, it was noted that there are significant relationships between certain levels of ARs

exposure and the incidence of notoedric mange (Serieys et al., 2015). Furthermore, teratogenic effects, and post-partum mortality after exposure to ARs have been reported in pregnant animals (Robinson et al., 2005; Fitzgerald et al., 2018).

CONCLUSION

AR are widely used in France and represent the chemical treatment of choice for rodent control. Their use, especially in the fields, inevitably exposes wildlife such as foxes or birds, sometimes leading to poisoning. Concerning our domestic animals, and in particular dogs and cats, anticoagulant rodenticides represent the first cause of poisoning in France (Berny et al., 2010). On the other hand, concerning the environmental exposure of these species, our study shows a limited exposure of dogs and cats, in contrast to wildlife, for which exposure is often high, in regions where rodent control is important. These results suggest a good efficiency of the mitigation measures put in place to protect these species.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

Conceptualization: VL and EB; methodology: TM, EK, LG, AM, CH, VL, and IF; data curation: AM, TM, LG, and VL; formal analysis and investigation: TM, EK, LG, AM, CH, IF, EB, SL, and VL; writing—original draft preparation: TM, VL, SL, EK, and EB; writing—review and editing: TM, VL, CH, and EB; funding acquisition: VL and EB; resources: VL; supervision: VL and EB.

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5.2.1.3 Article :

Accidental chlorphacinone exposure of lactating ewes : Clinical follow-up and human health dietary implications

Accidental chlorophacinone exposure of lactating ewes: Clinical follow-up and human health dietary implications

Meg-Anne Moriceau^{1,2} , Sébastien Lefebvre¹ , Isabelle Fourel¹ , Etienne Benoit¹ , Barnett A. Rattner³ , Virginie Lattard¹

Abstract

Anticoagulant rodenticides are widely used for rodent control in agricultural and urban settings. Their intense use can sometimes result in accidental exposure and even poisoning of livestock. Can milk, eggs or meat derived from such accidentally exposed animals be consumed by humans? Data on the pharmacokinetics of chlorophacinone in milk of accidentally exposed ewes were used to estimate the risk associated with its consumption. Three days after accidental ingestion, chlorophacinone was detected in plasma of 18 ewes, with concentrations exceeding 100 ng/mL in 11 animals. Chlorophacinone was detected in milk on day 2 post-exposure and remained quantifiable for at least 7 days in milk of these 11 ewes. Concentrations in milk were much lower than in plasma and decreased quickly (mean half-life of 2 days). This study demonstrated dose-dependent mammary transfer of ingested chlorophacinone. Variation in prothrombin time (PT) on Day 3 suggested that some of the ewes that ingested chlorophacinone may have been adversely affected, but PT did not facilitate estimation of the quantity of chlorophacinone consumed. Using safety factors described in the literature, consumption of dairy products derived from these ewes after a one-week withdrawal period would pose low risk to consumers.

Keywords

Chlorophacinone residues ; Ewes ; Food safety ; Intoxication ; Mammary transfer ; Ovis aries ; Prothrombin time ; Sheep

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Introduction

Anticoagulant rodenticides (ARs) are widely used for pest control in urban and agricultural settings [1]. They disrupt the vitamin K cycle, antagonizing vitamin K epoxide reductase in liver and other tissues [2, 3], and thus interfere with the synthesis of active forms of blood clotting factors II, VII, IX and X. Their action results in coagulopathy that can lead to death of target rodents in several days[4].

As these products are widely used, they are all too frequently involved in domestic animal poisoning, especially in dogs (*Canis lupus familiaris*). For example, the National Centre for Veterinary Toxicological Information data from 2007 to 2017 (CNITV – Lyon, France) indicate that ARs are the main category of potential poisoning incident calls among all pesticides (40.4%), with dogs being 82.8% of incidents [5]. Ruminants are apparently less often exposed than other domestic species (only 2.2% of calls about rodenticide poisoning are for ruminants), and display clinical signs less frequently (only 10.5% of exposed animals displaying clinical signs according to CNITV data). This decreased incidence of clinical signs is possibly due to a lower sensitivity to ARs [6].

Clinical signs of rodenticide intoxication are similar for higher vertebrate species: hemorrhagic syndrome, with main localizations being respiratory and digestive tract, and less specific symptoms including lethargy, anorexia, or ataxia [4, 7–9]. Clinical onset is usually delayed from 2 to 10 days post-ingestion [5, 10]. No simple and definitive diagnostic procedures are available, and clinical suspicion is based on coagulation testing by measuring prothrombin time (PT) which is the clinical standard for diagnosis of AR intoxication [4, 11, 12]. Exposure can be definitively assessed by concentration of the ARs in liver and plasma [5]. Another issue with ARs lies in the dairy production field, as they undergo mammary transfer [13]. Residues can be found in the milk of an exposed and potentially intoxicated dairy animal, resulting in human exposure to ARs. The milk transfer rate of ARs in lactating animals has not been adequately documented in the literature, although it is mentioned in some older studies investigating the risks of vampire bat (*Desmodus rotundus*) mitigation campaigns in Latin America involving intraruminal injection of ARs in cattle (*Bos taurus*) [14, 15]. Many older publications report dose-dependent effects related to AR transfer through milk. For example, administration of massive doses of dicoumarol [16] or warfarin [17] to lactating rats (*Rattus norvegicus* - *Rattus norvegicus domestica*) produced hypoprothrombinemia in suckling young. Administration of diphenadione (i.e., diphacinone) to lactating cows resulted in increased PT in suckling calves [18]. Investigations have also been conducted in lactating women receiving anticoagulant drugs, allowing study of the effects of anticoagulant therapy on breastfed infants [19–21]; these children were usually asymptomatic [19, 20], with no residues of the drug in their mother's milk [21].

As there is no established Maximum Residue Limit (MRL) for these products in animal tissues, no withdrawal period has been defined. This brings into question the safety of dairy products for human consumption in suspect dairy livestock exposure incidents. Chlorophacinone (CAS number: 3691-35-8) is a first-generation AR, derived from indanedione. In Europe this product is no longer used as a plant protection agent but is still approved as a biocide (EU regulation n°528/2012) for control of pest rodents (e.g., brown rat, *Rattus norvegicus*; Black rat, *R. rattus*; house mouse, *Mus musculus*). It can

be hazardous for domestic animals (ACTA, 2018; Standing committee on Biocidal Products, 2009), and to a lesser degree a public health issue if accidentally ingested by dairy animals. Herein we report results of an investigation on the pharmacokinetics of chlorophacinone in milk of accidentally exposed dairy ewes to estimate the risk associated with its consumption by humans.

1. Material and methods

1.1 Animal population, case history

In May 2017, a flock of 23 healthy Lacaune adult lactating ewes (*Ovis aries*), of standard weight for the breed (70–80 kg), from a farm of the french department of Creuse (23), accidentally ingested an entire 20-kg bag of grain bait (ROZOL GRAIN'TECH - Liphatech SAS) containing 50 mg/kg of chlorophacinone (i.e. 1 g of chlorophacinone in total). Exposure distribution among the flock was obviously unknown. A veterinarian was contacted about 12 h post-ingestion, and all animals were asymptomatic at that time.

1.2 Samples

Samples collection was conducted under a veterinary license issued by France. For each animal, milk and blood samples were collected. Milk was obtained daily in dry tubes by the farmer from days 2 to day 7 post-ingestion. Whole blood was drawn by the attending veterinarian from the jugular vein into 10-mL tubes containing citrate on day 3 post-ingestion from all animals, and on day 11 from the subset of animals with plasma chlorophacinone concentration >100 ng/mL in the first blood sample.

1.3 Determination of AR concentrations in plasma and milk

Liquid-Liquid Extraction (LLE) was used to extract ARs from plasma and milk of the sheep. Briefly, 200 μ L of blood plasma was homogenized with 4 mL of acetone using a rotator for 10 min. The mixture was centrifuged for 5 min. The liquid fraction was separated and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residues were reconstituted and mixed in 0.2 mL of acetonitrile. For milk, 1 mL was homogenized with 4 mL of acetone using a rotator for 30 min. The mixture was centrifuged for 5 min. The liquid fraction was separated and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residues were resuspended in 4 mL of acetone using a rotator for 10 min, then centrifuged (2000g) for 5 min. The supernatant was resuspended in 200 μ L of acetonitrile, vortexed for 10 s, then placed in an ultrasonic tank for 5 min and again vortexed for 10 s. In order to freeze the lipidic portion, the sample was placed in the freezer for about 10 min. The supernatant was recovered and purified through a 0.2 μ m filter.

AR residue analysis was carried out by using the LC-MS/MS method previously described [22]. The ARs analysed with this method are three first-generation ARs: warfarin, coumatetralyl, chlorophacinone and 5 s-generation ARs

potentially used in European countries: bromadiolone, difenacoum, brodifacoum, flocoumafen and difethialone. The chromatographic separation was achieved with a semi-porous Poroshell 120 StableBond C18 column (2.1 × 100mm, 2.7 μm) and MS/MS detection was carried out by a 6410B Triple Quadrupole from Agilent Technologies (Palo Alto, CA, USA) equipped with ElectroSpray Ionization source in negative mode. Two fragment ions were recorded in dynamic Multiple Reaction Monitoring mode for each analyte. Limits of quantification (LOQ) were 0.1 ng/mL for milk and 2 ng/mL for plasma.

1.4 Prothrombin time determination

Blood samples were collected into citrated tubes, then centrifuged at 2000 g for 10 min. The PT (in seconds) was assessed in duplicate using a Biomerieux Option 2 Plus (Behnk Electronick, Norderstedt, Germany) with the Neoplastin CI, INR Determination kit (Diagnostica Stago, Asniere, France). The PT was determined in 100 μl samples of plasma with thromboplastin, according to the manufacturer’s instructions. Each value was the mean of 2 measurements.

1.5 Data analysis

Statistical analyses were also performed using GraphPad Prism 7.04 (GraphPad Software, La Jolla CA, USA) and SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Pharmacokinetic calculations were performed using the noncompartmental approach. Chlorophacinone concentrations in milk were used to estimate the elimination rate constant and half-life from daily samples collected between 48 and 168 h post-exposure, except for one animal having a concentration below the LOQ for the 168-h time point. The half-life of elimination ($t - \frac{1}{2}(el)$) was calculated using a linear regression.

For residues in milk (<50% of samples under the LOQ), the Kaplan-Meier method was used to estimate the extremes of the mean in the whole flock[23]. Lowest bound was calculated replacing values under the LOQ by 0.001 ng/mL, while highest bound was obtained by replacing those values by the value of the LOQ (i.e. 0.1 ng/mL).

2. Results

2.1 Clinical description of the flock

None of the ewes exhibited overt signs of intoxication at the time accidental chlorophacinone exposure was discovered. Vitamin K1 was not administered, and in the month following ingestion no clinical signs appeared in any of the animals.

2.2 Post-exposure measurements during week 1

On day 3 post-exposure, chlorophacinone was detected at quantifiable concentrations (LOQ = 2 ng/mL) in plasma of 18 of the 23 ewes (78.3%). Plasma concentrations (median = 47.6 ng/mL) are presented in Fig. 1A. Seven ewes had plasma chlorophacinone concentrations > LOQ to ≤50 ng/mL, and 8 were between >50 and 500 ng/mL. Three ewes had plasma

levels >500 ng/mL, with a maximum concentration of 1800 ng/mL found in 2 animals.

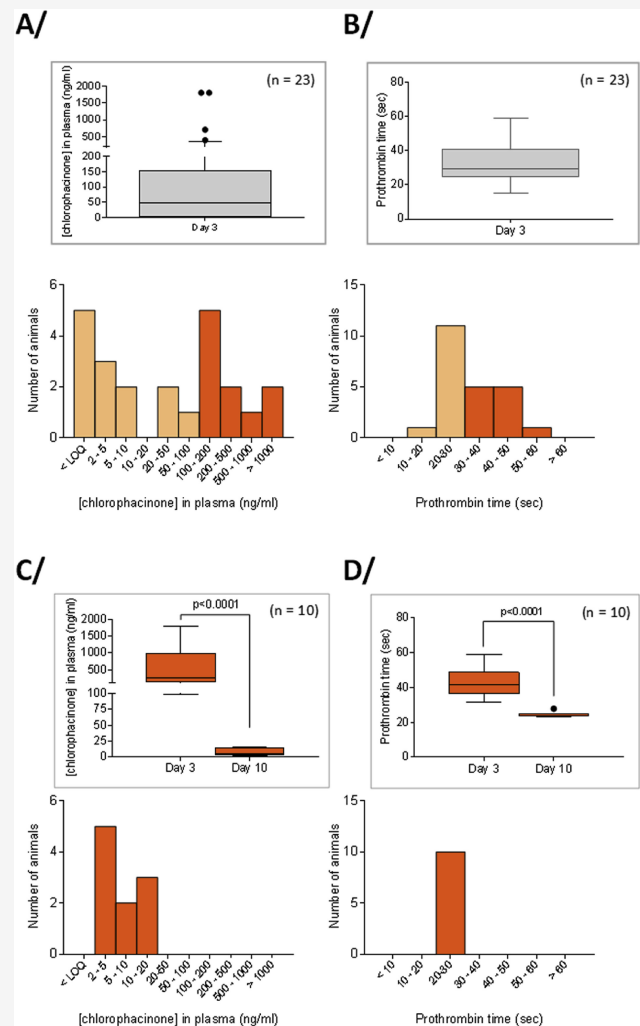


Figure 1. Distribution of chlorophacinone concentrations in plasma and prothrombin time on Days 3 and 11. A/Box plot and distribution of chlorophacinone in plasma on Day 3. B/Box plot and distribution of prothrombin time on Day 3. C/Box plot and comparison of chlorophacinone on Days 3 and 11, and distribution of chlorophacinone in plasma on Day 11. D/Box plot and comparison of prothrombin time on Days 3 and 11, and distribution of prothrombin time on Day 11.

On day 3 post-exposure, PT was determined for each animal (Fig. 1B). Median value in the flock was 29.3 s, and all values were within 15–60 s. Clotting time was compared among groups of ewes with plasma chlorophacinone concentrations assigned the LOQ or in the ranges of >LOQ to 50 ng/mL, >50–500 ng/mL, and >500 ng/mL, and differences were apparent (analysis of variance $F_{3,19} = 23.08$, $p < 0.0001$). On day 3, ewes with plasma chlorophacinone >50 ng/mL exhibited prolonged PT (Tukey’s honestly significant difference, $p = 0.025$) compared to those with plasma chlorophacinone assigned the LOQ (Table 1).

Table 1. Prothrombin time and plasma chlorophacinone concentration of accidentally exposure flock ewes on day 3.

Plasma chlorophacinone	N	Prothrombin time (mean ± SD in sec)
LOQ	5	27.45 ± 4.46 ^a
>LOQ to 50 ng/mL	7	23.68 ± 1.92 ^a
>50–500 ng/mL	8	28.02 ± 10.67 ^b
>500 ng/mL	3	50.83 ± 7.47 ^d

Means with different letter superscripts are different by Tukey’s HSD test ($p < 0.025$).

Chlorophacinone was detected at quantifiable concentrations in milk in 13 of 18 animals sampled on day 2 and remained quantifiable in 12 of these animals for 5 additional days (Table 2). For these 12 animals, the mean chlorophacinone value in milk on day 3 was 3.37 ng/mL, and all values were ≤ 8.9 ng/mL. Notably, for these 12 animals, plasma concentrations were near or >50 ng/mL (one individual at 47.59 ng/mL) on day 3. In milk of the other 11 animals, chlorophacinone was not detected or detected at concentrations ≤ 1 ng/mL on day 3. The frequency distribution of chlorophacinone concentration in milk of all ewes on day 3 is presented in Fig. 2. Seven days after accidental exposure, chlorophacinone concentration in milk of all animals was <1 ng/mL.

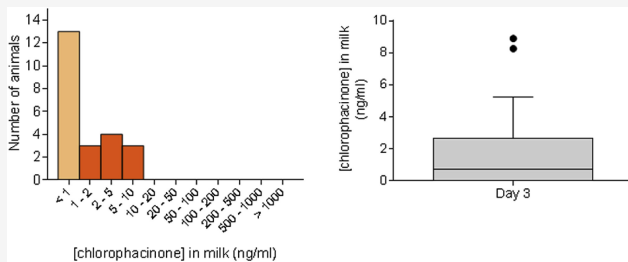


Figure 2. Distribution and box plot of chlorophacinone concentrations in milk on Day 3 ($n = 23$ animals).

2.3 Post-exposure measurements day 11

On day 11 (i.e., 8 days after the initial sample collection), chlorophacinone concentration was measured in plasma of 10 animals that exhibited concentrations ≥ 100 ng/mL on day 3 post-exposure. Values ranged from 2.01 to 16.37 ng/mL (Fig. 1 C) and were significantly lower (paired t-test, $p = 0.0256$) than those collected on day 3. For each individual, the remaining fraction of initial plasma concentration varied between 0.80 and 4.12%, with a mean value of 1.93% (SD = 0.99).

Prothrombin time values in the samples collected 11 days after chlorophacinone ingestion were distributed in a narrow range (23.2–27.85 s), with a mean value of 24.25 s (SD: 0.44) (Fig. 1D), and did not differ from PT values of the 10 of 23 animals with plasma chlorophacinone concentration below 10 ng/mL on day 3 (i.e., animals with low exposure to chlorophacinone on day 3)(unpaired t-test, $p = 0.58$).

2.4 Pharmacokinetics of chlorophacinone in milk

Chlorophacinone was quantifiable in milk of 12 ewes from days 2 through 7. Two ewes exhibited an unexpected increase in the concentration of chlorophacinone in milk on day 4 and those animals were excluded from pharmacokinetic calculations (i.e., R^2 values of 0.44 and 0.24 for excluded individuals compared to $R^2 > 0.85$ for other ewes; Fig. 3 and discussed in section 3.1). Using simple regression modelling of chlorophacinone elimination from milk, elimination half-life for each animal was estimated (mean $t_{1/2} = 48$ h) (Table 3). Extrapolated initial concentrations in milk varied between 1.60 and 30.12 ng/mL (Table 2). Kinetics were similar for all tested animals (Fig. 3 – right: mean $K_{el} = 0.01489$; $R^2 = 0.9676$) over a wide range of initial concentrations in plasma and milk.

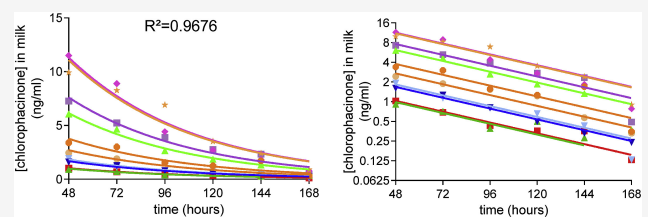


Figure 3. Changes in chlorophacinone concentrations in milk over time (mean $K_{el} = 0.01489$; $R^2 = 0.9676$, $n = 10$ animals)..

2.5 Relationship between chlorophacinone concentration in plasma and milk

A positive relation between the residues in the plasma and the milk was observed, as determined by linear regression ($R^2 = 0.9603$, $p = 0.002$; Fig. 4). Such a correlation suggests a dose-dependent mammary transfer.

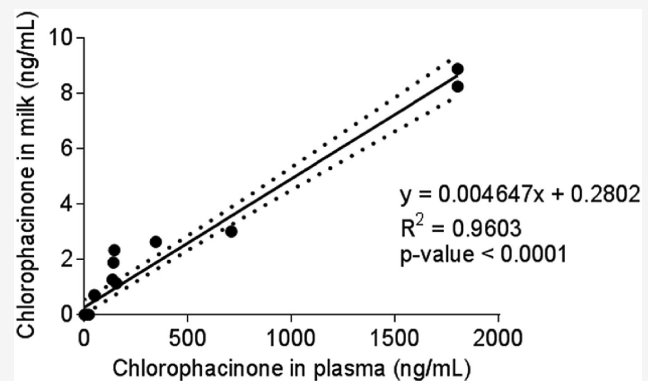


Figure 4. Relation between chlorophacinone residues in milk and plasma. Dotted lines represent the 95% confidence intervals for the linear regression model. Statistical analysis was done to test the hypothesis of a slope significantly non zero.

3. Discussion

Table 2. Concentration of chlorophacinone in milk (>LOQ) of ewes between days 2 and 7 post-accidental exposure for animals with detectable chlorophacinone and for the entire flock.

Day post-exposure	2	3	4	5	6	7
<i>Animals with detectable chlorophacinone in milk^a</i>						
Number of animals	13	12	12	12	12	12
[chlorophacinone] in milk (ng/ml)	3.84 [0.40–11.50]	3.37 [0.69–8.89]	2.57 [0.39–6.91]	1.53 [0.36–3.52]	1.08 [0.28–2.41]	0.37 [0.13–0.90]
<i>Entire flock (n = 23)^b</i>						
[chlorophacinone] in milk (ng/ml)	2.17–2.22 [LOQ–11.50]	1.76–1.80 [LOQ–8.89]	1.45–1.50 [LOQ–6.91]	0.80–0.85 [LOQ–3.52]	0.57–0.61 [LOQ–2.41]	0.19–0.24 [LOQ–0.90]

^a Values are mean and [range of values] for animals with detectable chlorophacinone in milk.

^b Values are Kaplan-Meier extremes of the mean and [range of values] for the entire flock.

Table 3. Changes in chlorophacinone concentrations in milk over time (mean $K_{el} = 0.01489$; $R^2 = 0.9676$, n = 10 animals).

	K_{el} (h^{-1}) [95% CI]	$T_{1/2}$ (h) [95% CI]	C_0 (ng/ml) [95% CI]	R^2
Animal 1	0.01277 [0.007909–0.01762]	54.26 [39.33–87.62]	4.68 [2.89–6.48]	0.951
Animal 2	0.01461 [0.009647–0.01957]	47.33 [35.41–71.83]	2.03 [1.27–2.78]	0.9591
Animal 3	0.01179 [0.003483–0.02011]	58.77 [34.46–198.9]	1.60 [0.57–2.63]	0.8891
Animal 4	0.01729 [0.01272–0.02186]	40.08 [31.70–54.48]	4.32 [2.93–5.71]	0.978
Animal 5	0.01424 [0.009652–0.01882]	48.66 [36.82–71.79]	14.58 [9.51–19.64]	0.9653
Animal 6	0.01706 [0.01255–0.02157]	40.62 [32.13–55.22]	14.26 [9.71–18.81]	0.9789
Animal 7	0.01557 [0.01222–0.01892]	44.50 [36.63–56.71]	3.53 [2.66–4.39]	0.9848
Animal 8	0.01913 [0.01324–0.02502]	36.22 [27.69–52.34]	30.12 [18.13–42.11]	0.9737
Animal 9	0.01258 [0.004087–0.02106]	55.08 [32.90–169.56]	6.44 [2.11–10.77]	0.8527
Animal 10	0.01386 [0.007176–0.02055]	50.00 [33.72–96.5]	20.70 [10.11–31.29]	0.9284
All animals	0.01489 [0.01321–0.01657]	47.55 [42.35–52.76]	/	

Results are means with 95% confidence interval. K_{el} : calculated elimination constant; $T_{1/2}$: calculated elimination half-life; C_0 : extrapolated initial concentration in milk.

3.1 Chlorophacinone transfer in milk

As previously reported in the literature [13] our findings demonstrate mammary transfer of ingested chlorophacinone to milk. However, only a small fraction of the chlorophacinone in plasma was found in milk, with proportions varying between 0.8 and 4.1%. Three days after accidental chlorophacinone ingestion, concentrations in milk were very low (<15 ng/mL) while plasma concentrations remained high (ranging up to 1800 ng/mL). Seven days after chlorophacinone ingestion, concentrations in milk were <1 ng/mL.

It should be noted that 2 ewes exhibited an unexpected increase in chlorophacinone concentration in milk on day 4. Two hypotheses might explain such elevated values: (i) possible new exposure, or (ii) a delay in absorption of chlorophacinone from the digestive tract of those two animals. A larger-scale study would be necessary to more fully examine inter-individual variability.

This mammary transfer appears to be dose-dependent, as suggested by the positive relation between the residues in plasma and milk (Fig. 4). This same dose-dependent transfer has been observed in cows intra-ruminally treated with another AR, diphenadione (i.e., diphacinone) [14]. Proportions of mean quantifiable plasma to milk concentrations in cows, calculated from the data provided at each time point, varied between 0.5 and 1%, a rate similar to that estimated in the present study.

One of the limitations of the present accidental exposure event was that the quantity of chlorophacinone ingested by each animal was unknown. It would be interesting to explore the possible relation between ingested amounts of chlorophacinone and concentrations in milk. The total ingested quantity of chlorophacinone was 1 g, and based on plasma concentrations at day 3, only 10 animals had values above 100 ng/mL, suggesting a significant quantity of chlorophacinone was ingested by those animals. Assuming that all of the AR was ingested by those 10 ewes, and was distributed equally among them, the dose per animal would be 1.4 mg/kg BW (using a standard weight of 70 kg). For sheep treated intra-ruminally with 1 mg chlorophacinone/kg BW [6], mean plasma concentration after 72 h was about 1000 ng/mL. In the present accidental exposure event, if in fact 1 g was ingested by only 10 ewes, the observed plasma concentrations at 72 h post-exposure (range 100–1800 ng/mL, mean = 570 ng/mL) were within the range of values reported by Berny and coworkers (2006) [6]. Thus, the present hypothesis of a mean ingested amount of 1.4 mg/kg BW per animal corresponds to a consumption of 98 mg per animal for a standard 70-kg ewe. In the present study, mean extrapolated initial concentration (C_0) in milk is 10.53 ng/mL. Assuming that daily milk production for one ewe is about 1.5L, at day 1 each animal would have 0.016 mg ($C_0 \times$ milk production) of chlorophacinone in its milk. Assuming that all chlorophacinone was transferred into milk in 24 h, this amount represents 0.00016% of the ingested quantity. Thus, only a small fraction of ingested AR undergoes mammary transfer.

3.2 Milk residues of chlorophacinone and human health issues

There is no MRL defined for ARs in feed, and only limited data are available regarding the toxicity of chlorophacinone in humans (Standing Committee on Biocidal Products, 2009). Data from literature indicate that acute ingestion of between 100 mg and 250 mg of chlorophacinone causes prolonged clotting time in adults, but these dosages are only based on a few clinical cases [21, 24]. Using these data for prolonged clotting time, we attempted to estimate the human health risk associated with consumption of milk produced by the exposed ewes.

Notably, risk estimations have been conducted for diphenadione (i.e., diphacinone; [14, 24]) that was used both as an AR and as a therapeutic agent in man, with a dosage between 2.5 and 30 mg per individual. It was estimated that an adult human would have to drink about 115L of milk containing the maximal quantity found in milk of ewes orally administered with 2.75 mg/kg BW of diphenadione (i.e., diphacinone, 21 ng/mL), to reach the therapeutic dosage of 2.5 mg [14]. However, such data are not available for chlorophacinone, making extrapolation challenging. A safety factor of 1000 was used to extrapolate the human health risk associated with consumption of game animals containing diphacinone employing toxicity data in rats [24]. If such a safety factor is applied to the LD50 of chlorophacinone in rats (3.15 mg/kg BW; ACTA, 2018), a dose with unacceptable risk for humans would be 0.003 mg/kg BW (i.e., 0.18 mg of chlorophacinone for a standard adult of 60 kg). The maximal concentration of chlorophacinone found in milk in our study was 12 ng/mL on day 2 post-ingestion (Fig. 2). If this chlorophacinone concentration in milk is used to assess the human health risk associated with consumption, one standard adult would have to drink 14.3L of milk to reach this dosage, or eat 2.4 kg of ewe cheese [(as 6 L of milk are needed to make 1 kg of cheese; this assumes that the AR would not affect the coagulation properties of milk, which may not be the case [25] and that cheese formation would not affect AR concentration]. To provide even a more accurate estimate, one should use the concentration found in the entire milk tank (i.e., a mixture of milk from all 23 ewes) rather than the greatest value found in a single animal at the beginning of the accidental exposure period. While this approach uses a large safety factor, risk assessments based solely on LD50 are not robust.

Another study presented the use of a safety factor of 300 to calculate the human health risk from the NOAEL of chlorophacinone after repeated oral exposure in rats (i.e., 0.005 mg/kg BW/day for 90 days; Standing committee on biocidal products, 2009). Using this factor, a standard adult weighing 60 kg would have to ingest 0.0010 mg/day, which corresponds to a daily consumption of 0.09L of the most contaminated milk or 17 g of cheese. Taking the regular safety factor generally used to calculate the Acceptable Daily Intake (ADI) of chemicals in feed (i.e., 100), exceedance would require more than 0.27L of milk or 51g of cheese daily for a standard adult. Such

estimations are a “worst-case scenario” that do not take into account previously described considerations (i.e., dilution in the tank by the addition of milk from unexposed ewes and declining chlorophacinone concentrations related to its 2 day elimination half-life in exposed ewes). If applying this same worst-case scenario at day 7 post-ingestion (with maximal measured concentration of 0.9 ng/mL), a standard adult could drink up to 1.1 L of the most contaminated milk daily for 90 days without exceeding the estimated ADI.

To obtain a more conservative estimate, this approach could be applied to the mean chlorophacinone concentration in milk from the entire flock ($n = 23$) at day 2 post-ingestion (i.e., 2.2 ng/mL). In this scenario, using a safety factor of 300, a standard adult weighing 60 kg (CVMP, 2018) could consume 0.45 L of milk or 76 g of cheese daily, and three times those quantities if using a safety factor of 100.

In this more realistic scenario, it is possible to estimate the “withdrawal period” for the milk from the entire flock (exposed and unexposed individuals) that was mixed in the tank. According to WHO’s model food basket (FAO/WHO Expert Committee on Food Additives, 2006), an adult consumes 1.5L of milk daily. Taking the dose of 0.0010 mg/day as a reference for the ADI, the “maximal residue limit” would be 0.7 ng/mL. According to Table 2, using the Kaplan-Meier method, the mean chlorophacinone concentration in the tank would be under this value at day 6. It is to be noted that this estimation is liberally adapted from the reference method to establish MRLs for veterinary drugs, and provided results are only rough estimations.

Thus, even in the worst-case scenario, the human health concern for consumption of chlorophacinone contaminated dairy products seems low. Still, this study is only a case report based on a small sample size, and not a robust controlled blinded study. It should be viewed as a preliminary risk assessment, and may only be valid for chlorophacinone.

3.3 Using clinical parameters to estimate residues levels in milk?

3.3.1 Species sensitivity to chlorophacinone intoxication

During the study, and in the absence of vitamin K1 administration, none of the ewes exposed to the AR chlorophacinone displayed overt clinical signs, even those with highest plasma levels. Those observations are in line with a suspected lack of sensitivity of ruminants to AR intoxication, as described in literature. For example, cattle experimentally administered doses up to 5 mg/kg BW of diphenadione (i.e., diphacinone) did not display any clinical signs [14, 15], and Texel sheep orally administered 1 mg/kg BW of chlorophacinone did not display overt signs of intoxication, but did exhibit a slight increase of bleeding time at the venipuncture site [6].

These findings are corroborated by statistics from the French National Center for Veterinary Toxicological Information (CNITV). Between 2007 and 2017, this poison call-center received 398 calls about possible or probable intoxications with AR in ruminants (cattle, goats, sheep - i.e., 2.2% of

the calls regarding this category of biocide). Total number of exposed animals was 2043 individuals, and only 216 of them (i.e. 10.5%), displayed clinical signs, without further details on the severity of those signs. This presumed lack of sensitivity could be related to a dilution effect in the rumen, endogenous production of vitamin K1 in the rumen, or dietary vitamin K in their feed composed of leafy greens. Thus, based on these results, overt symptoms are not a good indicator of the quantity of AR ingested in ruminants.

3.3.2 Evaluation of the relationship between PT and residues in plasma and milk

An increase in PT was observed in some of the “exposed” ewes (plasma chlorophacinone concentration > LOQ: mean PT value = 34.1 s, $n = 18$ animals) when compared to “seemingly unexposed” individuals (assigned LOQ: mean PT value = 27.45 s, $n = 5$ animals). Mean clotting time on day 3 increased by about 137% in ewes with plasma chlorophacinone between >50 and 500 ng/mL, and by about 185% in animals with plasma chlorophacinone >500 ng/mL. This variation is similar to the one observed in cattle [14, 15], sheep [6] or goats receiving pindone [13]. In the study by Berny and coworkers [6], baseline PT was around 13.25 s (SD = 0.52), increasing up to 27–40.7 s after administration of the AR. No definitive reference baseline value could be found for PT in sheep, and values may differ among breeds and the analytical method used [26]. Median values found in the literature varied from 7.31 s (Leite Carlos et al., 2017[27] – $n = 20$ “mixed breed” ewes) to 40 s (Siller-Matula et al., 2008[28] – $n = 6$ Austrian Mountain ewes). In our study, a control group (known to be unexposed) would have permitted determination of a baseline value for the breed. However, the mean PT value of “unexposed” animals is a good proxy of such a reference value.

Despite the observed increase, no simple linear relation exists between PT and concentrations of chlorophacinone in plasma and milk (Fig. 5), but a “threshold-level effect” is seemingly apparent on day 3 at plasma concentrations >50 ng/mL and milk concentrations >2 ng/mL. Thus, while PT can serve as an indicator of exposure and adverse AR effects, it cannot be used to predict the residue levels in the milk of dairy animals intoxicated with chlorophacinone, highlighting the need for better indicators of exposure.

4. Conclusion

This study demonstrates minimal mammary transfer of ingested chlorophacinone in accidentally-exposed ewes, and seemingly low risk for consumers of their dairy products after a one-week withdrawal period. Based on plasma chlorophacinone levels, transfer to milk is dose-dependent and suggests that milk could serve as a non-invasive proxy for AR exposure. However, clinical markers used for routine monitoring in animals (overt signs of intoxication, changes in PT) did not permit estimation of the magnitude of the primary exposure of ewes, and therefore the potential risk related to consumption

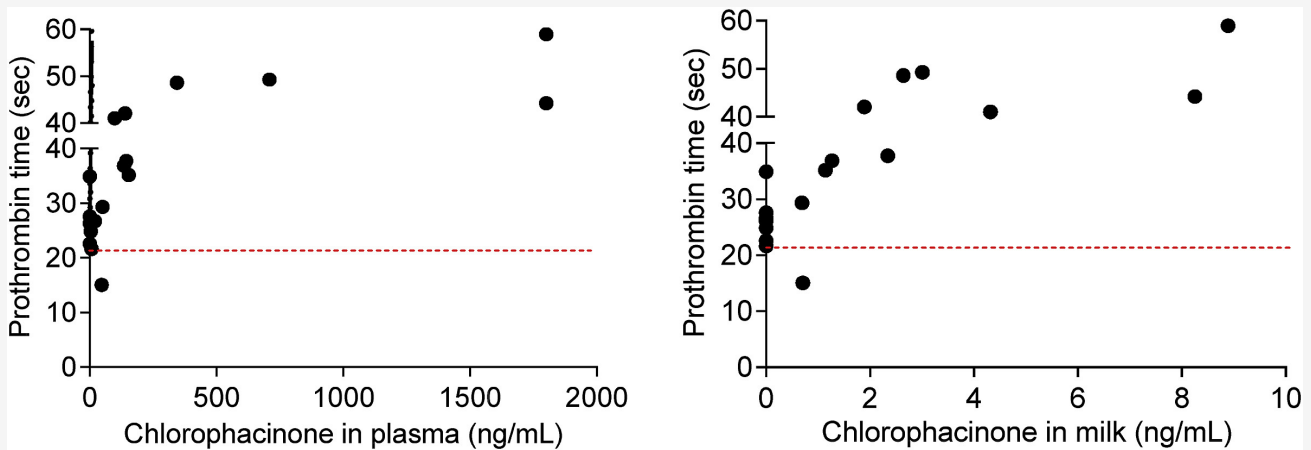


Figure 5. Prothrombin time versus chlorophacinone concentration in plasma (A) and milk (B).

of their dairy products.

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5.2.1.4 Article :

Exposure of predatory and scavenging birds to anticoagulant rodenticides in France : Exploration of data from French surveillance programs

Exposure of predatory and scavenging birds to anticoagulant rodenticides in France: Exploration of data from French surveillance programs

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Abstract

Wild raptors are widely used to assess exposure to different environmental contaminants, including anticoagulant rodenticides (ARs). ARs are used on a global scale for rodent control, and act by disruption of the vitamin K cycle that results in haemorrhage usually accompanied by death within days. Some ARs are highly persistent and bioaccumulative, which can cause significant exposure of non-target species. We characterized AR exposure in a heterogeneous sample of dead raptors collected over 12 years (2008-2019) in south-eastern France. Residue analysis of 156 liver samples through LC-MS/MS revealed that 50% (78/156) were positive for ARs, with 13.5% (21/156) having summed second-generation AR (SGAR) concentrations >100 ng/g ww. While SGARs were commonly detected (97.4% of positive samples), first-generation ARs were rarely found (7.7% of positive samples). ARs were more frequently detected and at greater concentration in predators (prevalence: 82.5%) than in scavengers (38.8%). Exposure to multiple ARs was common (64.1% of positive samples). While chlorophacinone exposure decreased over time, an increasing exposure trend was observed for the SGAR brodifacoum, suggesting that public policies may not be efficient at mitigating risk of exposure for non-target species. Haemorrhage was observed in 88 birds, but AR toxicosis was suspected in only 2 of these individuals, and no difference in frequency of haemorrhage was apparent in birds displaying summed SGAR levels above or below 100 ng/g ww. As for other contaminants, 17.2% of liver samples (11/64) exhibited Pb levels compatible with sub-clinical poisoning (>6 µg/g dw), with 6.3% (4/64) above the threshold for severe/lethal poisoning (>30 µg/g dw). Nine individuals with Pb levels >6 µg/g dw also had AR residues, demonstrating exposure to multiple contaminants. Broad toxicological screening for other contaminants was positive for 18 of 126 individuals, with carbofuran and mevinphos exposure being the suspected cause of death of 17 birds. Our findings demonstrate lower but still substantial AR exposure of scavenging birds compared to predatory birds, and also illustrate the complexity of diagnosing AR toxicosis through forensic investigations.

Keywords

Raptors; Biomonitoring; Exposure; Anticoagulant rodenticide

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Introduction

Wild raptors are widely used in Europe for biomonitoring trends in environmental contamination, natural resource management and as sentinels for human health, although harmonization of pan-European monitoring programs has yet to be achieved [1–3]. In free-ranging raptors, many studies have assessed exposure to various environmental contaminants, including lead (Pb) [4–8], veterinary drugs (e.g. diclofenac) [9–12], pesticides (e.g., organophosphorus and carbamates) [13, 14], and anticoagulant rodenticides (ARs) [15–20]. Anticoagulant rodenticides have been widely used since the 1950's for pest control [21]. Widespread resistance of rodents to some first generation ARs (FGARs; e.g., warfarin) became apparent in the 1950's, which eventually resulted in the development of second-generation compounds (e.g., bromadiolone, brodifacoum, difenacoum, difethialone and flocoumafen; [22–25]). ARs disrupt the vitamin K cycle by inhibiting vitamin K epoxide reductase (VKOR), leading to impaired blood coagulation that can be fatal to both target pest species and non-target species (reviewed in [26]).

Second-generation ARs (SGARs) are persistent, bioaccumulative and toxic compounds, and are currently the most commonly used products to control rodent populations. Non-target exposure to ARs may be primary (e.g., direct consumption of baits) or secondary (e.g., consumption of intoxicated or dead rodents that had been exposed to ARs), and can include transfer through food webs [27]. Multiple AR residues reflect repeated exposure that could lead to adverse effects [19] and also reflect the complexity of exposure pathways [27, 28]. Contamination of non-target raptors with SGARs is globally widespread [15–20, 29]. Prevalence of ARs exposure in wild predatory birds and mammals was recently estimated as 58%, reviewing data from North America (Canada, USA), Europe (UK, Denmark, France and Spain) and New Zealand between 1984 and 2015 [30]. Data are more scarce for scavenging birds.

In France, ARs are authorized as biocides to be used by professionals and the public in urban and suburban settings (European Biocidal Product Regulation N°528/2012) and are also used as plant protection products (PPP, e.g. for the control of the European water vole *Arvicola amphibius* in crops and meadows up to 2020) [31]. Chlorophacinone was used as a PPP in France till the end of 2010 (Ministère de l'Agriculture et de la Pêche, 2007), and bromadiolone was authorized for this use till its recent withdrawal (21 February 2020 - see https://www.anses.fr/fr/system/files/phyto/decisions/SUPERCAIDA_PREX_2015-1399_D.pdf) by the French Agency for

Food, Environmental and Occupational Health and Safety. Other ARs are only authorized as biocides. Regulatory measures were also implemented in an attempt to reduce SGAR exposure of non-target fauna through biocidal use. Starting in 2013 within the European Union, rodenticide baits to be placed indoors or in and around buildings must be in tamper-resistant bait stations. These stations limit primary exposure of non-target species by physically preventing access to baits [32–34], but regrettably still may permit bait access of non-target species of a similar size as pest rodents. To our knowledge, the success of these public policies has not been thoroughly assessed.

Diagnosis of AR lethal intoxication is complex, generally associating post-mortem lesions suggestive of AR poisoning (i.e., haemorrhages) and quantifiable residues in the liver [35]. However, there is no consensus on the toxicity threshold to distinguish between sublethal exposure and poisoning [19]. An often cited toxicity threshold for SGAR levels of >100 to 200 ng/g liver wet weight (ww) has been suggested to be potentially lethal [36], although both field observations and modeling efforts indicate that SGAR toxicity and lethality can occur at much lower levels (Thomas et al., 2011), and species-specific differences in sensitivity are notable [19]. It was also recently suggested that due to large differences in molecular weight and VKOR inhibitory potency, summing of ARs should be limited to second-generation ARs, and it may be appropriate to present exposure data on both a summed mass basis and summed molar basis [19, 37].

Herein we present findings characterizing prevalence and factors affecting AR exposure in raptors sampled in France, through analysis of residues in liver of dead predatory and scavenging birds collected over a 12-year period by several bird of prey surveillance programs. The effects of public policies to mitigate risk to non-target fauna were also assessed. We examined diagnosis of AR intoxication using both hepatic AR concentration and molar values that may better represent exposure. As these avian samples were part of broad biomonitoring programs, results of other toxicological analyses (lead, some pesticides and pharmaceuticals) are briefly mentioned to emphasize concomitant exposure to multiple contaminants in wild birds.

1. Material and methods

1.1 Collection of postmortem data: use of a French surveillance database

Raptors (in most instances carcasses) were collected over a 12 year period (2008–2019) from several bird of prey surveillance programs [e.g., LIFE GYPCONNECT that include partnerships with associations such as the Bird Protection League (LPO), and the National Centre for Veterinary Toxicological Information (CNITV); Supplemental Table 5]. Sampling was principally focused on mountainous areas of southeastern France, from the eastern Pyrenees to Alps, in the regions of Auvergne-Rhône-Alpes, Occitanie and Provence-Alpes-Côte d'Azur, especially in the regional natural parks of Baronnies,

Grands Causses, Vercors and Verdon, and southern department of Aude, which are the working areas for LIFE GYP-CONNECT (see Fig. 1 for the summary of origins of all individuals). Scavenging birds accounted for the majority of samples (see Table 1 for the number of individuals per species collected).

Carcass collections were performed by field agents, usually on the day of the animal death or within a few days thereafter. Carcasses were frozen (-18 degC), and subsequently thawed and necropsied within a variable time frame (several days to a month) by veterinarians of CNITV. While those veterinarians were not pathologists, necropsies were supervised by a senior veterinarian with over 20 years of experience in bird of prey necropsy. For each specimen, history of the carcass (collection location, date of discovery, description of the area) was provided, and information on the species, sex, as well as descriptions of the external aspects and of all organs were compiled during necropsies. Radiographs were performed, and gastric content, liver, kidney, muscle and bone were sampled. Only liver samples were used for this study.

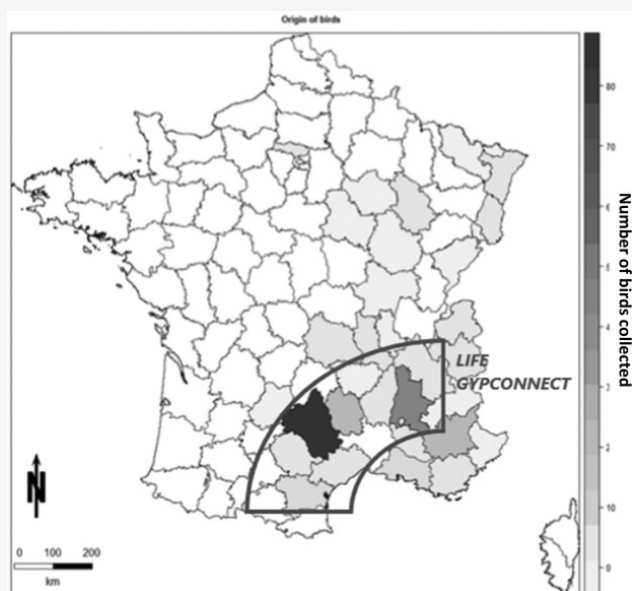


Figure 1. Number of birds collected per department (n = 245) (gray frame: working area of LIFE GYPCONNECT).

Findings were coded in a database, with details on lesions, results of toxicological analysis and possible cause of death. All necropsy reports in the database were analysed to identify elements for further exploration. Parameters which were not systematically documented (e.g. age of birds and state of decomposition) were not coded for further analysis. Presence/absence of haemorrhages was coded based on keywords in the report (“haemorrhage”, “bleeding”, “anaemia”, “bruising”, “haematoma”, “blood”), and reports with at least one of these keywords were read to determine whether or not the animal had displayed haemorrhages. Conclusions of reports were analysed, with cause of death classified in six categories [e.g., Electrocutation, Trauma, Intoxication, Other (external

cause; suspected drowning, poisoning), Other (internal cause; suspicion of a tumoral process, cardiopathy, infectious disease, etc.), and Unknown].

1.2 Sample analysis

AR residue analysis of liver samples was conducted using a previously described liquid chromatography with tandem mass spectrometry (LC-MS/MS) method [38]. Analytes quantified included 3 first-generation ARs (i.e., warfarin, coumatralyl, chlorphacinone) and 5 s-generation ARs (SGARs) used in Europe (i.e., bromadiolone, brodifacoum, difenacoum, flocoumafen and difethialone). Limit of quantification (LOQ) was 2 ng/g ww. All analyses (samples collected between 2008 and 2019, stored at -80 °C) were performed between 2016 and 2020 using the same analytical procedures.

As collected birds were part of broad toxicological surveillance programs, it should be noted that some of the samples were also sent to other external laboratories for lead (Pb) analysis (French departmental laboratory of Drôme, LDA 26) by inductively coupled plasma mass spectrometry (ICP-MS) and for a broad toxicological screening with a panel of more than 500 environmental contaminants (mostly pesticides and pharmaceuticals, laboratory Ecoloxie-La Voulte) using LC-MS/MS. Those labs are accredited by a French committee (COFRAC). Results from those other analyses will only be briefly mentioned in this paper.

1.3 Statistical analyses

Descriptive statistics and graphs were generated with R software (version 3.5.1) associated with RStudio (version 1.1.453). Concentrations of ARs in liver were expressed as ng/g ww, and also converted to pmol/g ww (or equivalent nmol/kg ww) using the molecular weight for each AR (PubChem: chlorphacinone = 374.8 g/mol bromadiolone = 527.4 g/mol, brodifacoum = 523.4 g/mol, difenacoum = 444.5 g/mol, difethialone = 539.5 g/mol). As warfarin, coumatralyl and flocoumafen concentrations were consistently below the LOQ, those ARs were excluded from statistical analyses.

For descriptive results of the levels of ARs (analytes with <50% of samples below the LOQ), the Kaplan-Meier method was used to estimate the extremes of the mean [39]. Lower bound was calculated replacing values below the LOQ by 0.02 ng/g ww, while upper bound was obtained by replacing those values by the LOQ (i.e., 2 ng/g ww). For each species, the range of values, number of animals above LOQ and number of animals with a sum of SGARs above the literature threshold of 100 ng/g ww[36] were described.

To assess AR exposure by feeding regimen, a simple classification was implemented. All cultures were categorized as scavengers, while other raptors, including facultative scavengers (e.g., Golden eagle, *Aquila chrysaetos*), were classified as predators. Chi-squared test was used to detect differences in AR exposure between scavengers and predators for prevalence data (e.g., AR detection rate) and t-test for continuously distributed variables (e.g. summed SGARs concentration). Other factors, such as presence of multiple ARs, species (retaining

Exposure of predatory and scavenging birds to anticoagulant rodenticides in France: Exploration of data from French surveillance programs — 4/19

Species	n	Foraging behavior	AR exposed	CHLORO	BROMA	BFC	DFC	DFT	Multiple ARs
			/N samples tested (%)						
			N exposed (% of tested samples N) (% of AR exposed)						
Griffon vulture (Gyps fulvus)	139	Scavenger	29/90 (32)	3 (3) (10)	17 (19) (59)	10 (11) (34)	10 (11) (34)	13 (14) (45)	13 (14) (45)
Red kite (Milvus milvus)	32	Predator	16/16 (100)	0	14 (87) (87)	15 (94) (94)	9 (56) (56)	7 (44) (44)	14 (87) (87)
Cinereous vulture (Aegypius monachus)	26	Scavenger	13/17 (76)	2 (12) (15)	10 (59) (77)	5 (29) (38)	6 (35) (46)	6 (35) (46)	7 (41) (54)
Bearded vulture (Gypaetus barbatus)	14	Scavenger	3/9 (33)	0	2 (22) (67)	1 (11) (33)	2 (22) (67)	2 (22) (67)	2 (22) (66)
Bonelli's eagle (Aquila fasciata)	10	Predator	5/7 (71)	0	4 (57) (80)	4 (57) (80)	5 (71) (100)	4 (57) (80)	4 (57) (80)
Golden eagle (Aquila chrysaetos)	10	Predator	7/7 (100)	0	6 (86) (86)	7 (100) (100)	6 (86) (86)	7 (100) (100)	7 (100) (100)
Common buzzard (Buteo buteo)	4	Predator	1/3	0	1	1	1	1	1
Black kite (Milvus migrans)	2	Predator	1/1	0	0	1	0	0	0
Eurasian eagle-owl (Bubo bubo)	2	Predator	2/2	1	2	2	2	2	2
Hen harrier (Circus cyaneus)	2	Predator	0/2	0	0	0	0	0	0
Common kestrel (Falco tinnunculus)	1	Predator	NA	-	-	-	-	-	-
Eurasian hobby (Falco subbuteo)	1	Predator	0/1	0	0	0	0	0	0
Peregrine falcon (Falco peregrinus)	1	Predator	NA	-	-	-	-	-	-
Short-toed snake eagle (Circaetus gallicus)	1	Predator	1/1	0	1	0	0	0	0
TOTAL	245		78/156 50	6 (4) (8)	57 (37) (73)	46 (29) (59)	41 (26) (53)	42 (27) (54)	50 (30) (60)

Table 1. Species, sample size, foraging behaviour and frequency of exposure to ARs of raptors collected between 2008-2019

only species with sample sizes ≥ 5 individuals), gender and area of discovery (department), were also tested. Parametric tests (t-test) were used for sample size ≥ 30 individuals, while non-parametric procedures (Wilcoxon or Kruskal-Wallis, depending on the number of categories) were used for smaller sample size comparison of AR concentration.

To evaluate the effect of change in public policies in France related to AR usage on detection frequency, samples were grouped into 3 different key temporal periods: 2008–2010 (end of chlorophacinone use as a plant protective product in 2010), 2011–2013 (new regulations on SGARs in 2013, brodifacoum, difenacoum and difethialone only to be used in bait boxes starting from 2013) and 2014–2019. Differences in exposure among those 3 key periods were assessed for each AR using Fisher's exact test.

The relation between levels of SGARs found in the liver samples and evidence of AR intoxication (i.e., presence/absence of haemorrhage, for each individual) was examined using non-parametric Wilcoxon test. As trauma and electrocution were considered as possible biases for the presence of haemorrhages, individuals with such mortality classifications were removed for additional analysis. To test the commonly reported threshold of 100 ng/g ww, birds positive for SGARs were then classified into 2 groups, namely those with summed hepatic SGARs < 100 ng/g ww or ≥ 100 ng/g ww, and compared using Chi-squared test (all individuals) or Fisher's Exact test (birds with "Unknown" cause of death), based on the presence/absence of haemorrhages. As trauma is a common cause for haemorrhage, relationship between AR exposure and presence of haemorrhage in birds that died from trauma was also tested through Fisher's Exact test.

For liver lead (Pb) concentrations, toxicity thresholds reported in literature were used to classify the results [subclinical poisoning (2 to < 6 $\mu\text{g/g}$ ww, corresponding to 6 to < 18 $\mu\text{g/g}$ dry weight), clinical poisoning (6–10 $\mu\text{g/g}$ ww, i.e., 18–30 $\mu\text{g/g}$ dw), and severe poisoning compatible with death (> 10 $\mu\text{g/g}$ ww or > 30 $\mu\text{g/g}$ dw)] [4, 40].

2. Results

2.1 Study population, causes of death

In total, 245 birds were necropsied, with the most represented species being Griffon vulture (*Gyps fulvus*; $n = 139$, 56.7% of samples), Red kite (*Milvus milvus*; $n = 32$, 13.1%), Cinereous vulture (*Aegypius monachus*; $n = 26$, 10.6%) and Bearded vultures (*Gypaetus barbatus*; $n = 14$, 5.7%). Scavengers accounted for the majority of collected samples (73.1%). Excluding cases for which the cause of death could not be determined (i.e., unknown, $n = 62$, 25.3%), the principal causes of mortality were trauma and electrocution ($n = 71$, 29% and $n = 50$, 20.4%, respectively). Two birds (one with trauma, one electrocuted) were found alive but were euthanised due to poor prognosis for survival. Other reported causes were internal disorders (mainly infectious processes, 13.1%), intoxication (lead or other contaminants, i.e., carbofuran, mevinphos or

pentobarbital, 9.4%) and other external causes (drowning, envenomation, 2.9%).

2.2 AR exposure

Liver samples were not available for all specimens recorded in the database (absent due to consumption of part of the carcass before collection or advanced decomposition), and thus only 156 samples were analysed for ARs (63.7% of individuals).

2.2.1 Prevalence and concentrations of ARs

Of the 156 liver samples analysed, 78 (50.0%) were found positive for at least one AR at a concentration exceeding the LOQ (Table 1). While the FGAR chlorophacinone was present in only 6 birds (4% of all samples, 7.7% of positive samples), SGARs were found in nearly all positive samples (76/78, 97.4%), with bromadiolone being detected most frequently ($n = 57$, 73.1% of positive samples).

Concentrations of individual compounds ranged up to 231.4 ng/g ww (442.1 pmol/g ww; brodifacoum, Red kite) (Table 3). Brodifacoum had the highest median concentration, followed in descending order by difenacoum, bromadiolone and difethialone (Fig. 2). However, concentrations for these 4 SGARs were not different (Kruskal-Wallis $X^2 = 5.40$, $df = 3$, $p = 0.1450$). Chlorophacinone levels were lower when compared to each of these SGARs (Kruskal-Wallis rank sum test, $p < 0.0001$ followed by pairwise Wilcoxon test with Bonferonni correction: chlorophacinone vs brodifacoum or bromadiolone, or difenacoum or difethialone, $p < 0.0001$ for each comparison).

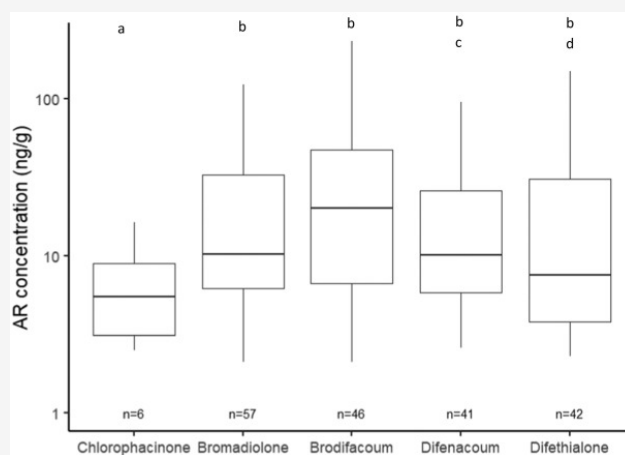


Figure 2. Distribution (boxplot: minimum, first quartile, median, third quartile, and maximum) of detected quantities of each AR in birds; compounds not sharing common letters are different ($p < 0.001$).

Feeding habit	Species	N	CHLORO	BROMA	BFC	DFC	DFT	ΣSGARS	
			Kaplan-Meier mean (in ng/g ww) Range (ng and pmol/g ww) Detects > LOQ					> 100 ng-g-1/detects	
SCAVENGERS	Griffon vulture (<i>Gyps fulvus</i>)	90	0.4-2.3 0.9-6.0 3/90	2.9-4.50 5.3-8.2 1/790	2.6-4.3 4.9-8.2 10/90	0.7-2.4 1.5-3.3 10/90	0.7-2.4 1.5-4.45 13/90	6.9-13.6 13.3-36.67 10/128.0/246.2 29/90 2/9	
		Cinereous vulture (<i>Aegypius monachus</i>)	17	0.7-2.5 2.0-6.6 LOQ-9.1/24.3 2/17	11.94-12.64 22.6-24.0 LOQ-61.0/115.7 10/17	5.0-6.2 9.5-11.9 LOQ-39.3/75.1 5/17	5.4-6.7 12.2-15.1 LOQ-54.6/122.8 6/17	8.4-9.7 15.6-18.0 LOQ-71.4/132.3 6/17	30.8-35.3 59.9-69.0 LOQ-169.6/338.12 13/17 2/13
			9	/ LOQ 0/9	2.4-3.9 4.5-7.4 LOQ-11.9/22.56 2/9	2.2-4.0 4.3-7.6 LOQ-19.9/38.0 1/9	1.7-3.2 3.8-7.3 LOQ-11.2/25.2 2/9	3.8-5.4 7.1-10.0 LOQ-27.7/51.3 2/9	10.1-16.5 19.7-32.3 LOQ-42.2/81.4 3/9
	Red kite (<i>Milvus milvus</i>)	16	/ LOQ 0/16	29.1-29.4 55.2-55.7 14/16	37.5-37.6 71.6-71.9 LOQ-231.4/442.1 15/16	20.0-21.0 45.5-47.1 LOQ-95.3/214.4 9/16	20.0-21.0 37.1-38.9 LOQ-122.3/226.7 7/16	106.8-108.9 299.4-213.6 2.4/4.2-565.3/705.4 16/16 7/16	
		Bonelli's eagle (<i>Aquila fasciata</i>)	7	/ LOQ 0/7	14.1-15.0 26.8-28.40 LOQ-35.4/67.1 4/7	12.8-13.4 24.5-25.6 LOQ-71.0/135.6 4/7	17.1-17.7 38.3-39.8 LOQ-88.0/198.0 5/7	10.9-11.7 20.1-21.7 LOQ-35.2/65.2 4/7	54.9-57.7 109.9-115.4 LOQ-224.0/455.5 5/7 1/5
			Golden eagle (<i>Aquila chrysaetos</i>)	7	/ LOQ 0/7	33.7-33.9 61.6-62.5 LOQ-112.0/212.4 6/7	53.1-53.1 101.4-101.4 3.1/5.9-98.0/187.2 7/7	24.2-24.5 54.5-55.7 LOQ-63.0/141.7 6/7	19.5-19.5 36.7-39.7 LOQ-53.9-253.1 21.3/36.3-204.4/410.0 7/7 6/7
	PREDATORS	Common buzzard (<i>Buteo buteo</i>)	3	/ LOQ 0/3	2.6-3.9 4.9-7.4 LOQ-7.7/14.6 1/3	20.8-22.1 39.7-42.2 LOQ-62.3/119.0 1/3	5.9-7.2 13.2-16.2 LOQ-17.6/39.6 1/3	49.8-51.1 92.3-94.8 LOQ-149.4/276.9 1/3	79.1-84.3 150.2-160.6 LOQ-237.0/450.1 1/1 /
			2	/ LOQ 0/2	/ LOQ 0/2	/ LOQ 0/2	/ LOQ 0/2	/ LOQ 0/2	/ LOQ 0/2
		Eurasian eagle-owl (<i>Bubo bubo</i>)	2	1.8-2.8 4.7-7.3 LOQ-3.5/9.3 1/2	88.4-88.4 167.6-167.6 71.6/135.8-105.2/199.5 2/2	44.4-44.4 84.7-84.7 LOQ-26.6-74.8/142.9 2/2	46.5-46.5 104.5-104.5 30.7/69.1-62.2/139.9 2/2	73.8-73.8 136.6-136.8 180.0/351.1-326.0/636.2 2/2 2/2	
Black kite (<i>Milvus migrans</i>)	1	/ LOQ 0/1	/ LOQ 0/1	/ LOQ 0/1	/ LOQ 0/1	/ LOQ 0/1	/ LOQ 0/1	/ LOQ 0/1	
Short-toed snake eagle (<i>Circus gallicus</i>)	1	/ LOQ 0/1	44.0 83.4 44.0/83.4 1/1	/ LOQ 0/1	/ LOQ 0/1	/ LOQ 0/1	/ LOQ 0/1	/ LOQ 0/1	
Eurasian hobby (<i>Falco subbuteo</i>)	1	/ LOQ 0/1	/ LOQ 0/1	/ LOQ 0/1	/ LOQ 0/1	/ LOQ 0/1	/ LOQ 0/1	/ LOQ 0/1	

Table 3. Hepatic residues of anticoagulant rodenticides (in ng/g ww or pmol/g ww) in raptors collected in France between 2008-2019^a
^a % were reported when sample size was greater than 5; abbreviations: chlorophacinone, CHLORO; bromadiolone, BROMA; brodifacoum, BFC; difenacoum, DFC; difethialone, DFT; not analyzed, NA.

Only 21 individuals (13.5% of samples) had summed SGAR concentrations in liver exceeding 100 ng/g ww (see Table 2 for the details of prevalence per species). When this 100 ng/g ww threshold of summed SGARs for all samples was examined on a molar basis (i.e., using brodifacoum as a reference, 100 ng brodifacoum/g liver ww = 191 pmol/g liver ww), these same 21 individuals had summed SGAR molar values exceeding 191 pmol/g ww. Only 7 birds had a concentration of an individual SGAR above the commonly used threshold of 100 ng/g ww (i.e., 9% of the positive samples).

2.2.2 Factors affecting AR exposure

Foraging behaviour

Anticoagulant rodenticide residues were more frequently detected in predatory birds (33 of 40 samples, 82.5%) than in scavenging birds (45 of 116 samples, 38.8%) (Chi-squared test, $X^2 = 21.01$, $df = 1$, $p < 0.001$). The summed concentration of SGARs was also greater in predators than in scavengers (t-test, $t = 5.45$, $df = 41$, $p < 0.0001$, 95% confidence interval (CI) for difference: 52.9–115.2 ng/g ww), averaging 94.57 ng/g ww in predators vs 10.48 ng/g ww in scavengers (Fig. 3 B). Nevertheless, while concentrations of SGARs were low for the majority of scavengers, levels of summed SGARs in 16 of 116 samples (13.8%) were outliers (values above Quartile 3 + 1.5x interquartile range), with 12 (10.3%) being extreme points (Quartile 3+ 3x interquartile range). For these 12 individuals with extreme values (6 Griffon vultures, 4 Cinereous vultures and 2 Bearded vultures), summed SGAR concentrations in liver ranged from 30.2 to 169.6 ng/g ww (57.7–338.1 pmol/g ww), with 4 individuals exceeding 100 ng/g ww (Supplemental Table 6).

Number of ARs detected

For liver samples with quantifiable AR residues, 64.1% ($n = 50$ of 78) contained two or more compounds. Multiple AR residues were more frequently present in predatory birds (28 of 33, 82.5%) than in scavenging birds (22 of 45, 48.9%) (Chi-squared test, $X^2 = 9.19$, $df = 1$, $p = 0.0024$). Summed SGAR concentrations were higher in birds with multiple ARs (Wilcoxon test, $W = 91.5$, $p < 0.0001$), and among the 21 birds (including 4 scavenger birds) with summed SGARs concentration above 100 ng/g ww, all had multiple SGAR residues, with 16 of them exposed to the four SGARs, and 2 of these 16 samples also containing chlorophacinone residues.

Species

The concentration of summed SGARs differed among species with sample sizes ≥ 5 individuals (Kruskal-Wallis rank sum test, $X^2 = 63.45$, $df = 5$, $p < 0.001$). Levels were greater in Golden eagles and Red kites compared to Bearded, Cinereous and Griffon vultures, respectively (pairwise Wilcoxon test with Bonferonni correction: Golden eagle vs vulture species $p = 0.0241$, 0.0223 and < 0.001 , and for Red kite vs vulture species $p = 0.0100$, 0.0440 and < 0.001) (Fig. 3 A), further supporting the aforementioned difference in exposure between predatory and scavenger birds.

Other factors

Concentrations of summed SGARs did not differ between

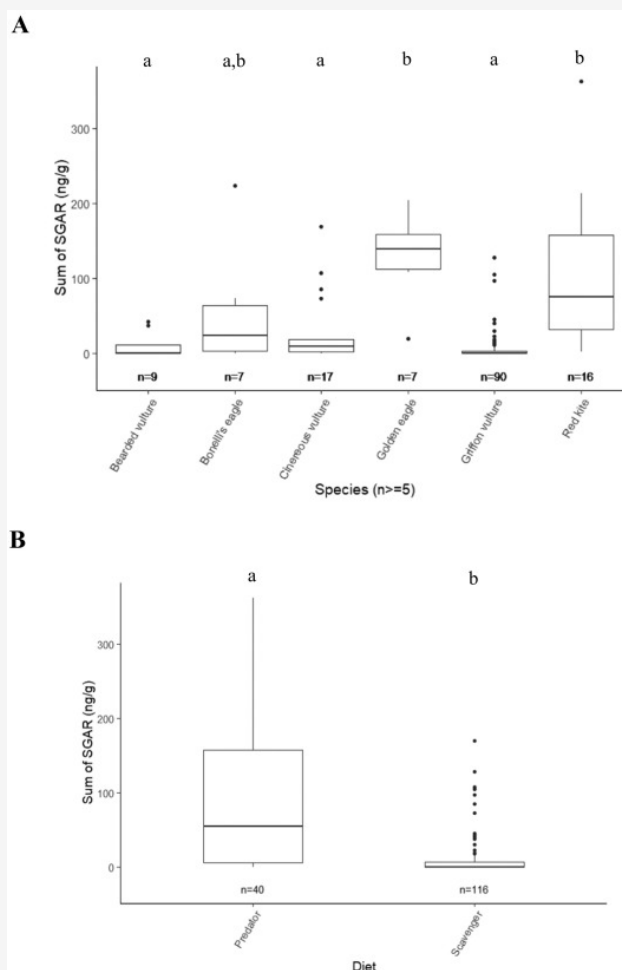


Figure 3. A/ Summed SGAR concentrations per bird species (boxplot) and B/ summed SGAR concentration by foraging behaviour (boxplot); individual SGARs < LOQ were assigned a value of zero. Species or foraging behaviour groups not sharing a common lower case are different ($p < 0.05$). SGAR, second-generation anticoagulant rodenticide; LOQ, limit of quantification.

sexes (t-test, $t = -1.014$, $df = 102.96$, $p = 0.3132$). Birds were categorized by the geographic region (i.e., department) where they were found, but there was no difference among departments (sample size ≥ 5 individuals) in summed SGAR concentrations for either scavenging (5 departments, Kruskal-Wallis $X^2 = 4.0515$, $df = 4$, $p = 0.3991$) or predatory birds (2 departments, Wilcoxon test, $W = 32$, $p = 0.3690$).

2.3 Temporal trends of AR exposure

A decreasing trend in the proportion of individuals exposed to chlorophacinone was observed (Fisher's exact test, $p = 0.0041$, $df = 2$) when birds were grouped according to three key periods of changing patterns of AR use ($n = 3$ of 17 for 2008–2010, $n = 2$ of 24 for 2011–2013, and $n = 1$ of 115 for 2014–2019), with a significant difference between 2008 and 2010 and 2014–2019 (pairwise comparison of proportions

using Fisher's test with Bonferroni correction, $p = 0.0213$, $df = 1$, Odds Ratio = 0.043 [0.0008-0.5747] (Fig. 4). In contrast, an increasing trend for brodifacoum was observed among these 3 key periods (Fisher's exact test, $p = 0.0398$, $df = 2$; but no difference for pairwise comparisons). For the other ARs, hepatic residue concentrations were similar among these 3 periods (Fisher's exact test for bromadiolone, $p = 0.5389$; for difenacoum, $p = 0.378$; for difethialone, $p = 0.2743$; for summed SGAR, $p = 0.2609$).

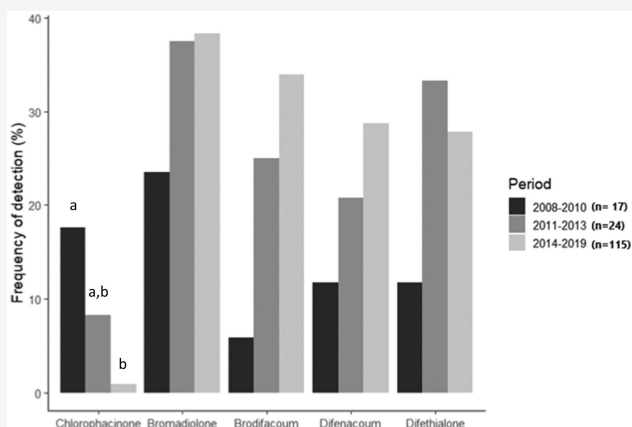


Figure 4. Detection frequency of each AR per time period; chlorophacinone detection frequency differed ($p < 0.05$) between 2008 and 2010 and 2014–2019 as indicated by different letters superscripts.

2.4 Evidence of AR intoxication

Of 156 birds tested for ARs, only 130 were examined for the presence of haemorrhage at necropsy because of carcass decomposition of the 26 other specimens. Eighty-eight (88) of those 130 birds displayed signs of haemorrhage (67.7%). None were initially diagnosed with AR toxicosis, but for 9 birds, cause of death was undetermined. Six (6) of those 9 birds were positive for ARs: 2 had low levels of summed SGARs (2.1 and 2.2 ng/g ww) and were not further considered, but the remaining 4 birds (all Red kites) had summed SGAR levels ranging from 27.3 to 191.9 ng/g ww. Levels of ARs and the clinical picture for those 4 individuals are described in Table 4 and Fig. 5, respectively. While the clinical picture does not favour coagulopathy in birds 1 and 2, lesions reported in birds 3 (Fig. 5) and 4, combined with high levels of summed SGARs, are suggestive of AR toxicosis in those birds (2/156, i.e., 1.3% of tested birds actually died of AR toxicosis).

There was no difference in summed SGAR concentrations between birds with or without haemorrhage (non-parametric Wilcoxon rank Test, $W = 1681$, $p = 0.3814$). For the remaining subset of birds excluding those with trauma and electrocution, summed SGAR concentrations did not differ between those with or without apparent haemorrhage for all birds (Wilcoxon rank Test, $W = 365.5$, $p = 0.2584$) and for the subset of species with ≥ 5 individuals (i.e., Griffon vulture, Cinereous vulture and Red kite - Wilcoxon rank test, $W = 103$, 6 and



Figure 5. Haemorrhagic lesions in bird n° 3. A: Cervical haematoma, surrounding the trachea - B: Blood in coelomic cavity - C: Haemorrhage of digestive tract - D: Haematoma on the surface of the skull.

10, respectively, and p -values = 0.4523, 1 and 1, respectively). When examining data for only those birds with cause of death classified as “Unknown” ($n = 20$; avoiding bias), summed SGAR concentrations did not differ between those with or without apparent haemorrhage (Wilcoxon test, $W = 49$, $p = 1$).

No difference was found between summed SGARs > 100 ng/g and presence of haemorrhages, for samples involving all individuals (Chi-squared test, $X^2 = 0.057$, $df = 1$, p -value = 0.8117) or when excluding bias and keeping only birds with “Unknown” cause of death ($n = 13$; Fisher's Exact test, OR = 0.527, $p = 1$).

Thirty-nine (39) of the 130 birds examined for the presence of haemorrhages died from trauma, and 35/39 displayed signs of haemorrhage (i.e., 89.7% of birds with trauma). AR exposure was detected in 15/35 birds, with 7 birds having summed SGARs concentrations ≥ 100 ng/g ww. No relationship was apparent between AR exposure and presence of haemorrhage in birds that died from trauma (Fisher's Exact test, OR = 0.756, $p = 1$).

2.5 Other contaminants

Lead concentration was determined in 64 liver samples representing 8 species. Evidence of elevated Pb exposure (> 6 $\mu\text{g/g dw}$) was apparent in 11 individuals (17.2%) and levels exceeding the threshold of severe/lethal poisoning (> 30 $\mu\text{g/g dw}$) occurred in 4 individuals (6.3%). In the necropsy reports, lead intoxication was described as the suspected cause of death for these 4 individuals. Of the liver samples with elevated Pb exposure (> 6 $\mu\text{g/g dw}$, $n = 11/64$), 9 (81.2%) were also positive for ARs. All ARs detected were SGARs, with summed concentrations ranging from 2.5 to 224 ng/g ww. Seven (7) individuals had multiple SGARs, and 3 of them displayed summed SGAR levels > 100 ng/g. Pb intoxication was suspected in 3 of those birds with multiple SGAR expo-

Bird	Species	Year	CHLORO	BROMA	BFC (ng/g ww)	DFC	DFT	ΣSGARs	Macroscopic haemorrhagic lesions at necropsy	Blood clots in cardiac cavity (Y/N/NA)
1	Red kite	2013	0	31,6	2,1	0	0	33,7	Subcutaneous hematoma of the thoracic area Serohaemorrhagic pericardial effusion	Y
2	Red kite	2018	0	2,1	25,2	0	0	27,3	Haemorrhage of the right eye Slight bleeding of lung surface Hematoma of right pectoral muscle and neck	Y
3	Red kite	2018	0	52,5	8	63,2	68,2	191,9	Blood in abdominal cavity Lung haemorrhage Haemorrhage of digestive tract Several hematoma on the surface of the skull bone	N
4	Red kite	2019	0	4,4	86	0	5,8	96,2	Subcutaneous hematoma of the thoracic area Lung haemorrhage Haemorrhage of pericardium	N

Table 4. Levels of ARs, haemorrhagic lesions and presence of blood clots in individuals suspected of AR toxicosis
Abbreviations: chlorophacinone, CHLORO; bromadiolone, BROMA; brodifacoum, BFC; difenacoum, DFC; difethialone, DFT

sure, and the 4 others died from electrocution (n = 3) or from trauma (n = 1).

Broad toxicological screening was performed on 126 birds, of which 18 exhibited positive results. For those birds, it was concluded that cause of death was consistent with pesticide or pharmaceutical intoxication (18/126, 14.3%). Carbofuran was detected in 13 samples (13/18, i.e., 72.2% of intoxicated birds – 10.3% of screened birds) and mevinphos in 4 samples (4/18, 22.2% of intoxicated birds – 3.2% of screened birds). One bird was positive for pentobarbital that had not been euthanised, potentially from consumption of tissue from euthanized livestock that had been mistakenly dropped off at a feeding station. Of these 18 positive samples, 12 had been analysed for AR residues and 9 (75.0%) of these were positive (SGARs present in 7, 58.3%). Summed SGAR concentrations ranged from 3.0 to 213.5 ng/g ww. Multiple SGARs were found in 5 samples.

3. Discussion

3.1 Causes of death of the study population

Scavengers, and especially Griffon vultures, were the most frequently represented samples. This is likely due to a sampling bias, as vultures are widely monitored in France, and Griffon vultures were the first to be reintroduced starting in the 1980s through several programs and National Action Plans (Ministère de l'environnement, de l'énergie et de la mer, 2017) resulting in a large population (n = 1544 reproductive pairs in 2014, with census in the Pyrenees counting 1254 reproductive pairs in 2019). Another likely source of bias was apparent when comparing numbers of samples collected over the years (more samples from 2016 to 2019) as the LIFE GYP-CONNECT program was most active doing carcass collection, necropsy and toxicological analyses during that period.

The principal causes of death reported for all birds were trauma (28.6% of all samples) and electrocution (20%). Trauma was often related to the collision of the bird with a wind turbine, vehicle or power line. Collision with wind turbines is a well-known threat to wild birds [41, 42], and was reported to account for up to 41% of casualties in areas directly surrounding wind farms. Collision with power lines results either in trauma or electrocution, and represents another major threat for wild birds [43, 44].

3.2 Characterizing exposure to ARs in wild birds

3.2.1 Prevalence of AR exposure in raptors from France

All available liver samples collected from dead raptors were systematically analysed, without any prejudgment of potential AR exposure. This systematic monitoring may allow less bias in the estimation of the prevalence of AR exposure than incident-based surveillance that is sometimes performed and certainly overestimates this prevalence [16–18, 45, 46]. Yet, half of the samples (78/156) were positive for ARs. This is consistent with the mean exposure rate calculated in the review from López-Perea and Mateo [30], who estimated a prevalence of ARs in raptors of 58% [30]. More recent data from the UK indicates a higher prevalence of 66.8% in common Kestrels [47]. However, both those rates are based on results obtained from predatory birds. In our study, prevalence of AR exposure in predatory birds (82.5%) was higher than previously reported rates, but was calculated based on a small sample size (n = 40). Prevalence in scavenger birds (38.8% - n = 116) was lower, but still quite substantial. To our knowledge, this is the first report of prevalence of AR exposure in scavenging birds calculated on a large sample size. Future work focusing on the prevalence of AR exposure in scavenging birds could add clarity this observation.

Detected ARs were mainly SGARs, which is consistent with their greater environmental persistence when compared to FGARs [22, 48]. Warfarin and flocoumafen were not detected in any of the samples, and they were not sold in France during the study period (<https://simmbad.fr/public/servlet/accueilGrandPublic.html>). While coumatetralyl is authorized for use in France, it was not detected.

Levels of SGARS were mostly low, with only 13.5% of samples above the commonly used threshold of 100 ng/g ww. This suggests a relatively prevalent exposure of wild birds in the zone of the study (i.e. southeastern France), but mainly at low levels.

3.2.2 Factors affecting AR exposure

Exposure of predatory vs scavenging birds: different pathways?

As expected, the proportion of AR-positive samples was greater in predators than scavengers. Predatory birds are likely to be directly exposed to ARs through consumption

of intoxicated rodents, which may be weakened and motionless, lose their nocturnal disposition and positive thigmotactic behaviour, and are therefore easy prey [49–51]. Exposure of predatory birds could also occur through the consumption of AR-contaminated non-target mammals, reptiles, and birds (e.g., passerines, waterbirds) [27, 28, 52]. It should however be noted that one limit of this study was that the opportunistic nature of some predator species (e.g., Golden eagle is a facultative scavenger obtaining much of its diet through predation) was not taken into account, as well as some species-specific feeding habits (e.g., Short-toed snake eagle whose diet is mainly reptiles, not rodents).

Even if prevalence of AR exposure was lower in scavengers, some of them (10%) displayed high levels of summed SGARs. The AR exposure pathway for scavengers is not obvious. In the areas of carcass collection, reintroduced scavengers forage at feeding stations stocked with dead livestock by local farmers and with butchery remains (including bones for Bearded vultures) by field agents. While accidental primary AR exposure of domestic ruminants can occur (e.g., recently described poisoning event in ewes)[53] based on the sources of food and vigilance, substantial AR exposure at such supplemental feeding “vulture restaurants” seems unlikely. As vulture populations are growing in the area studied, feeding stations may not provide adequate food resources, resulting in some birds feeding on wild carrion. Presence of numerous illegal hunting “dumps” have been reported in the study area (Florence Buronfosse, National Centre for Veterinary Toxicological Information [CNITV], oral communication, 2021), and scavenging of the remains of large wild hunted mammals (e.g., boars) that may have consumed AR baits [54] could be a source of exposure. This could especially be the case for Griffon vultures that are keen on viscera. Rodents might occasionally be part of the diet of Cinereous vultures (Xiao-Ti, 1991)[55], and of young Bearded vultures, when food resources are scarce (Florence Buronfosse, National Centre for Veterinary Toxicological Information [CNITV], oral communication, 2021). Further investigation of feeding habits of vultures in the study area are needed to better understand AR exposure pathways.

Acute vs chronic multiple exposure

Less than 10% of positive samples displayed a concentration of an individual SGAR above the commonly used threshold of 100 ng/g ww, and none of the sampled birds presented evidence of AR toxicosis, suggesting that massive acute exposure was uncommon in raptors in our study [30]. The fact that high concentrations of a single ARs are rarely found could also suggest that much of the ingested AR is quickly eliminated, with only a small fraction persisting [26]. This deserves further investigation through toxicokinetic studies.

In contrast, all birds with summed SGARs above 100 ng/g ww had multiple AR residues (i.e. up to 4 SGARs) and more than half of positive samples had residues of 2 or more ARs. This is consistent with other publications (Albert et al.,

2010; Stone et al., 1999; Thomas et al., 2011)[15, 20, 56], and suggests that chronic repeated exposure is a more prevalent pathway of exposure in wild birds, which can lead to sublethal adverse effects[19].

Other factors affecting AR exposure

Sex ratio of adult bird populations, including raptors, has received limited attention, but there is some evidence that in free-ranging populations males outnumber females [57]. Notably, for the Andean condor (*Vulture gryphus*), an age-dependent skewed sex ratio favoring mature males has been described, with a proposed explanation that immature females forage in plains which might enhance exposure to human threats such as environmental contaminants [58]. However, in the present study, involving many different species of raptors, no such sex-based pattern of AR exposure was apparent, which is generally similar to observations in another AR exposure study [16].

While human population density, urbanization, density of livestock and acreage of crops have been observed to influence the magnitude of SGAR exposure [59, 60], no geographic trend for scavenging or predatory birds was apparent in the present study. This is likely a deficiency of recordkeeping for habitat type where carcasses were discovered (e.g., urban, suburban, agriculture, rural, natural area) could be addressed in this monitoring scheme, using available public data on land use.

3.3 Effect of public policies on AR exposure

Following the restriction of chlorophacinone use as a PPP in agricultural settings at the end of 2010, the frequency of detection in liver of raptors decreased. However, this trend should be viewed with caution as it is based on few detections. Furthermore, chlorophacinone is seemingly less persistent than several SGARs in liver [61] which may limit its probability of detection. Bromadiolone was the most frequently detected SGAR in this study, and it may partly be explained by the fact that between 2010 and 2020 it was the only AR authorized in France as a PPP. Continued monitoring of bromadiolone since its 2020 withdrawal as a PPP would reveal how frequency of detection and residue concentrations change over time in non-target raptors.

While from 2013, use of bait stations was made mandatory for ARs used as biocides[32–34], the trend observed in the present study seemed to be an increase in the frequency of detection of SGARs (Fig. 2), with this being significant for brodifacoum. These findings suggest that further measures may need to be considered to mitigate the risk of exposure in non-target fauna to SGARs used as biocides. It also suggests that exposure to PPP in agricultural settings is not the only pathway for AR exposure of wild birds, especially considering that their use is currently banned in the EU (EU Pesticides database, https://ec.europa.eu/food/plants/pesticides/eu-pesticides-database_en, access on 14 October 2021); use of AR biocides in urban areas and cattle farms could even be the principal exposure route

for wildlife[60]. Such an increase in the use of SGARs might be related to the occurrence of resistance to ARs in rodents pest species [22, 24, 25, 62–64], and to the absence of efficient alternatives for pest control. Differences in potency and toxicity between stereoisomers of SGARs could be a promising direction for investigation to develop effective and more “eco-friendly” rodenticides [65–68].

3.4 Diagnosis of AR toxicosis

While many birds were exposed to ARs, toxicosis was suspected in only 2 of 156 individuals evaluated for rodenticide residues (i.e., 1.3% of sampled birds, 2.6% of birds with detectable AR residues). Suspicion of AR toxicosis in those 2 birds was established as they displayed macroscopic haemorrhage and clearly elevated AR residues (i.e., 96.2 and 191.9 ng summed SGARs/g ww) [35], and no other obvious cause of death was described in necropsy records. Using these criteria, 2 other birds might be suspected of succumbing from AR toxicosis (Table 3), yet residue concentrations were low and signs of haemorrhage were less apparent, and clotted blood was observed in the cardiac cavities of both individuals. This rate of toxicosis is consistent with prevalence reported in a recent study in Bald and Golden eagles (4% of eagles tested actually dying from AR toxicosis, Niedringhaus et al., 2021 [69]), suggesting that mortality directly related to AR consumption is rather low in non-target birds of prey. However, other studies in the northeastern United States involving several species of raptors indicate a greater frequency of AR toxicosis, with incidence of exposure increasing in recent years [16–18].

In contrast to these few suspected cases of AR toxicosis, the presence of haemorrhage was observed in a high proportion of birds (88/130, 67.7%), but most (71/88) were diagnosed with causes of death that result in haemorrhage (e.g., trauma, electrocution, infectious processes). No excessive bleeding from minor wounds was documented in any of the cases, that might be suggestive of impaired coagulation [35]. While some publications may suggest increased susceptibility to trauma in AR-intoxicated animals [70, 71] no relationship was apparent between levels of AR exposure and presence of haemorrhage in birds that were classified as dying from trauma.

Our observations illustrate the difficulty of diagnosing AR toxicosis during post-mortem examination, especially for heterogeneous samples of wildlife with a high prevalence of other causes of death involving haemorrhage. No histopathological examination was performed on the samples to detect microscopic evidence of haemorrhage or hypoxic damage to tissues, as suggested by several previous studies [16, 35, 69, 72]. However, this type of examination is not appropriate if carcasses are not fresh. Moreover, the codification of the presence/absence of haemorrhage was based on the personal interpretation of the senior author’s review of descriptive data in each necropsy report based on keywords (i.e., “haemorrhage”, “bleeding”, “anaemia”, “bruising”, “haematoma”, “blood”). Efforts are underway to improve the coding of necropsy obser-

vations in our internal wildlife mortality database.

To assist with diagnosis of AR toxicosis, many diagnosticians were led to the implementation of a toxicity threshold for summed SGARs, with a commonly reported threshold of 100 ng/g ww [73]. However, in the present dataset this threshold concentration did not seem to reveal many individuals that died from AR toxicosis. In addition, no significant difference could be found when excluding potentially confounding factors (e.g., trauma and electrocution as cause of death) or by the grouping of samples by species to account for potential species-specific differences in AR sensitivity that limit the utility of this threshold [19]. Drawbacks of the present study and dataset that limit our availability to examine the AR toxicity threshold for wild birds include small sample size for many of the species, and unlike other studies (e.g., Thomas et al., 2011; Murray, 2020 [18, 20]), few individuals which seemingly died from AR toxicosis sequelae including coagulopathy. Moreover, carcasses could be more or less decomposed, possibly affecting AR concentration estimates in sampled livers [74]. It is noteworthy that some have suggested that such a residue threshold may not be a relevant diagnostic tool for AR toxicosis, as it does not address sublethal effects and the potential consequences of bioaccumulation from repeated exposures[19, 75]. Development of additional biomarkers for AR effects could be useful in monitoring the effect of rodenticides on non-target fauna [37].

3.5 Concomitant exposure to other contaminants

In our study, most of the samples displaying elevated Pb exposure ($>6 \mu\text{g/g dw}$) were also positive for SGARs. The majority of those birds with exposure to both AR and Pb died from either trauma or electrocution. Combined sequential exposure to both lead and AR could have increased the risk of mortality in those birds, as these contaminants may impair flight coordination and/or spatial recognition [16, 72, 76]. Furthermore, through different mechanisms of action, both lead and ARs can cause anaemia [77]. Further investigations are needed to characterise such potential “cocktail effects” of environmental contaminants commonly encountered by wild birds.

4. Conclusion

This study aimed at characterizing exposure to ARs in a large yet heterogeneous sample of birds of prey from southeastern France collected over a 12 year period. Results were generally consistent with other reports, documenting a rather high prevalence of AR exposure (mainly SGARs), usually low levels of individual ARs and summed of SGARs, and occasionally with a few birds seemingly succumbing to AR toxicosis. Multiple exposure was quite frequent, raising the issue of sub-lethal chronic effects related to repeated exposure. As expected, levels of ARs were higher in predators, but some scavengers also displayed high levels for which a pathway of exposure could not be fully elucidated. It was also noted that current public policies had a limited effect on mitigating AR exposure; the

development of more eco-friendly ARs could be an approach to limit AR contamination of non-target fauna. The study also confirmed that diagnosis of AR toxicosis is a complex issue, and establishing a threshold like the widely used value of 100 ng/g ww may not be ideal. Future research exploring biomarkers of effect rather than just exposure, could permit discrimination of AR exposure and toxicosis. The presence of both ARs and Pb in some samples demonstrates multiple contaminant exposures that could have the potential to result in additive or synergistic effects.

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5. Supplementary materials

Name of the project	Number of birds provided for the study
LIFE GYPCONNECT (https://gypaetebarbu.fr/life-gypconnect/)	118
VIGILANCE POISON (Berny et al. 2015)	47
PNA Cinereous Vulture (https://www.ecologie.gouv.fr/sites/default/files/PNA_Vautour-moine_2011-2016.pdf (2nd PNA))	34
PNA Red Kite (https://www.ecologie.gouv.fr/sites/default/files/PNA-Milan-Royal-2018-2027.pdf)	30
LIFE GYPHELP (https://gypaete-barbu.com/10/le-projet-life-gyphelp/58/pourquoi-ce-projet.html)	9
Unknown project	7
TOTAL	245

Table 5. Surveillance programs implicated in the study, and number of birds provided by each program.

Outlier	Species	BROMA	BFC (ng/g ww)	DFC	DFT	ESGARs (ng/g ww)
1	Cinereous vulture (<i>Aegypius monachus</i>)	61	39,3	6,7	0	107
2	Cinereous vulture (<i>Aegypius monachus</i>)	24,2	19,4	54,6	71,4	169,6
3	Griffon vulture (<i>Gyps fulvus</i>)	86	22,6	10,1	9,3	128
4	Griffon vulture (<i>Gyps fulvus</i>)	53	45,6	2,6	3,4	104,6

Table 6. SGAR levels in outlier scavengers (n=4). Individual SGARs \leq LOQ were assigned a value of zero.

5.2.2 Différencier exposition et intoxication

5.2.2.1 Article :

Biomarkers Potency to Monitor Non-target Fauna Poisoning by Anticoagulant Rodenticides



Biomarkers Potency to Monitor Non-target Fauna Poisoning by Anticoagulant Rodenticides

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The widespread use of pesticides to control agricultural pests is a hot topic on the public scene of environmental health. Selective pest control for minimum environmental impact is a major goal of the environmental toxicology field, notably to avoid unintended poisoning in different organisms. Anticoagulant rodenticides cause abnormal blood coagulation process; they have been widely used to control rodents, allowing inadvertent primary and secondary exposure in domestic animals and non-target predatory wildlife species through direct ingestion of rodenticide-containing bait or by consumption of poisoned prey. To report toxic effect, the most common approach is the measurement of liver or plasma residues of anticoagulant rodenticides in dead or intoxicated animals showing clinical symptoms. However, one major challenge is that literature currently lacks a hepatic or plasma concentration threshold value for the differentiation of exposure from toxicity. Regarding the variation in pharmacology properties of anticoagulant rodenticides inter- and intra-species, the dose-response relationship must be defined for each species to prejudge the relative risk of poisoning. Beyond that, biomarkers are a key solution widely used for ecological risk assessment of contaminants. Since anticoagulant rodenticides (AR) have toxic effects at the biochemical level, biomarkers can serve as indicators of toxic exposure. In this sense, toxicological knowledge of anticoagulant rodenticides within organisms is an important tool for defining sensitive, specific, and suitable biomarkers. In this review, we provide an overview of the toxicodynamic and toxicokinetic parameters of anticoagulant rodenticides in different animal species. We examine different types of biomarkers used to characterize and differentiate the exposure and toxic effects of anticoagulant rodenticide, showing the strengths and weaknesses of the assays. Finally, we describe possible new biomarkers and highlight their capabilities.

Keywords: biomarkers, rodenticides, primary exposure, secondary exposure, non-target, antivitamin K anticoagulant, toxicokinetics

INTRODUCTION

Pesticides are today the main means of pest control, making it possible to mitigate the economic, environmental and health consequences when pest population outbreak occurs (1). Nevertheless, legitimate environmental concerns relative to the use of all kind of pesticides (insecticides, herbicides, fungicides, rodenticides, fumigants) are growing. One of this concern involves their impact on non-target living species since more than 50 years already. A central question on this

topic is: “How to differentiate sub-toxic exposure to sublethal effects or lethal exposure?” (2–5). Indeed, in order to monitor the ecotoxicity of molecules and their effects on non-target wildlife and thus adapt pesticide use practices, it is necessary to clearly establish the causal link between the death of a non-target species or negative fitness effects on non-target species and pesticide exposure. Due to the diversity of non-target species, from small mammals to birds of prey, with for each species particular sensitivities or resistances to the pesticide in question, it is difficult to establish a level of exposure that may lead to the death. Nevertheless, for animal species, one powerful way to answer this question is through detection and/or quantification of biomolecules or compounds characteristic of a non-toxic or a toxic exposure, the so-called biomarkers, associated with clinical signs and near-environmental analysis. In this review, we will focus on summarizing current knowledge on biomarkers and associated parameters differentiation of sub-lethal exposure effects vs. lethal exposure for anticoagulant rodenticides (AR), that are also a hot topic of environmental concerns since decades (6–9).

More and more primary or secondary non-target intoxications with anticoagulant rodenticides are reported in the literature including mostly birds, especially raptors, but also a minor part of domestic animals such as cats, dogs and horses, and other wildlife species such as deers, polecats, martens, foxes, and very relevant literature reviews are already available on this topic (10–13). Few studies deal with determination of parameters of poisoning in different species (14) and the way to treat non-target poisoned animals is still relatively obscure while many questions on this topic still need answers, such as how long does the treatment should last, what is the frequency and the quantity of vitamin K needed as a function of the species or even based on the race (15). From this lack arise many questions about the follow up of AR and their impact on environment, notably: how to attribute the cause of death of an AR exposed animal? Considering the diversity of exposed species with specific susceptibility to AR and the diversity of AR molecules with different pharmacodynamic and pharmacokinetic parameters and the frequent detection of multiple contaminants for the same animal, the simple level of exposure of the animals seems to be insufficient to point at AR as sole responsible of the death. No literature review on the specific biomarkers of the sub-lethal or lethal exposure to anticoagulant rodenticides in non-target species is available until now. The aim of this review is to outline existing and propose new biomarkers, in order to allow a better follow-up of anticoagulant exposure and intoxication.

ANTICOAGULANT RODENTICIDES USE AND PROPERTIES

Importance of Anticoagulant Rodenticides Use and Animal Exposure

Use of Anticoagulant Rodenticides

Rodents control using anticoagulant rodenticides was introduced officially in 1948 (16), answering to the demand of authority, general population (17, 18) and mostly farmers and of all

the production and supply chains against tremendous damage caused by rodents all around the world, sometimes resulting in the loss of 100% of a production (19, 20). Anticoagulant rodenticides provide strong advantages among all chemical methods, e.g., they are slow acting compounds, they have a safe and very common antidote, the vitamin K, and they can be used at low concentration.

The risk of a pesticide used to control target species by causing their death is to select living target species that are less or not sensitive to this pesticide, the latter specimens are said “resistant.” In 60’s; the first resistant rodent population has been described (21). To deal with the emergence of resistant rodent populations resulting from the use of these first generation of anticoagulant rodenticides (FGAR) (warfarin, diphacinone, chlorophacinone) a second generation of anticoagulant rodenticides (SGAR) has been developed (22–24). Second generation molecules (bromadiolone, brodifacoum, dicoumarol, difenacoum, difethialone...) are more efficient against resistant rodents and effective at a lower dose. Despite these abilities, resistance to some SGARs has been reported in different areas due to massive use (25, 26). Currently this second generation is the most used in developed countries.

Anticoagulant rodenticides are included in baits of different types and forms (grains, pellets, solid blocks) depending on the application site and the targeted rodents (27). The presence of a carbohydrate source in bait formulation is important to ensure high palatability to attract rodents. Unfortunately, they are also palatable to other non-target species, even if a bittering agent is usually added to the bait. Besides the physical and chemical properties of bait formulations, its consumption depends on the affluence of traditional food sources that interest rodents (28–30). In European Union, rodenticide baits must be disposed in secured bait station since 2013. These bait stations are supposed to avoid primary exposure of non-target species by physically preventing the access to baits and their spread through water (27, 31, 32). However, their use by amateurs is not mandatory and non-target animals having similar size to target rodents can still access to the bait.

Animal Exposure to Anticoagulant Rodenticides

Primary and secondary exposure to anticoagulant rodenticides

There are two types of exposures to consider. Primary exposure occurs when a non-target animal directly eats the bait, while secondary exposure occurs when a predator ingests preys previously exposed to AR. Prevention is different for each case. Considering the primary exposure, the goal is to avoid the access to the bait by ensuring their correct storage and using them in secured bait station as previously discussed. Considering the prevention of the secondary exposure, the matter is more complex. Indeed, AR is a long-acting poison and targeted rodents may die within 2–6 days after ingestion. Moreover, rodenticides may influence gradually rodents’ behavior. Actually, poisoned rodents become weakened with reduced appetite and motionless, lose their nocturnal disposition and positive thigmotactic

behavior; they are therefore more prone to predation (33–35). Thus, to mitigate secondary exposure, AR have to be less persistent in the target animal body.

Domestic animal exposure to anticoagulant rodenticides

In domestic animals, the majority of exposures are primary ones. They are often the consequences of a misuse or an improper storage of baits allowing domestic animals to access to AR. According to a French veterinary poison control center (CNITV), AR exposures represent 10% of the total call to the center and dogs accounted for 82.8% of AR exposure incidents, followed by cats with <10% (36). The pet exposures are mainly accidental. Indeed, the few studies that assess the exposure of healthy dogs without AR intake mentioned by the owner show that they are not chronically exposed to AR. Indeed, <2% of dogs are positive to AR (37, 38). Cats would be more prone to secondary exposure to AR. However, to the knowledge of the authors only primary exposure cases have been observed and studied in cats (36, 39). For pets the incidence of exposure seems to be seasonal, with an increase at the beginning of autumn (36, 39). Considering livestock, the primary exposure is mainly due to an improper storage of baits that makes them reachable. The incidence of these exposures seems to be low compared to pets (<2.2% of AR exposure cases) (36). Nevertheless, livestock exposures raise public health concerns discussed below.

Since the majority of AR exposures are accidental, owners notice the event and treatment is administered before symptoms occur. According to the CNITV data, SGAR are reported in more than 60% of exposure cases (in 22%, the AR molecules are not identified) (36). This incidence is due to the highest efficiency of SGAR compared to FGAR, that are now less used. Moreover, the number of cases started to decrease after 2013 with the regulatory obligation to use bait station.

Livestock can also be exposed and intoxicated to natural anti-vitamin K like dicoumarol after the ingestion of moldy clover fodder (40, 41) or like ferprenin and ferulenol after giant fennel intake (42, 43).

Wildlife exposure to anticoagulant rodenticides

Depending on their size and dietary regimen, wild animals can be primary or secondary exposed to AR. The mandatory use of secured bait stations for the application of AR during a biocidal use may decrease the primary exposure of mammals larger than rats although some large mammals like wild boars seem to be highly primary exposed to AR (60%) in suburban area (44). However, small rodents can still access to baits. Thus, in the 20 meters perimeter around bait boxes, about 50% of non-target small mammals can be exposed to AR (45). This exposure affects all *taxa* and decreases with the distance to the bait boxes (45, 46). The small mammal exposure is a concern for both small-mammal populations themselves (notably for endangered species) but also for their predators.

Predators can be secondary exposed to AR from target and non-target species, according to their diet (47). The exposure of birds of prey, foxes, racoon dog, marten, bobcat, or polecat varies from 20 to up to 90% (48–53). This prevalence is strongly linked to the proximity of an area of human activity such as urban

areas or farms (51, 54, 55). Similar to domestic animals, wildlife predators are exposed mostly to SGAR. SGAR are composed of asymmetric molecules called stereoisomers, but predators like red foxes are more exposed to some stereoisomers than others (56). While predator exposure to AR is high, it is more rarely linked to their death (57–60). However, it raises many questions about the consequences of this chronic exposure on the wildlife. Some mitigation risk measures to protect predator fauna have been tried as the removal of dead rodents (61). However, new approaches are needed to mitigate efficiently the secondary exposure of predators. The design of new “eco-friendly” SGAR, based on the different properties of AR stereoisomers, might be interesting and is discussed below (62). Finally, some mammals and birds might also be exposed by eating invertebrates that would have entered the bait box (63).

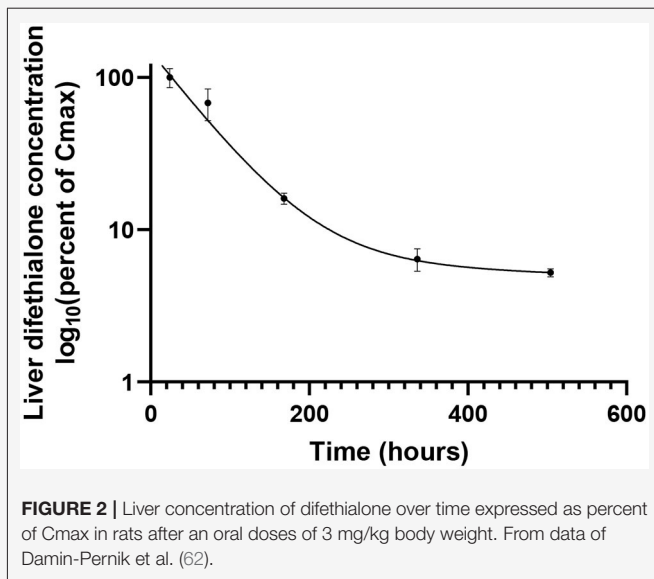
Anticoagulant Rodenticides Properties

General Mechanism of Action of Anticoagulant Rodenticides

Vitamin K in its hydroquinone form (VitK_{HQ}) is a cofactor of Gamma-Glutamyl Carboxylase (GGCX) enzyme that performs a gamma-carboxylation of glutamate residue of some proteins called vitamin K dependent proteins (VKDP). VKDP have to go through this post-translational gammacarboxylation to be able to chelate calcium and activate their physiological properties (64–67). During the reaction, VitK_{HQ} is oxidized and oxygenated into vitamin K epoxide (VitK_{OX}). Since dietary intake is often insufficient to meet the vitamin K need (66), VitK_{OX} has to be recycled in VitK_{HQ} to maintain the gamma-carboxylase activity. The recycling is performed by the vitamin K epoxide reductase enzyme (VKORC1) and it takes place in two stages (**Figure 1**): (1) a deoxygenation of VitK_{OX} catalyzed by the Vitamin K Epoxide Reductase (VKOR) enzyme leading to the formation of Vitamin K Quinone (VitK_Q) and (2) a bielectronic reduction of VitK_Q in VitK_{HQ} mostly catalyzed by the VKORC1 enzyme and as an alternative minor pathway by the consensual NAD(P)H:Quinone Oxidoreductase 1 (NQO1) enzyme that is not the sole alternative reduction path (69–71).

Anticoagulant rodenticides are inhibitors of VKORC1 (72). When their concentration reaches a sufficient threshold, vitamin K recycling is stopped. Four clotting factors, the factor II, VII, IX, and X, synthesized by the liver, are among the VKDP. Consequently, under the effect of AR, these vitamin K dependent clotting factors are no longer activated by gamma-carboxylation and the blood concentrations of active clotting factors decrease leading to delayed uncontrol bleeding and sometimes death (64–67). This delay in action prevents the rodent to associate the effects with the ingestion of baits and explains the efficiency of these rodenticides. Moreover, in case of accidental exposure, the delay of action eases the implementation of a treatment.

SGAR molecules are little more potent than FGAR ones to inhibit VKORC1 of susceptible rat (73). Nevertheless, SGAR are more than 10 times more efficient than FGAR on resistant rodents (73). This potency to inhibit recycling mechanism of resistant rodent is one aspect of the effectiveness of SGAR. Moreover, it seems that this characteristic is shared among stereoisomers of a same molecule (74).



are for some molecules of more than 100 days. Moreover, these results are inconsistent with other pharmacokinetic studies that report half-lives in hours (62). These discrepancies can be explained by a bi-phasic elimination with an initial- and a terminal-half-life. **Figure 2** shows the hepatic pharmacokinetic of difethialone in rats. During the first decay phase the initial half-life is of 44 h while during the terminal phase the half-life is of 74 days like previous reported data in the table. However, when slow decay begins 95% of liver difethialone has been yet eliminated. Thus, final half-lives have to be interpreted with care. Nevertheless, the difference between the first and the second generation is clear and well-known, SGAR are more persistent than FGAR. This persistence explains, in part, the efficiency of SGAR. Indeed, while the baits including FGAR require several ingestions over several days to be effective, baits including SGAR require theoretically only one ingestion. Nevertheless, this advantage is also certainly responsible for SGAR high exposure and bioaccumulation in non-target species.

As presented above, all stereoisomers of SGAR are not found in exposed non-target animals (56). This observation can be explained by the different persistence between stereoisomers. Indeed, while some stereoisomers are still quantified for many days after a lethal exposure to SGAR, others are not because they have a very short hepatic half-life (62, 74, 93). For example, the four stereoisomers of difethialone have initial half-life of, respectively, 6, 25.4, 69.3, and 82.3 h (74). Thus, only the last two stereoisomers contribute to the secondary exposure of non-target species.

AR are metabolized through cytochrome P450. Cytochromes involved in the AR degradation are different according to the nature of the molecule and its stereochemistry (94). These specific pathways for each stereoisomer could be the source of their different half-lives (95). If a part of some FGAR can be eliminated through urine (96, 97); the majority of AR are mainly or exclusively eliminated through feces (86, 87, 98, 99).

Idiosyncratic Susceptibility of Individuals to Anticoagulant Rodenticides

Not all individuals respond equally to exposure to AR. According to their species, sex or genetic, animals will be more or less susceptible to AR. **Table 2** presents LD₅₀ of some AR according to the species exposed. This table pinpoints the wide discrepancy between species considering AR susceptibility. Moreover, some species are more susceptible to some molecules without a correlation with their generation. For instance, pigs tolerate difethialone more than brodifacoum or warfarin (106). In this part, we focus on the reasons of these differences.

Variations of Susceptibility to Anticoagulant Rodenticides Related to Pharmacodynamics

A large part of the differences of susceptibility to AR might be explained by a difference of VKORC1 sequence among species. **Figure 3** shows a multiple sequences alignment of the VKORC1 amino acids. While the active site is well-conserved between species, there are multiple changes among other parts of the protein. The sequence identity (conserved amino acids) between the 15 sequences presented is only of 60% and their homology (conserved amino acids and amino acids with similar chemical properties) is of 83%. The change of only one amino acid may induce a major resistance to AR of the VKORC1 enzyme. Indeed, VKORC1 amino acid changes have been demonstrated to be involved in the difference of susceptibilities among same species, as described in mice, rat and human (23, 73, 107, 108) and the intensive use of AR has selected the most resistant variants of VKORC1 among rodent populations (23, 73). However, this difference of susceptibility to AR differs according to the mutation and to the AR considered (73). For many species, VKORC1 polymorphisms and their consequences on the susceptibility to AR are unknown.

Another possible source that may explain differences in sensitivity is the vitamin K dependent clotting factors pool and its rate of decrease after the inhibition of VKORC1 by AR. This mechanism is proved in the difference of susceptibility between female and male rats (66). Indeed, females have higher basal pool of factor VII and the decrease of plasmatic factor II and factor X are slower than males. Moreover, there is a lag time of 4 h before factor VII begins to decline in females. Thus, female rat coagulation is slower impaired by AR than male one (66). The difference of susceptibility according to the sex differs among species. For instance, female mice resist less to AR than males.

Finally, food could be a pathway of resistance. Indeed, a vitamin K rich diet might reduce the action and the efficiency of AR exposure by providing the antidotes to the animals. However, this kind of resistance has not been clearly demonstrated or identified in wildlife populations (109, 110).

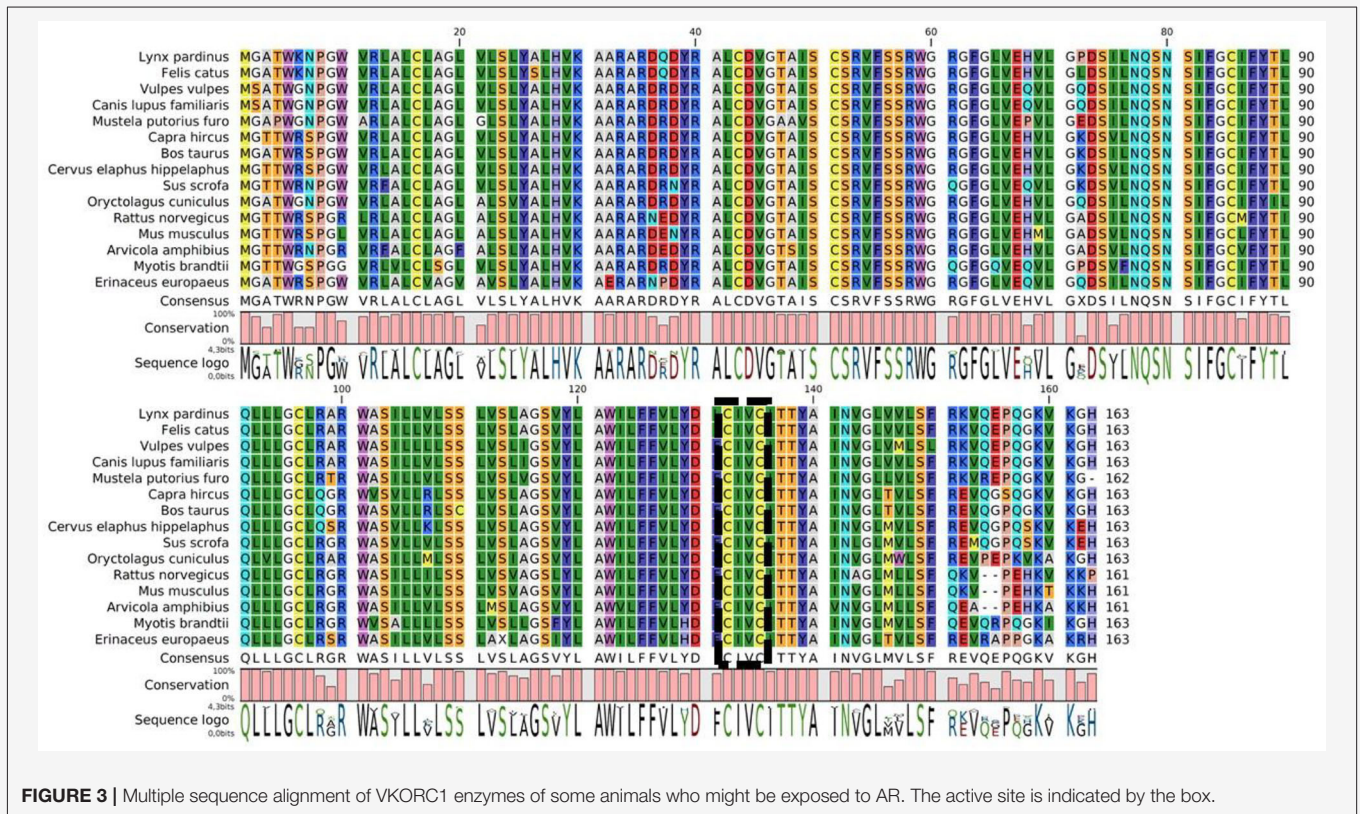
Variations of Susceptibility to Anticoagulant Rodenticides Related to Pharmacokinetics

Pharmacokinetics may be another source of susceptibility or resistance to AR. As discussed previously, the hepatic half-lives of rodenticides differ widely between rats and mice. Moreover, there is no general rule to convert rat half-life to mice one, pharmacokinetic parameters should be determined

TABLE 2 | Single-dose oral LD50 (mg/kg body weight) in different animals.

	Chlorophacinone	Diphacinone	Difenacoum	Brodifacoum	Difethialone	Bromadiolone
Rat	2–20.5	0.3–30	1.8	0.27–0.5	0.56	0.56–1.12
Mouse	1–20.5	141–340	0.8	0.4	1.29	0.99–1.75
Guinea pig	–	–	50	0.28	–	2.8
Rabbit	50	35	2	0.2–0.3	5.3	1
Dog	–	0.88–15	50	0.25–1.0	4	8.1–10
Cat	–	5–15	100	0.25–25	–	25
Pig	–	150	80	10	2–3	3
Chicken	–	–	50	3.15–20	–	5
Sheep	–	–	100	10	–	–
Mallard duck	100	3,158	2	4.6	–	–
Wild birds	430	–	–	–	–	–
Quails	–	–	–	–	–	1,600

Modified from: (10, 100–105).



for each pair molecule-specie. Moreover, some bird species like *Gallus domesticus* showed greater metabolic activity of warfarin compared to rat whereas very little warfarin metabolism was demonstrated in owls which can explain the recurrence of reported poisoning in these species (111). In addition, compared to red deer and pigs, cattle has different pattern of distribution and metabolization of diphacinone with rapid initial phase of decline and longer terminal hepatic half-life suggesting a more developed binding of diphacinone in the liver of cattle or advanced enterohepatic circulation (112). More generally, since expression of cytochrome P450 and xenobiotic metabolism

pathways differ among species (113), it is not possible to generalize the elimination rate of AR for all species. This elimination rate defines the persistence of the molecule in non-target species and therefore its ability to bioaccumulate.

Among a same species, some lines of animals have a better metabolism of AR that may lead them to resist to some rodenticides. It is the case of a line of roof rat (*Rattus rattus*) (114) in Japan, of the Berkshire line of brown rats (*Rattus norvegicus*) (115) and of a population of California ground squirrels (*Otospermophilus beecheyi*) (116). The overexpression of P450 cytochromes involved in AR metabolism was often

undefined or suspected. As these expressions are also sex dependent, it could be linked to the AR susceptibility difference between male and female. Indeed, in many species P450 cytochromes are overexpressed in female (116, 117). Despite that pharmacokinetics are not always associated with susceptibility or resistance, in Lefebvre et al. (66) liver difethialone area under the curve and maximum concentration are almost twice higher in female rats than males, yet females are less susceptible than males.

Anticoagulant Rodenticides Exposure, When to Suspect It?

Considering the extent of AR exposure in domestic and wildlife fauna, it is essential to monitor it. The first clues of an animal exposure are clinical signs suggesting of an acute exposure. However, the consequences of chronic exposure, even if less known, as well as animals that are asymptomatic but at a high risk of exposure must also be considered in AR exposure monitoring.

Acute Clinical Signs That Are Suggestive but Not Specific

Clinical signs of rodenticide intoxication are quite similar for higher vertebrate species: hemorrhagic syndrome, with main localizations being respiratory (coughing/hemoptysis, nasal bleeding, pulmonary oedema, intrapulmonary, and pleural hemorrhages) and digestive (hematemesis, hematochezia, and melena) tracts, and less specific symptoms including lethargy, anorexia, pallor or ataxia, that are usually the first signs to be noticed (118–122). Clinical onset is usually delayed from 2 to 10 days post-ingestion (36, 121).

Numerous case reports in dogs provide extensive descriptions of the clinical signs observed. Apart from general and non-specific signs, and respiratory and digestive bleeding disorders, tachycardia and dyspnea with polypnea are reported, as well petechiae and ecchymoses on the skin and mucosa, and pallor (11, 119). Some reports mention also subconjunctival hemorrhages (123). At a later stage of the intoxication (after 1 week), a bilateral symmetrical enlargement of the abdomen can also be observed in certain cases, frequently accompanied by prolapse of the ventral abdominal wall and lordosis of the vertebral column, resulting from hemorrhagic effusion (ascites). The formation of massive hematomas after venipuncture is also quite characteristic (11).

Ruminants are apparently less often exposed than other domestic species (only 2.2% of calls to the CNITV about rodenticide poisoning are for ruminants), and display clinical signs less frequently (only 10.5% of exposed animals displaying clinical signs according to CNITV data—without further details on the severity of those signs) (36). Case reports in ruminants (cattle, sheep) mention that overt clinical signs of intoxication are usually absent (85, 124), with only a slight increase of bleeding time at the venipuncture site in some cases (125). This decreased incidence of clinical signs is possibly due to a lower sensitivity to AR, that could be related to a dilution effect in the rumen, endogenous production of vitamin K1 in the rumen, or dietary vitamin K in their feed composed of leafy greens (85, 125). Still, AR intoxication might be more severe in young animals, as one case report mentions fatal hemorrhages in lambs following

exposure to chlorophacinone (126). Reports on horses are less frequent, but still some cases of AR intoxication are described, with similar symptoms to other species (127, 128). In one case, AR toxicosis is suspected to have led to the abortion of the mare (129).

AR toxicosis in wild animals, especially birds of prey, is also quite well-described in literature. Similar signs are reported, observed ante- or post-mortem, with a great focus on overt hemorrhagic lesions (e.g., bruising, bleeding from orifices, hemorrhages of cavities, and gastro-intestinal tract) (130, 131). In some cases, there might be no frank evidence of bleeding, and microscopic hemorrhages in tissues (heart, liver, kidney, lung, intestine, skeletal muscle) should be sought by histological examination to confirm diagnosis (132).

In laboratory rats, transient early hemoglobinuria (associated with oxidative stress) with consecutive late hematuria (associated with anticoagulation) were observed after poisoning with brodifacoum, that could be used as novel clinical biomarkers of AR poisoning, if generalizable to other species (133). Moreover, in human treated with warfarin soft vascular calcification may occur (134). This adverse effect of anti-vitamin K anticoagulant has been confirmed in rat model (135). Nevertheless, to the knowledge of the authors, such calcifications have not been suited in AR exposed fauna but would deserve to be studied.

Thus, hemorrhage are important markers of acute AR toxicity in higher vertebrate species. Moreover, they can be used in both alive and dead animals. Nevertheless, hemorrhages are not specific of AR intoxication, as they might be the consequence of trauma (consecutive to AR intoxication or not) or diseases like leptospirosis (136) or other causes of bleeding. Thus, if the presence of hemorrhages is an important clue in favor of AR poisoning, it is not sufficient to conclude.

When hemorrhages occur in living animals, the therapeutic response to vitamin K injection may allow to reinforce or reject an AR intoxication hypothesis. However, false positive occurs in the case of vitamin K deficiency (due to an unbalance diet) or of genetic VKORC1 activity deficiency (137). However, in the case of an exposure of a large and or wild animal the cost of the treatment may raise the question of its necessity. Is the exposure sufficient to threaten the animal health and when should we stop the treatment? Indeed, because of the persistence of SGAR, treatment lasts at least 5 or 6 weeks, and in human medicine its courses averaged 168 days, is it sufficient or too long? The current method to set the duration of treatment is, to stop it after 1 month of treatment for 48 h then to assess the prothrombin time (PT) (36). Is there a biomarker that can predict if the treatment is necessary and when to stop it without discontinuing the treatment?

Clinical Signs Suggestive of a Chronic Exposure to Anticoagulant Rodenticides

Clinical signs of a chronic exposure are currently not sufficiently characterized to be used to suspect an AR exposure at the individual scale. More and more studies suggest that the effects of AR are not confined to coagulation alone. Indeed, the activity of VKDP unrelated to coagulation may also be impacted by AR. More precisely the bone metabolism may be influenced by the decrease of osteocalcin carboxylation and vascular

calcification prevention by that of MGP. Decrease in bone density and increased risk of fractures (by under-carboxylation of osteocalcin), have been investigated in AR-exposed predatory birds, without any conclusive result (138). However, studies based on rodent model show teratogenic activities of some AR with consequences on bone formations (139, 140). Conversely, the relationship between chronic exposure to warfarin and valvular and coronary calcification has been widely described in human (as part of anticoagulant treatment) and in rodent model (135, 141). To the author knowledge vascular calcification have not been studied in exposed wildlife population.

Beyond effect related to VKDP, AR are suspected to increase susceptibility of bobcats (*Lynx rufus*) to notoedric mange, with some fatal outcomes, by interfering with the immune system of the exposed animals (142, 142). The effect of AR on immunity has been confirmed in rats and on human cells (81, 143, 144). Another studies show that chronic exposure could have an effect on genetic expression of bobcat (145). In addition, as described in rodent, AR might be a source of behavior modification (33–35). Thus, the consequences of chronic exposure on the health of non-target animals could be very diverse and should be better studied. Indeed, with this lack of characterization it may be difficult to distinguish the effect of AR and non-AR molecules in the case of multiple contaminations.

As the clinical signs of a chronic exposure are not well-established, AR exposure should be suspected at animal population level. The sign of appeal might be, bone deformation or abnormalities (notably in new-born), unusual infectious susceptibility for no obvious reason (immunodeficiency virus...) and vascular calcification at necropsy. Moreover, chronic exposure with only slight clinical signs may not be identified, the criteria for suspicion of asymptomatic exposure are also relevant.

Asymptomatic Exposure to Anticoagulant Rodenticides

Due to the difference of susceptibilities of animal species to AR, to the level of exposure or to the delay after exposure (few hours to few days), some animals may not present any clinical sign whereas they have been exposed to AR. If it is not a problem for the animal itself; it can be, depending on the situation, for other individuals, for animals in the food chain or even for humans. This animal can be a source for secondary AR exposure. Indeed, this animal or its products can be consumed.

In the case of domestic animals, as exposures are mainly primary and occurs in a controlled environment, asymptomatic exposure to AR is suspected when animals were able to get access to the bait. There are few studies on the risk of the consumption of products from exposed animal. However, as AR can be excreted in milk and eggs (84, 85), it is advisable to withdraw these products from human and animal consumption and to contact a veterinary poison control center.

Considering wildlife, it is more complicated as it is not possible to suspect if the animals have been exposed (no controlled environment). It is an issue for the wildlife, as exposed animal can be a source of secondary exposure for predator. Thus, it is a major concern for hunted species like wild boar which can be a source of human exposure. According to the exposure

studies on small mammals, predators and wild boar, wild animals should be suspected of being exposed when they are found close to areas with significant human activity (urban area, farms...) (44, 46, 49, 51). Moreover, in some countries, they can be both a pest (and treated with AR) and a food source (146). This makes it necessary to provide pedagogical support to the population and a risk-benefit approach to the use of AR.

In the presence or absence of clinical signs, a suspicion of exposure to AR should be confirmed and its biological consequences assessed, in order to measure the impact of these molecules on non-target species. This monitoring will make it possible to measure the effectiveness and relevance of new practices and methods for controlling rodent populations with a view to reducing the exposure of non-target populations.

BIOMARKERS FOR ANTICOAGULANT RODENTICIDES EXPOSURE ASSESSMENT

Definition and Efficiency Criteria

Biomarkers are measurable parameters at biochemical, histological, immunological, physiological, or organismic levels, that are used as indicators of certain biological states or conditions (147). They are relevant in many scientific fields, especially in ecotoxicology, but also in the biomedical field.

In the field of ecotoxicology, there is a constant need to detect and assess the impact of pollution, particularly in cases of low concentrations of increasingly complex mixtures of contaminants. This has led to an increasing number of studies on the development of indicators of the biological effects of contaminants on organisms.

A common dichotomy for biomarkers in ecotoxicology is between biomarkers of exposure, and biomarkers of effect. While biomarkers of exposure are relevant for hazard identification (e.g., for xenobiotics, identification of the parent compound, or derived metabolites), biomarkers of effect are used for hazard assessment, as they assess the response of the exposed organism to the particular xenobiotic or complex mixture (147). A third category would be biomarkers of susceptibility, used for indicating the inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic substance.

Biomarkers are sought at different levels of the organisms (148, 149). They may be inducible enzymes playing a role in the xenobiotics elimination (i.e., phase I biotransformation enzymes—such as isoforms of cytochrome P450—and phase II enzymes such as glutathion-S-transferase), but also oxidative stress parameters, biotransformation products (e.g., metabolites levels in body fluids), stress proteins (heat-shock proteins), hematological parameters (e.g., delta-aminolevulinic acid dehydratase—ALAD—as a biomarker applicable to lead exposure), immunological parameters, reproductive and endocrine parameters, neuromuscular parameters (e.g., acetyl cholinesterase inhibition by organophosphorus and carbamate agricultural pesticides), genotoxic parameters (e.g., DNA adducts), physiological and morphological parameters (e.g., asymmetric development, organ size variations), or even

proteomics and genomics, which provide an integrated picture of the way an organism responds to a changing environment (150).

It is worth noting that misapplication or misinterpretation of biomarkers in ecotoxicology may lead to erroneous conclusions. The first potential biases arise from handling conditions of samples, including their collection and storage that might affect the final quantitative and qualitative assessment of the biomarker. Sample relevance may decrease due to biological (decomposition process including other living organisms) and physicochemical (oxidation, denaturation, hydrolysis...) factors that cause potential instability of the biomarkers. In general, ante-mortem samples are better processed because they are taken fresh from a live animal. This does not apply to those taken from animals found in the wild without knowing the time of their death therefore ignoring the post-mortem interval before their collection. Carcasses should ideally be collected upon animal death and specimens should be fixed in liquid nitrogen transported and stored at -80°C to ensure biomarker preservation. For an accurate interpretation of circumstantial changes, a thorough understanding of the post-mortem process of the biomarker in different organs regarding to its constancy over time and environmental factors (temperature, photoperiod, etc.) is necessary.

Interspecies extrapolation is not always possible, as well as translation from the laboratory to the field situation, and the impact of non-pollution-related variables (health condition, gender, age, nutritional status, metabolic activity, migratory behavior, reproductive and developmental status, population density, season, ambient temperature, heterogeneity of environmental pollution) should be considered as confounding factors (148). The relevance of the use of biomarkers is therefore sometimes questioned (151), as they may be useful as indicators of exposure and for the formulation of hypothesis in carefully controlled experiments, but might not be expected to provide useful predictions of relevant ecological effects, which does not meet the aims of environmental monitoring and ecological risk assessment to detect and/or predict adverse chemical effects on populations, communities, and ecosystems. Still, those markers may be relevant for long-lived species, or rarer species, for which non-invasive indicators are needed.

For non-target fauna poisonings by anticoagulant rodenticides (AR), while clinical biomarkers are relevant for the identification of individual poisonings in domestic and wild animals, ecotoxicological biomarkers are needed to assess the extent of exposure to AR and the sublethal effects of these contaminants on wildlife populations. Based on the criteria that have been defined to identify relevant biomarkers in the environmental risk assessment (ERA) for chemicals (149), we propose seven criteria for assessing the relevance and condition of use of AR biomarkers:

- Development stage: is the biomarker only an idea, or used for research purposes or sufficiently standardized to be used on the field?
- Dead or alive: does the biomarker is usable for a dead animal, a living animal or both?
- The availability of usual values or thresholds for the considered species.

- Compatibility with treatment with vitamin K: can biomarker be used to assess AR exposure or potential effects during treatment?
- Sensitivity: does biomarker can detect limited exposure to AR or confirm a symptomatic exposure?
- Specificity: are there confounding factors for the biomarker?
- The toxicological significance of the biomarker (relationship between response and impact to the organism) must be demonstrated.

Table 3 gathers and assesses the main biomarkers identified in this review according to previous criteria.

Exposure Biomarkers: the Concentrations of Anticoagulant Rodenticides Molecules

Non-invasive detection and quantification of AR can be performed in plasma (85, 152–155), milk (85, 124), and feces (37, 154, 156, 157), while invasive analysis can also detect AR in target organs, especially in the liver, that is widely used for forensics investigation especially for wildlife (61, 130, 131, 158–160), but also in muscle, fat, or bone (155). Analyses are usually performed by high performance liquid chromatography that can be coupled with UV, fluorescence (152) or mass spectrometry (157, 160–162) detection.

AR screenings can be relevant to confirm and quantify exposure to these chemicals. For domestic animals, it can be a valuable tool for confirming the diagnosis of AR intoxication in cases where ingestion has not been observed, (152) but it is not always really relevant, as blood parameters are quite sufficient to determine the need of treatment, and as it is not a possible routine exam in clinical settings. Furthermore, severity of clinical signs or extent of PT prolongation do not seem to be correlated to AR concentration in dogs (152) or sheep (85).

As feces are the main route of AR elimination, their use to monitor exposure to AR has been widely investigated, both for domestic species (e.g., sheep, dogs) (154, 155) and wildlife, with investigations on foxes (157, 163). Further research is still needed on the elimination pattern for each AR, to accurately monitor exposure to those products, and some limits for this method to be used on the field is that AR are not stable in feces when outdoor, so only fresh samples (<5 days) should be used for monitoring (163). Moreover, plasma and fecal levels of AR are not correlated with liver levels (37, 154, 164). Some AR such as bromadiolone circulate poorly in the blood. Thus, the assessment of exposure through plasma or fecal material may lead to false negative results or underestimated exposure.

Investigations on AR levels in the liver of necropsied birds of preys have been widely conducted, with the purpose to determine a threshold that could allow to differentiate between simple environmental exposure and real intoxication on those animals, and thus to better assess the ecological impact of AR on the wildlife population. A repeatedly cited toxicity threshold is above 100–200 ng/g wet weight (165), but was determined rather as a potential lethal range, and derived from a single species, the barn owl (*Tyto alba*). More recent research has been conducted by combining both published data and results from surveillance programs in Canada and using logistic regression

TABLE 3 | Biomarker assessing the exposure and the effect of anticoagulant rodenticides in animals.

Biomarker	Tissue	What is dosed	Dosing methods	Development stage (experimental, research, available)	Dead or alive	Stability of sample over time	Available usual value	Compatibility with treatment	Sensitivity	Specificity	Toxicological significance
AR dosing	Liver (organs)	Levels of AR in the tissue—detection can be stereoisomer-specific	Usually LC-MS/MS	Available	Dead (or through biopsy)	Really stable	NA	Yes	High	High	Proposed threshold for birds of prey, to be further investigated
	Plasma				Alive						No
	Feces				Alive or freshly dead	Quick decrease over time when exposed to weathering	NA	Yes	Low	High	No
Prothrombin time	Plasma on citrated tube	Ratio based on coagulation time of a citrated plasma, without platelets, with calcium and thromboplastin	PT assay	Available	Alive	NA	Available for domestic animals	No	Medium	Medium	High in acute exposure
Vitamin K dependent clotting factors	Plasma on citrated tube	Activity of clotting factor	Chromogenic assay	Research	Alive	NA	No in animals	No	Medium	High	High in acute exposure
ucOC	Plasma	Undercarboxylated osteocalcin as the percent of the total osteocalcin in the sample	Radioimmunoassay (RIA) or ELISA	Experimental	Alive	NA	NA	Unknown	Unknown	Unknown	Unknown
ucMGP	Plasma and some tissues	Undercarboxylated, inactive species of MGP	Radioimmunoassay (RIA) or ELISA	Experimental	Both depending on the tissue considered	NA	NA	Unknown	Unknown	Unknown	Unknown
VKOR activity from liver extract	Liver	Ability to produce vitamin K quinone from epoxide form	Enzymatic assay	Research	Dead	Has to be immediately store at -80°C	NA	NA	NA	NA	Assess one mechanism of resistance
VKOR activity from VKORC1 expressed in yeast or cell	DNA sample or VKORC1 sequences	Enzymatic assay : Ability to produce vitamin K quinone from epoxide form Cell assay : ability to produce carboxylated proteins	Enzymatic or cell assay	Research	Alive	Long	NA	NA	NA	NA	Assess one mechanism of resistance
Vitamin quantification	Liver, kidney or lung	Vitamin K1, and MK-4, in quinone and epoxide forms	HPLC-fluorescence	Experimental	Dead	Fairly stable at room temperature	Epoxide forms are absent in healthy animal	Yes	Unknown	Quite high (author data)	High in acute exposure, unknown in chronic exposure

NA, not available.

to estimate the probability of toxicosis associated with different liver AR residues. It described a significant likelihood of toxicosis in 5% of individuals with liver concentrations of 20 ng/g wet weight and in 20% of individuals with concentrations of 80 ng/g wet weight (160). Still, one of the limitations pointed out in the study, as well as in some other publications (159), is the species-specific sensitivity that prevents any extrapolation of a potential threshold, especially as a discrepancy exists between birds tested in captivity which might be more tolerant to AR than free-ranging birds with more complex daily activities and environmental stressors. Some authors thus consider that such a parameter may not be a relevant diagnostic tool, as it is neither accounting for the impacts of sublethal effects on reproduction and non-target mortality, nor for bioaccumulation of repeated sublethal exposures (166). Indeed, it has been demonstrated that subsequent exposures to AR may lead to increased adverse effects on the contaminated birds (159). Finally, it has also been suggested that the use of total sum of AR to assess the extent of exposure should rely on a quantification expressed in nmol/g instead of ng/g, that would better reflect the inhibitory potency on VKOR for each AR (159).

Beyond the assessment of exposure, liver AR dosing and more specifically the dosing of stereoisomers may provide information on the type of exposure and in the case of primary exposure may help to estimate the time from bait ingestion to death. Because stereoisomers have different elimination rates, the longer the time between ingestion and death, the greater the proportion of persistent enantiomers in the total residue (62, 74, 93). Conversely, the shorter the delay, the closer the ratio of enantiomers will be to the initial bait. This clue on time between ingestion and death might be useful to determine the cause of death. Indeed, it is unlikely that an AR can kill in <2 days. Moreover, a ratio of stereoisomers close, to that of the bait is in favor of a primary exposure.

Effect Biomarkers: Highlight Anticoagulant Rodenticides Effects Blood Clotting Assessment

As anticoagulant rodenticides main action is on coagulation cascade, blood clotting assays are obvious and relevant diagnostic tools.

Prothrombin time (PT) measured on citrated plasma samples is considered a sensitive test to confirm AR contamination, as it is the first coagulation parameter to be altered after AR exposure, due to the short half-life of factor VII (6.2 h) (167). It should be noted, however, that in animals with clinical bleeding both PT and aPTT (activated Partial Thromboplastin Time) are prolonged, while thrombin time remains normal. Still, since assesses the extrinsic—or tissue factor—pathway of coagulation, it remains a more specific marker of AR intoxication, and is considered as a clinical standard for the diagnosis of AR intoxication (36, 120, 121, 152, 168).

PT as a diagnostic tool for AR contamination is widely described in dogs (118, 119, 121–123, 152, 168). Basal value for dogs is around 6–10 s, and is greatly prolonged after AR exposure, with reported median values between 40 (119) and

52.3 s (152). A commonly used threshold is a 25% increase over a control animal.

In ruminants, same mechanisms are expected, and there are also some reports of increased PT after AR exposure in cattle (124), sheep (85, 125), and goats (169), with similar variations. In one study, baseline PT for sheep was around 13.25 s (SD = 0.52), increasing up to 27–40.7 s after administration of the AR (125). As reference baseline values cannot be easily found for those animals, and values may differ among breeds and the analytical method used (170) [e.g., for sheep median values vary from 7.31 s in “mixed breed” ewes (171) to 40 s in a study with Austrian Mountain ewes (172)], the use of a control animal may be beneficial (85).

While PT seems a good biomarker of AR intoxication in domestic animals, no linear correlation was found between AR concentrations and extent of PT prolongation neither in dogs (152) nor in sheep (85).

It is worth noting that some research has previously been performed on the use of Proteins Induced by Vitamin K Antagonism or Absence (PIVKA) for the sensitive diagnosis of AR intoxication in dogs (173). The PIVKA test was originally designed to monitor human patients under treatment with coumadin and is still use in human medicine for diagnosis of e.g., hepatocellular carcinoma, pancreatic, or biliary tumors. This test is quite similar to the PT-test, using a diluted plasma sample and an “altered” thromboplastin that delays the *in vitro* clot formation, and thus may be more sensitive in detecting prolongation of clotting time by AR. Still, as the PT variation after AR exposure is usually marked, and with the availability and standardization of PT reagents, the PIVKA test was quite abandoned as a diagnostic tool, especially as results of this test can also be increased by deficiencies in factors II, VII, and X because of heredity and acquired coagulopathies other than anticoagulant poisonings, and is sensitive to the presence of heparin (168, 173).

Blood assays are also used for diagnosis of AR exposure in wildlife animals, especially birds of prey (61, 132, 174–176). In those animals, lengthening of PT by more than 25% or two standard deviations above baseline values is considered as suggestive of AR exposure (61). Although blood clotting assays seem promising for diagnosis of AR exposure in wildlife, there is still a need to establish species specific reference values and standardize assay methods among testing facilities (175). Among observations in wild birds, some studies showed that far greater PT values were observed in birds undergoing subsequent challenge exposure to AR than the ones observed in previously unexposed birds, suggesting a potentiation of AR adverse effects after repeated exposure as a sub-lethal effect of AR (158, 159). One limit of the PT assay in birds is the recommended use of homologous (i.e., avian) thromboplastin, not readily available, to efficiently trigger *in vitro* coagulation. Great variations (up to three-fold increase of PT, and even higher increase for aPTT) can occur when using human or mammalian (rabbit, bovine) thromboplastin for avian clotting assays (177–179). To complete avian blood coagulation assays, another test, Russel's Viper Venom Time, is routinely used, (159, 174–176), with observed increases of both “adapted” PT time and Russel's Viper Venom Time within 48–96 h following the exposure to AR

(174). Russell's viper venom is known to activate factor X in the common pathway of the clotting cascade (180). Finally, thrombin generation test and thromboelastometry can also be used, even if they don't bring any added value (181, 182).

Vitamin K-Dependent Proteins (VKDP) Dosage

Vitamin K-dependent proteins (VKDP) dosing might be an important source of biomarkers as the inhibition of the recycling mechanism of vitamin K has direct consequences on those proteins. Nevertheless, the challenge is not only to dose VKDP but also to dose separately carboxylated and under-carboxylated VKDP (ucVKDP). Indeed, AR molecules act on the post-translational gamma-carboxylation mechanism but not on the genetic expression of VKDP.

Vitamin K-dependent clotting factors

The dosing of vitamin K dependent clotting factors might be a good indicator of AR intoxication. These dosages are based either (i) on the evaluation of the activity of the clotting factor under consideration through a reaction cascade (close to the coagulation cascade) leading to the production of a chromogenic factor and where the dosed factor is the limiting factor, or (ii) on the supplementation of plasma depleted in the factor considered. Only carboxylated clotting factors should be assessed. As carboxylated clotting factors decrease faster than increase in clotting times arises, their dosing allows early detection of intoxication. For example, in rats and after an ingestion of a lethal dose of AR; the factor VII and factor X activity are halved in about 4 and 5 h, respectively, while prothrombin time doubles in 10 h (182). However, as the assessed clotting factors have to interact with reagent component optimized for humans, it is not obvious that this method is conceivable for all species. Moreover, there are not usual values for animals.

Other vitamin k-dependent proteins

If vitamin K dependent clotting factors are linked to the acute toxicosis of AR, other VKDP might be linked to their chronic effects. The two major non-clotting VKDP are osteocalcin (OC) and Matrix gla protein (MGP).

Osteocalcin limits bone formation without impairing bone resorption or mineralization (76). Under-carboxylated osteocalcin circulates naturally in bloodstream and seems to have positive effects on insulin sensitivity (183, 184). Moreover, several factors influence serum under-carboxylated osteocalcin levels notably osteoblastic synthesis, hormonal status, renal function, age, sex, timing of blood sampling, and specificity of the radioimmunoassay (185–187). Recently, a study showed that under-carboxylated osteocalcin increases in resistant rodents under vitamin K deficient diet (188). But currently there is no link between osteocalcin and chronic AR exposure effects. So, further research is needed to determine if carboxylated osteocalcin or under-carboxylated osteocalcin can be useful as biomarker of AR effects.

Conversely, under-carboxylated MGP (ucMGP) is well-known to be in favor of vascular calcification (189, 190), which is one of the suspected effects of chronic exposure to AR. In rats an increase of ucMGP resulting from a vitamin K deficiency

is associated to vascular calcification (191). Further studies are needed to confirm this association and the link with chronic AR exposure, but circulating ucMGP might be a good candidate as biomarker.

MGP and OC are small proteins currently measured by immunoassays. Their sequences are variable between species, which raises the question of assay methods. Their assay will require the development of specific antibodies in the absence of methods to evaluate their activity. Moreover, as they are proteins, their stability at room temperature in animal cadavers will be a challenge. These biomarkers will eventually be used as research tools, but their use in surveillance studies seems difficult in the future. On the other hand, they could be dosed on live animals as well as on dead animals, from blood (OC and MGP), bone (OC), vascular, pulmonary and renal tissues (MGP).

VKOR Activity

In mammals, two different enzymes possess Vitamin K epoxide Reductase activity (VKOR) and are coded by paralogous genes. The enzyme called VKORC1 is mainly located in the liver and is the main contributor to the recycling of Vitamin K needed for activation of clotting factors, while the enzyme called VKORC1L1 does not sustain coagulation but maintain the Vitamin K cycle for carboxylation of other VKDP (192, 193).

In human, sensibility to warfarin before an anticoagulant treatment can be assessed by testing alleles coding for VKORC1 to detect the presence of potential variants susceptible to adapt the proper dose of warfarin (194, 195). These are consequently biomarkers of the susceptibility to a rodenticide and this pharmacogenomic approach could be used as a preventive measure.

Thus, the study of the differences in VKOR activity for each species can be useful to evaluate their sensibility to AR. This assessment is currently based on enzymatic assays (73, 108), that implies either to sample fresh liver from considered species or to use heterologous expression of VKORC1 in yeast or eukaryotic cells. Then the inhibition capacity of each AR molecule can be tested *in vitro* against VKORC1 of each species. Two methods are currently described to measure VKOR activity, the DTT-driven VKOR assay (73) and the cell-based VKOR assay (196). The first method allows the characterization of the susceptibilities of VKOR activity from animal tissues or recombinant VKORC1 enzymes. The second method allows the characterization of VKOR activity only from overexpression of VKORC1 enzymes and required thus to know the amino acid sequence of the VKORC1. Unfortunately, VKORC1 sequence is known only for few species. Nevertheless, characterization of VKOR activity by *in vitro* assays assesses only the susceptibilities linked to VKORC1 and is cumbersome to implement.

Vitamins Quantification: Picture of the VKOR Activity

Vitamin K is available in organisms under different forms, called vitamers. A distinction can be made between vitamin K1, also called phylloquinone, of vegetal origin, and menaquinones (MK), from bacterial origin. All those vitamers share a common 2-methyl-1,4-naphthoquinone cycle, corresponding to vitamin K3, derived from synthesis or catabolism. Vitamin K1 has a

phytyl side chain at the 3-position, while MKs have several repeating prenyl units, the number of units being given as a suffix (menaquinone-n or MK-n) (197). Menaquinone-4 seems to have a major but not fully elucidated physiological role, and is the main form of vitamin K in the organism. It can be used in humans as drugs or supplements for newborns (198). MK-4 can be synthesized in the body, from vitamin K1 of feed origin or from vitamin K3, by a biosynthetic enzyme localized in the endoplasmic reticulum and ubiquitously expressed, known as UBIAD1 (199). MK-4 is also from microflora origin. Vitamin K1 is mainly found in liver and heart, with high storage capacities, while MK-4 is preferentially found in extra-hepatic tissues, especially pancreas and testis (198). Nevertheless, from the authors' experience, some species have hepatic MK-4 concentrations higher than K1.

VKORC1 is partly responsible for the recycling of vitamin K: when the VKOR activity is impaired by AR, it can be assumed that it has an impact on the concentrations of those vitamins, with a possible shift between epoxide forms and quinone forms. A dose-response relationship was suggested between ratio of epoxide and quinone forms of vitamin K1 in plasma and concentrations of two AR, phenprocoumon and warfarin (200). It was also proposed to use measurements of this ratio for diagnosis of AR intoxication in dogs (201). Moreover, during a treatment with vitamin K1, if an animal is intoxicated by rodenticides the epoxide forms will increase the same way as the quinone form, while if it is not, only the concentration of vitamin K quinone will be increased. Thus, this method could be used to assess an intoxication while under treatment. Further studies will be necessary to explore vitamin K and vitamin K epoxide concentrations as potential markers of AR exposure. Nevertheless, because differences in vitamin K concentrations between species are predictable due to differences in diet and microflora, determination of usual values would be necessary for each species. One way to overcome this difficulty may be to assess the ratio between quinone and epoxide forms.

The measurement of vitamin K forms is usually performed by chromatography (liquid, high performance), that can be coupled with fluorescence (152) or mass spectrometry (157, 160–162) detection. The determination of vitamin K concentrations can be done in plasma, as the different forms circulate in the plasma in live animals. Nevertheless, their concentration in the plasma could be biased by the last meal. The determination of vitamins K can also be done post-mortem from different tissues, liver, kidney, etc. Nevertheless, the relevance of this assay in different tissues in relation to AR concentrations will require further studies. Nevertheless, vitamin K concentration measurement appear to be a promising route to provide relevant biomarkers. Vitamin K forms appear to be rather stable when the samples are protected from light.

Toward New Tools to Develop Biomarkers for Sublethal Effects of AR? Cytochrome P450 Pattern or Activity

Cytochromes P450 (CYPs) are a superfamily of enzymes containing heme as a cofactor that function as monooxygenase.

CYPs are the major enzymes involved in drug metabolism, accounting for about 75% of the total metabolism of xenobiotics. Most xenobiotics are inactivated by CYPs, although some are bioactivated. The detection and quantification of specific metabolite(s) resulting from this metabolization can be a biomarker of exposure. In the case of AR and vitamin K, the pathways of metabolization are not really well-defined. Vitamin K1 metabolism would involve different CYPs. In humans, CYP4F11 and F2 have been identified. The metabolism of warfarin in humans involves CYP2C9, but also CYP1A2, 2C19, and 3A4. In rats CYP1A1, CYP2B1, CYP2C6, CYP2C11, and CYP3A2 (202) are involved in the oxidation of warfarin. The metabolism of other ARs is currently poorly characterized. Nevertheless, the intervention of CYP is probable because of the lipophilic nature of these molecules and the low number of functionalized groups present on the molecules (except for the OH group in position 4, only bromadiolone has a second hydroxy group). In addition, different arguments are present in the literature suggesting a CYP450-dependent metabolism. It has been shown that resistance to difenacoum or chlorophacinone may be caused by increased catabolism through Cytochrome P450 in exposed rodent population (115, 203). Bromadiolone also seems impacted by P450-promoted-catabolism level (117, 204).

Many drugs may increase or decrease the activity of various CYP isozymes either by inducing the biosynthesis of an isozyme (enzyme induction) or by directly inhibiting the activity of the CYP (enzyme inhibition). Many studies deal with Cytochromes P450 as biomarkers of xenobiotic exposure in the liver but also in the blood (easy ante-mortem analysis) thanks to plasma exosomes [e.g., (205–214)]. This enzyme family is really well-studied and known, and methodologies already exist for this kind of application, thus it is a promising approach. However, as Cytochromes P450 responsible of AR catabolism differ according to species and to AR molecules, extensive research is needed to implement this method.

Metabolomics and Genomics

Metabolic profiling, also called metabolomics, consists in a screening of a maximum of metabolic parameters, in a fresh biological sample, that should as far as possible be relevant to the precise state or condition of the organism from which the sample come from at the time of the sampling. The usual but non-exhaustive parameters studied are: the evolution of protein expression level, of metabolites, of biological parameters (lipids, proteins, carbohydrates, salts, vitamins...), of cell number and relative types, of redox and acido-basic parameters, etc. The parameters are as well qualitative for example by studying the structure of a molecule or the morphology of a cell, as quantitative, either relatively to a given parameter, either through absolute quantification (215).

One classical use of metabolic profiling is the research of very specific biomarkers for disease or exposure to xenobiotics (216–219). It could therefore be used to discover new specific biomarkers for rodenticides exposure. For example, an ethanolamide plasma lipids regulated by warfarin was found during a metabolomic study and could be used as biomarker of warfarin exposure (220). Moreover, metabolomic is also a

screenshot at a given time of many parameters from an organism and combined together, the data can provide a very precise map of the condition of the organism including several parameters (221, 222) converging toward an exposure at anticoagulant rodenticides and permitting differentiation between various levels and nature of exposure as shown already in one study with brodifacoum (223).

The limitation of this method is its current technical difficulty and the requirement of very advanced data analysis (215), including most likely the need of a significant pre-existing comparison database. However, this methodology could become easy, fast, and very reliable through automatization using artificial intelligence combined with deep learning algorithms.

On the other side, genomics is the study of global gene expression patterns. It addresses all genes and their inter relationships in order to identify their combined influence on the organism (224), and relies on DNA or RNA sequencing. A recent study on AR-positive bobcats (*Lynx rufus*) used RNA sequencing on whole blood to investigate genes and cellular processes affected by sublethal exposure to AR. Differential expressions of genes involved in xenobiotic metabolism, endoplasmic reticulum stress response, epithelial integrity and immune function were identified (145). In particular, simultaneous immune dysregulation and disruption of epithelial integrity was suspected to predispose bobcats exposed to AR to opportunistic infections by ectoparasites, e.g., notoedric mange caused by *Notoedres cati*, with an increased mortality rate. Beyond those conclusions, genomics appears as an interesting tool to study

all metabolic pathways impaired by AR exposure, allowing for the development of new indicators (e.g., biological parameters related to those metabolic functions) to assess the sublethal effects of AR.

CONCLUSION

If rodenticide anticoagulants are currently essentials to manage pest rodent populations, wildlife and domestic animal exposure to AR is a major concern. The exposure level of wildlife is important close to area with human activities. However, it is difficult to assess the consequences of exposure on the non-target animal population, since there are great differences of susceptibilities between species. The biomarkers can be useful tools in this task by highlighting AR effect on non-target species and thus to open the way to an evaluation of pest control practice in order to decrease AR impact. While some biomarkers seem promising, they are currently underdeveloped for AR monitoring purpose. Some of the biomarkers proposed in this study could be developed for systematic use in AR monitoring exposure in the field, while others, which are more complex to implement, could certainly only be developed for research purposes.

AUTHOR CONTRIBUTIONS

AR, M-AM, XS, SL, and VL: conceptualization, writing—original draft preparation, and review. All authors contributed to the article and approved the submitted version.

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5.2.2.2 Article :

Interest of the Faecal and Plasma Matrix for Monitoring the Exposure of Wildlife or Domestic Animals to Anticoagulant Rodenticides

Interest of the Faecal and Plasma Matrix for Monitoring the Exposure of Wildlife or Domestic Animals to Anticoagulant Rodenticides

Antoine Rached¹, Tarek Mahjoub^{1,3}, Ambre Fafournoux¹ Brigitte Barbier¹, Isabelle Fourel¹, Hervé Caruel², Sébastien Lefebvre¹, Virginie Lattard¹

Abstract

Anticoagulant rodenticides (ARs), particularly second-generation compounds (SGAR), are known to be a potential threat to unintended species due to their tissue persistence. The liver is the storage tissue of ARs and is a matrix of choice in diagnosing exposure and intoxication of non-target fauna. However, it is only available on dead animals. Blood and faeces can be used on living animals. These two biological matrices were compared in terms of their relevance to exposure to ARs. In addressing this question, we compared the faecal, plasma and liver concentrations of bromadiolone, one of the SGAR frequently implicated in wildlife exposure. We studied this comparison at the individual level and at the population level, considering three influencing factors: dose, sex and time. Our findings demonstrate that faecal analyses are more valuable than plasma analyses for monitoring AR exposure of domestic and wild animals, even if faecal concentrations cannot be correlated with liver concentrations.

Keywords

Anticoagulant rodenticides, Exposure, Biological matrix, Faeces, Plasma, Liver

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Introduction

Anticoagulant rodenticides (hereafter ARs) have been widely used as an effective chemical solution to control rodent populations. These compounds were developed in two generations: first-generation ARs (FGARs), including warfarin, coumatralyl and chlorophacinone, for which resistance has been a limitation to their use, and second generation ARs (SGARs), such as bromadiolone, brodifacoum, difenacoum, difethialone, and flocoumafén, which were developed to improve efficacy and overcome FGAR resistance. Although developed in two generations, all of these compounds are vitamin K antagonists that cause coagulopathy in vertebrate animals due to a deficiency of vitamin K, required for activation of clotting factors II, VII, IX and X [1].

Anticoagulant rodenticides have been used for many years either as plant protection products or as biocides in and around buildings. Numerous studies mention exposure of non-target

species either through these uses allowing animals to come into direct contact with the baits (primary exposure) or through the food chain by consumption of poisoned prey or carrion (secondary exposure). Accidental exposures and poisonings to ARs have been widely documented worldwide in humans, pets, livestock, and wildlife, posing a public health concern due to this non-selectivity [2]. The involvement of the different ARs in the exposure of non-target wildlife has been reported to differ depending on the properties of the ARs and their generation, with longer tissue persistence and stronger anticoagulant potency for SGARs [3, 4]. Concurrently, the consequences of exposure to ARs have been suggested to vary between species with species more sensitive and more frequently exposed to ARs [5], but also according to different individual factors such as sex, diet, physiological and nutritional status. The main signs associated with AR exposure are hemorrhagic, that can lead to the death of the animal. Nevertheless, other adverse effects have been suggested during sublethal exposure to ARs [6–8].

The increasing detection of AR in wildlife [9–16] (and in domestic animals [17–24]) has raised awareness of the environmental risks posed by these compounds. This has prompted authorities to regulate the use practices in many countries, but also to implement exposure monitoring to prevent poisoning. To carry out this monitoring, the systematic question of the choice of the matrix to be used has often arisen. The liver has of course been the chosen matrix, since it is the target tissue and the storage tissue of ARs [25] with a proposed toxic threshold of 100 to 200 ng per gram of liver [26]. However, as this matrix is only available on dead animals, it does not allow for the assessment of the level of exposure on a population scale in order to prevent the mortality of animals due to a source of contamination present in their close environment. In this perspective, two biological matrices, blood and faeces, have been used on living animals in various scientific studies (for plasma, [24, 27–30]; for faeces, [31–33]). Nonetheless, few data are currently available concerning their relevance to reflect the exposure of an animal to ARs. Considering the aforementioned difficulties in diagnosing AR poisoning versus simple exposure, it was necessary to determine whether the residues detected in the faeces or the plasma reasonably reflect those present in the liver. In addition, it is also necessary to assess if AR residues in these matrices provide valuable information to distinguish primary from secondary exposure of non-target species.

Hence, we conducted a study in rats exposed to bromadiolone, the latter being one of the ARs most frequently implicated in wildlife poisonings [25] i/ in order to compare quantitatively and qualitatively the hepatic, plasma and faecal residues, and ii/ to evaluate the conclusions that may be reached from these determinations

1. Material and methods

1.1 Chemicals

Bromadiolone (3-[3-[4-(4-bromophenyl)phenyl]-3-hydroxy-1-phenylpropyl]-4-hydroxychromen-2-one) was provided by Liphatech (Pont-du-Casse, France). It was a mixture containing 85 % of the trans-isomers in racemic proportion (1S, 3R and 1R, 3S) and 15 % of the cis-isomers in racemic proportion (1S, 3S and 1R, 3R). Isoflurane® and vitamin K1 were purchased from Alcyon (Miribel, France).

1.2 Animals

Seven-week-old male (n=30) and female (n=30) OFA-Sprague Dawley rats were obtained from a local commercial breeder Charles Rivers in l'Arbresle, France. Animals were housed three per standard cage (Eurostandard, Type IV, Tecniplast, Limonest, France) for rats in the institutional animal facility and kept in a controlled environment (ambient temperature $20\text{ °C} \pm 1\text{ °C}$ and 12-h light/dark cycle). They were given standard laboratory food (Scientific Animal Food and Engineering, reference A04) and water ad libitum. Animals were acclimated for at least one week prior to use and were maintained in accordance with the European and French legislation guidelines on animal studies.

1.3 Animal study and sample collection

Experimental research on the rodents was carried out according to an experimental protocol following international guidelines and approved by the ethics committee of the Veterinary School of Lyon (authorization n°201704190941578). Male and female OFA-Sprague Dawley rats received through per os administration 0,5 mg/kg and 3 mg/kg of bromadiolone dissolved in 5 % DMSO and 95 % vegetable oil. Rats were maintained alive by daily subcutaneous administration of vitamin K1 ($10\text{ mg}\cdot\text{kg}^{-1}$). At 24, 72, 168, 336 and 720 hours after bromadiolone administration, 3 rats were anesthetized with isoflurane, and blood was drawn by cardiac puncture into citrated tubes. Then, rats were euthanized with CO₂ and the liver of each rat was immediately excised and taken. Faeces of the day were collected directly from the cage at the same time points. Samples were weighed and stored directly at -20 °C until analysis.

1.4 Analysis

1.4.1 Determination of bromadiolone concentration in the liver

The method of [34] was used in this study. Briefly, a solid-liquid extraction was performed to extract bromadiolone from rats' liver. The sample (1 g of liver tissue) was homogenized with 10 mL of acetone using an Ultra Turrax tissue disperser. The extract was subsequently centrifuged ($2817.6 \times g$ at 4 °C) for 10 min. The resulting supernatant was transferred in a test tube and evaporated at 60 °C under a gentle nitrogen flow. The test tube was rinsed and cleaned up with 2 mL of methanol and evaporated to dryness. Dry extract was then reconstituted in acetonitrile/hexane (50 % / 50 %). The top layer was removed, and the rest was dried at 60 °C . The final dry extract was dissolved in 1 mL methanol and the

bromadiolone concentration was analyzed by LC-MS/MS as previously described [33]. The extraction recovery of AR in liver using this protocol has been previously reported to be above 70%[35].

1.4.2 Determination of bromadiolone concentration in faeces

Faecal samples were extracted according to the protocols described by [31] and [32]. Briefly, the faecal samples were first dried for 48 h in an oven at 50 °C. An aliquot of 0.50 ± 0.01 g of crushed dried faeces was weighed and placed in a 50 mL polypropylene Falcon tube containing 10 mL of acetone. The whole was homogenized by shaking with a vortex. The homogenate was left for one hour without agitation. Then the samples were placed in a rotator PTR-60 during 10 min and were then centrifuged ($2817.6 \times g$ at 4 °C) for 5 min. Six millilitres of supernatant was transferred to test tubes and evaporated to dryness under nitrogen stream at 40 °C. The residue was taken up with 1 mL of acetonitrile, shaken twice for 10 s with a vortex. Another 1 mL of hexane was added to each tube and left for a minimum of 30 minutes. The upper layer was discarded, and the remainder was evaporated to dryness. The analytes were resuspended with 200 μ L of methanol, vortexed for 20 s. and filtered through a 0.2 μ m phenex filter (Phenomenex, Torrance, CA, USA) before LC-MS/MS analysis as detailed in [35]. The extraction recovery of AR in faeces following this protocol has been previously reported to be above 70% [35]. Bromadiolone concentrations in faeces were expressed as μ g bromadiolone per g dry matter.

1.4.3 Determination of anticoagulant concentration in the plasma

Liquid-liquid extraction was used to extract anticoagulant rodenticide from rat plasma. A volume of 200 μ L of plasma was mixed with 4 mL of acetone using a programmable rotary shaker in a sequence of cycles with orbital, reciprocal, and vortex rotation for 10 minutes. The mixture was centrifuged ($2817.6 \times g$ at 4 °C) for 5 minutes. The liquid phase was collected and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The extraction recovery of AR in plasma following this protocol was above 70%. The dry extract was reconstituted with 200 μ L of methanol and filtered through a 0.2 μ m phenex filter (Phenomenex, Torrance, CA, USA) before LC-MS/MS analysis [36, 37].

1.5 Data analysis

Data handling and statistical analyses were conducted with GraphPad Prism 8 software (GraphPad, San Diego, CA, USA). Data are presented as the median and its 95% confidence interval as box-and-whisker plots. Statistical analysis was done by non-parametric analysis using the Tukey's multiple comparisons test or the Mann Whitney tests. $P < 0.05$ was the accepted level of significance.

2.1 Comparison of faecal and plasma versus liver bromadiolone concentrations at the population level

Faecal, plasma, and liver concentrations were compared from samples taken at the same time from rats exposed to bromadiolone (containing 85% trans and 15% cis isomers) at lethal or sublethal concentrations, 24 to 720 hours prior to sampling. Bromadiolone was detected in all faecal samples ($n=60$) in which bromadiolone was present in the liver ($n=60$), whatever the dose and the post-administration period. In contrast, no bromadiolone was detectable in 3 plasma samples out of 58 (for 2 animals, blood collection was not successful). The median value of liver concentrations was 4.90 μ g/g, 20 times higher than that of faeces and 350×10^3 times higher than that of plasma (Table 1). The concentration ratios between liver and faeces ranged from 0.86 to 52.74, between liver and plasma from 30×10^3 to 8597×10^3 (Figure 1). Bromadiolone residues found in faecal samples were all composed of cis- and trans-isomers (Table 1, $p < 0.0001$ for cis and trans-isomers between liver and faeces), while 4 liver samples did not contain cis residues. The concentration ratios between liver and faeces ranged from 0.00 to 18.03 for cis-isomers; from 0.87 to 79.13 for trans-isomers (Figure 1). For plasma in which bromadiolone was present, 6 of 55 samples did not contain cis-isomers; 9 of 55 were negative for trans-isomers. The concentration ratios between liver and plasma (including only those containing cis and/or trans-residues) ranged from 5.4×10^3 to 1113×10^3 for cis-isomers; from 60×10^3 to 4617×10^3 for trans-isomers (Figure 1).

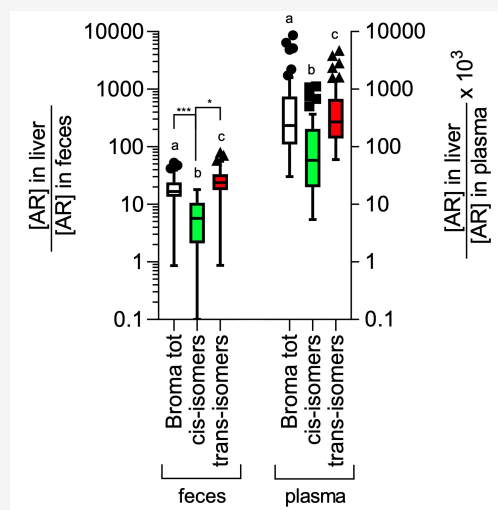


Figure 1. Ratio of AR concentrations between liver and corresponding faeces or plasma (in white, total bromadiolone; in green, cis-bromadiolone; in red, trans-bromadiolone). Statistical analysis was done using a non-parametric Tukey's multiple comparisons test. $P < 0.005$ was the accepted level of significance. The same letter indicates a statistical difference between plasma and faeces. *, indicates a statistical difference within the same matrix.

2. Results

	Bromadiolone	Cis-bromadiolone	Trans-bromadiolone
Liver ($\mu\text{g/g}$)			
Median [95% CI]	4.90 [3.67-7.60]a,b	0.65 [0.38-1.02]d,e	4.16 [3.07-6.02]g,h
Lower value	1.67	0.00	1.67
Upper Value	69.44	10.05	59.39
Feces ($\mu\text{g/g}$)			
Median [95% CI]	0.25 [0.20-0.34]a,c	0.08 [0.07-0.10]d,f	0.17 [0.13-0.27]g,i
Lower value	0.12	0.06	0.07
Upper Value	20.1	1.89	18.25
Plasma (ng/mL)			
Median [95% CI]	0.014 [0.006-0.039]b,c	0.007 [0.002-0.014]e,f	0.009 [0.003-0.033]h,i
Lower value	0.00	0.00	0.00
Upper Value	1.63	1.20	0.43

Table 1. AR concentration in liver, feces and plasma in rat population. Statistical analysis was done using a non-parametric Tukey's multiple comparisons test. $P < 0.005$ was the accepted level of significance. The same letter indicates a statistical difference.

2.2 Factors influencing liver/faeces concentration ratios at the population level

2.2.1 Depending on the post-exposure period

Liver/faeces concentration ratios were grouped according to the time between exposure to bromadiolone and the time of sampling (from 1 day to 30 days) (Figure 2). The ratio of liver to faecal bromadiolone residue concentrations increased significantly from 24 hours postadministration to later time points. One day post administration, the mean value of the liver to faecal total bromadiolone concentrations ratio was 6.6 ± 5.1 , increased to 26.6 ± 11.9 at 7 days post-administration and then stabilized. (Figure 2 A). The variations observed were due to variations in liver and faecal trans-bromadiolone concentrations, whereas the ratios of cis residues did not change with post-exposure times.

2.2.2 Depending on the dose of bromadiolone administered

Ratios of liver and faecal concentrations were grouped according to the dose administered, i.e. sublethal or lethal. No change in ratios was observed as a function of the dose, for total bromadiolone residues and their trans-isomers. However, a significant difference was observed for cis-isomers with a ratio decreasing by a factor close to 3.5 when the dose increased from 0.5 mg/kg to 3 mg/kg.

2.2.3 Depending on the gender

Ratios of liver and faecal concentrations were grouped by gender (Figure 4). A significant difference between males and females was observed for total bromadiolone residues and their cis-isomers with a higher ratio in females.

2.3 Comparison of faecal and hepatic concentrations at the individual scale

Liver concentration of each individual was presented according to its corresponding faecal concentration (Figure 5). No correlation was observed between faecal and liver concentrations. The highest faecal concentrations did not correspond to

the highest liver concentrations, faecal concentrations below $1.0 \mu\text{g/g}$ corresponded to liver concentrations ranging from $1 \mu\text{g/g}$ to $20 \mu\text{g/g}$ without any possible modelling (Figure 5, insert). Nevertheless, the liver concentrations could be grouped into two statistically different groups based on a threshold faecal concentration of $0.5 \mu\text{g/g}$ (Figure 6). For cis- and trans-isomers, similar profiles were observed (data not shown) with no correlation between faecal and liver concentrations.

2.4 Comparison of the diastereomeric composition of faecal and hepatic residues at the individual level

Diastereoisomeric composition of the hepatic residues of each individual was analyzed according to the diastereoisomeric composition of the residues of the corresponding faecal sample. Diastereoisomeric percentages were similar between liver and faeces for samples collected less than 48 hours after bromadiolone administration; they were statistically different ($p < 0.0001$) for samples collected more than 48 hours after bromadiolone administration (Figure 7).

3. Discussion

Our study first demonstrates the a meaningful comparison between the potential of the faecal matrix compared to the plasma matrix regarding extrapolation to liver values. For this, it was necessary to be able to compare the concentrations of AR on the three matrices in parallel (liver, blood, faeces) in order to evaluate the reflectiveness of the concentrations of AR in faeces or plasma with regard to those measured in the liver. This comparison was carried out on laboratory rats exposed to bromadiolone, since it is difficult to obtain in the field enough individuals of the same species exposed to the same anticoagulant, and especially at a known date and in a known amount of AR, to have statistically analyzable and interpretable data. These rats were exposed to bromadiolone (until very recently, this AR was used as plant protection product in Europe and has been widely reported in non-target

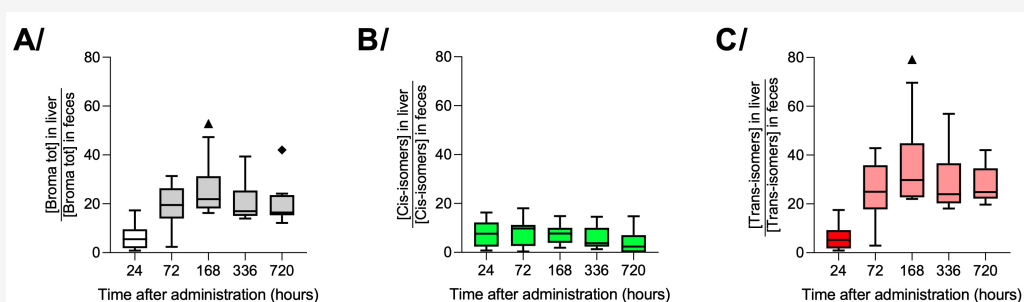


Figure 2. Ratio of AR concentrations between liver and corresponding faeces according to the delay post-exposition. In A/ ratio for total bromadiolone residues, in B/ ratio for cis-isomers of bromadiolone, in C/ ratio for trans-isomers of bromadiolone. Statistical analysis was done using a non-parametric Tukey’s multiple comparisons test. $P < 0.005$ was the accepted level of significance. In the same panel, different colors within the same graph are statistically different.

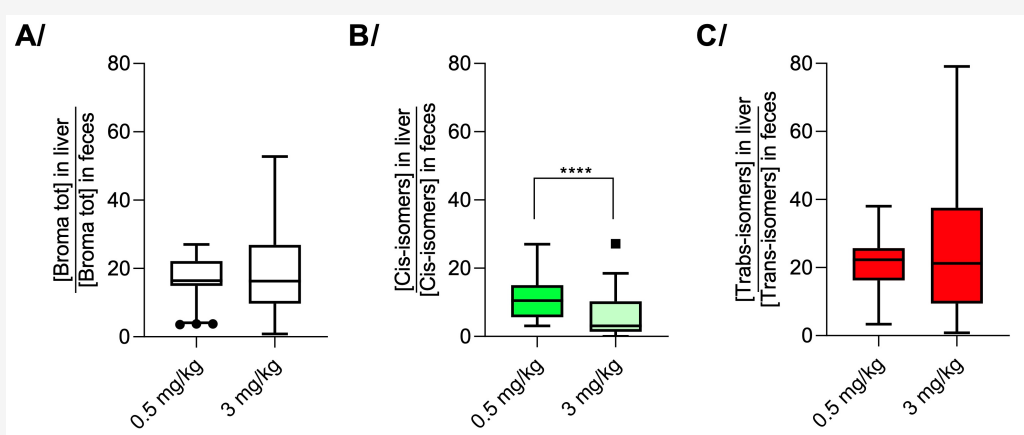


Figure 3. Ratio of AR concentrations between liver and corresponding faeces according to the concentration of bromadiolone administered. In A/ ratio for total bromadiolone residues, in B/ ratio for cis-isomers of bromadiolone, in C/ ratio for trans-isomers of bromadiolone. Statistical analysis was done using a non-parametric Mann-Whitney test. $P < 0.005$ was the accepted level of significance. *, indicates a statistical difference between doses.

wildlife) by gavage to control the administered dose. These rats were exposed to two different doses, one lethal and one sublethal, to rule out any dose effect. The population of exposed rats consisted of an equal number of females and males to avoid the influence of the sex, as the metabolism of xenobiotics being largely influenced by the sex of the animal. Finally, to consider the different situations in the field, the period between exposure and sampling was different for each animal (from 1 to 30 days with 3 animals per period), in order to have recently exposed animals as well as animals that have been exposed for a long time, as animals can survive in the field after ingestion of ARs.

While detection of ARs in plasma has been suggested as a possible indicator of toxicity [38], the use of the faecal matrix seems more relevant for exposure assessment. Although bromadiolone residue concentrations are consistently lower in faeces and plasma than in liver (while extraction rates are comparable between matrices), the concentration ratios between liver and faeces are always much lower, by a factor of nearly 50, than the concentration ratios between liver and plasma. Residues in plasma are therefore minor compared to those

in liver and even those in faeces. Consequently, in our population, a diagnosis of exposure based on the plasma matrix generates 8% of false negatives, whereas based on the faeces matrix, no false negatives are detected. Only the detection and quantification of the parent compound was investigated in this study. However, second generation anticoagulants have been reported to be not or only slightly metabolized [39]. Our study was performed using LC-MS/MS. This technology allows reaching extremely low limits of quantification of the order of a few ng/g for all ARs [40–42]. Of course, the false negative percentages on our sample would be much higher if other detection methods had been used. Indeed, different technologies are used for the detection and quantification of ARs, by liquid chromatography coupled to mass spectrometry as performed in our study, but also by liquid chromatography with UV detection [43, 44] or fluorescence detection [45, 46], leading to much higher detection limits (of the order of a few 100 ng/g, with variations depending on the AR) [25, 42]. These latter methods of detection are not very suitable for ARs quantification on faecal or plasma matrices.

Let us now compare the results obtained on faeces and

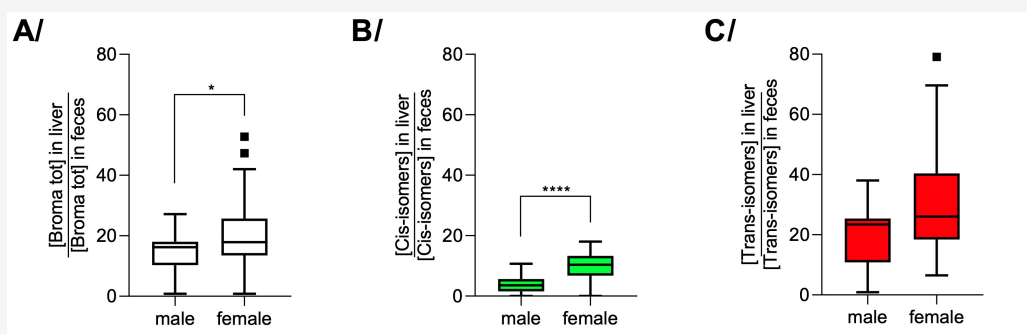


Figure 4. Ratio of AR concentrations between liver and corresponding faeces according to the gender of animals. In A/ ratio for total bromadiolone residues, in B/ ratio for cis-isomers of bromadiolone, in C/ ratio for trans-isomers of bromadiolone. Statistical analysis was done using a non-parametric Mann-Whitney test. $P < 0.005$ was the accepted level of significance. *, indicates a statistical difference between gender..

plasma at the finer scale of ARs stereoisomers. Indeed, the ARs are molecules containing one or two asymmetric carbons, at the origin of stereoisomers of configuration [47]. Thus, bromadiolone contains two asymmetric carbons and consists of a mixture of four stereoisomers, easily separable on achiral column by group of two isomers, called diastereomeric pair or cis and trans isomers [48]. Separation into four stereoisomers requires a chiral separation [49] that is more complex to perform routinely on field samples from various biological matrices. We will therefore focus on the diastereomeric scale considering that the administered bromadiolone contains 85% trans isomers and 15% cis isomers. For trans isomers, an exposure diagnosis using plasma generates, in our population, more than 23% of false negatives, while the use of the faeces matrix generates none. For cis-isomers, 11% of false negatives are observed among our samples using the plasma matrix versus 0 using faeces. Nevertheless, the use of faeces leads to the observation of 6% of false positives among our sampling, i.e. containing cis-isomers in faeces whereas they are undetectable in the liver. Here again, it is important to note that the use of liquid chromatography coupled to mass spectrometry leads to very few false positives because the specificity of the analyses is high due to the detection principle (based on the molar mass and fragmentation of the molecule) [50–52]. The use of liquid chromatography coupled with UV or fluorescence detection would certainly lead to observe many more false positives, especially with faeces generating a very high background noise level (mass effect) with these detection methods [41, 53, 54].

How to explain the high proportion of false negatives via the use of the plasma matrix? Bromadiolone is a second-generation AR molecule, like difenacoum, difethialone, brodifacoum or flocoumafen [1]. These molecules, unlike the first-generation molecules, are characterized by a very long persistence in the body, up to several hundred days according to some studies [3]. Their distribution is essentially intracellular [55] with a very strong hepatic extraction coefficient, the proportion of the molecule distributed in the plasma being infinitesimal, of the order of 0.4 of the total quantity for

bromadiolone [55]. This plasma circulation is low, but also very transient, whereas the presence of the molecule persists for several weeks or even months in the liver. Thus, previous studies show that after a single or repeated administration, bromadiolone becomes undetectable in plasma about 7 days after administration [41, 56]. In our study, it becomes undetectable in plasma between 14 and 30 days after initial administration. Because the distribution of bromadiolone is similar to other second-generation molecules [3, 57]), it is highly likely that using the plasma matrix to track exposure to other SGARs would lead to the same biases. As the pharmacokinetics of first generation ARs (chlorophacinone, coumatetralyl, warfarin) are different with a higher plasma distribution [55], the plasma matrix might be more relevant for monitoring exposure to these molecules. Nevertheless, since their use tends to decrease due to resistance in rodent populations [58, 59] and their tissue persistence is lower, monitoring the exposure of wildlife to these molecules is of less importance. The observation of false negatives in the field is problematic because it minimizes the prevalence of exposure. The purpose of field monitoring is to assess and prevent ecotoxicological risk to the non-target populations present. According to our results, the use of the faecal matrix avoids or, at least, minimizes the proportion of false negatives, making this matrix more relevant. Indeed, faecal excretion is the major route of elimination of most SGAR. Faecal residues consist of either direct eliminated non absorbed compound by the gastrointestinal tract or excreted unchanged unbound compounds. Biliary excretion and enterohepatic circulation also contribute to the continued elimination in faeces over a long period [39, 56, 60].

If the faecal matrix appears to be more relevant for monitoring the exposure of domestic and wild animals, is it possible to extrapolate the information from the analysis of this matrix to liver contamination? Quantitatively, faecal concentrations are much lower than liver concentrations. It is not feasible to correlate faecal concentrations with liver concentrations. This lack of correlation of these concentrations seems to be due mainly to three factors. Firstly, the post-exposure period, with a more rapid decreasing of the faecal concentrations of AR

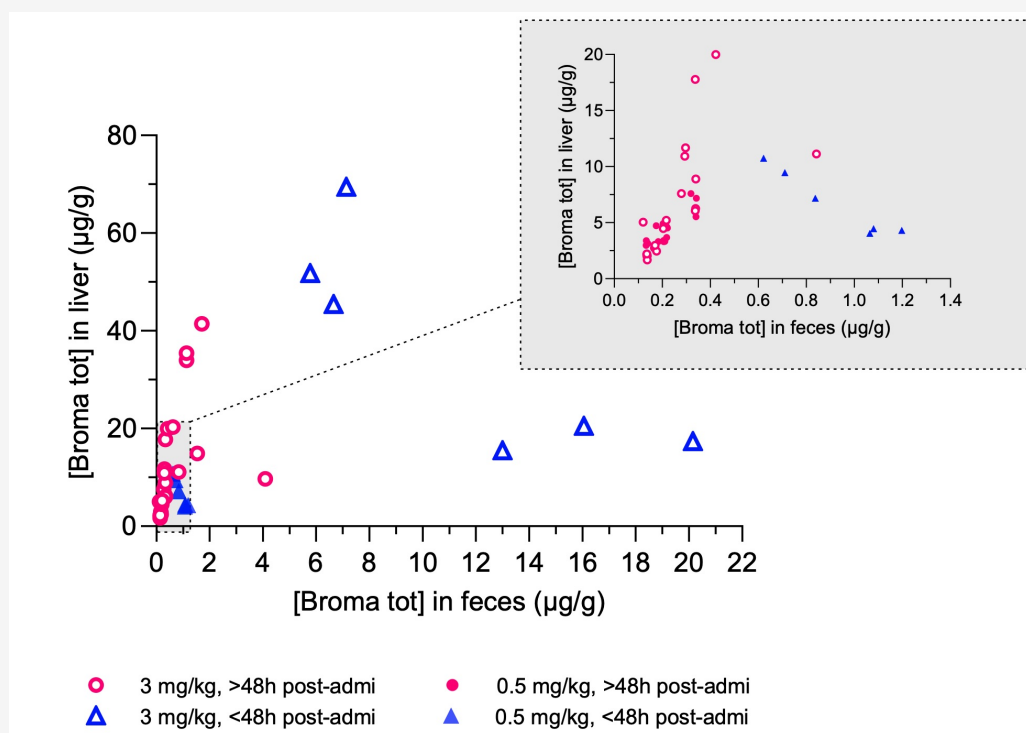


Figure 5. Hepatic bromadiolone concentration in function of its corresponding faecal concentration for each individual of the population (n=60), according to the dose and delay between bromadiolone administration and sampling. In Insert/ Focus on faecal concentrations between 0 µg/g and 1.5 µg/g.

than the hepatic ones. This is valid for total bromadiolone, for trans residues, but not for cis residues, whose hepatic persistence is lower [57]. The second factor contributing to this lack of correlation between faecal and hepatic concentrations relies on differences in metabolism depending on the sex of the animal. Indeed, it seems that bromadiolone, particularly the cis-isomers and eventually the trans-isomers, are more persistent in the liver of females than males. This finding is not surprising since the metabolism of xenobiotics is known to be largely sex-dependent [61–63]. This difference was recently illustrated for difethialone [64]. The third factor seems to be the ingested dose with possibly a rate of elimination depending on this concentration. However, there is not enough data to confirm this hypothesis.

From a qualitative standpoint, are the faecal bromadiolone residues similar to the hepatic residues? Cis- and trans-isomers were systematically detected in liver and faeces, regardless of sex and dose (except for four individuals without cis isomers in the liver, for whom samples were taken 30 days after exposure). For recent exposure (i.e. within the first 48h post-exposure), the qualitative composition of the faecal residues is completely similar to that of the liver. However, beyond 48h post-exposure, we observe an increase in the proportion of cis-isomers in the faecal matrix while at the same time the proportion of trans-isomers increases in the liver. This is related to the greater hepatic persistence of trans-isomers compared to cis-isomers. This evolution is responsible for the

occurrence of the 4 false positives for cis-isomers observed in our population. Despite this drawback, the qualitative analysis of isomers present in faeces and liver could have several major interests: dating of exposure and possible differentiation between primary and secondary exposure. Indeed, similar diastereoisomeric proportions between liver and faeces suggest a recent exposure and the more these proportions deviate, the older the exposure. Dating an exposure is important in the field to motivate the search for the source of contamination in the area where the dead, weakened or sentinel animal is found. Identifying whether the exposure is primary or secondary is also crucial to identifying the origins of an exposure. Finding only trans-isomers of bromadiolone in liver and faeces suggests secondary exposure via ingestion of an animal that has eliminated or nearly eliminated cis-isomers, which are less persistent than trans-isomers [57]. The presence of cis-isomers of bromadiolone in faeces and even in the liver would suggest primary exposure. Larger studies will be necessary to develop these hypotheses. In parallel, it will be necessary to verify the conservation of the diastereoisomer proportions in faeces and liver, depending on their state of conservation [65], as well as the homogeneity of concentrations and proportions of diastereomers in faeces. Indeed, previous studies suggest that ARs are not homogeneously distributed in the latter [32]. In our study, faeces samples were mixed and homogenized prior to bromadiolone extraction, which does not allow us to understand this methodological aspect.

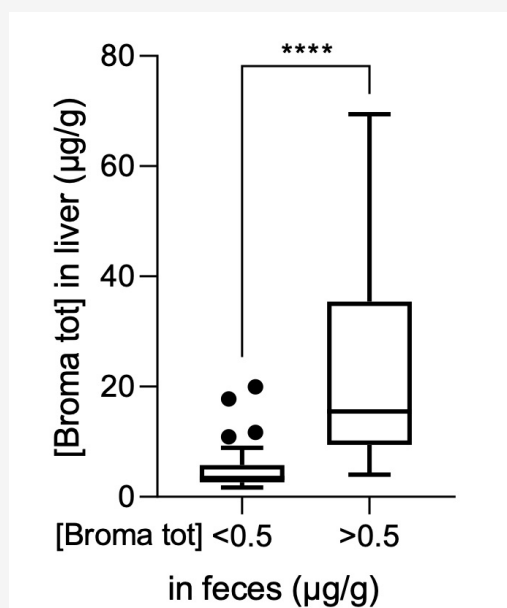


Figure 6. Hepatic concentration of bromadiolone based on a faecal threshold concentration of 0.5 µg/g. Statistical analysis was done using a non-parametric Mann-Whitney test. $P < 0.005$ was the accepted level of significance. *, indicates a statistical difference.

4. Conclusion

This study underlines the potential of faecal samples for meaningful monitoring of the exposure of non-target animal populations to ARs. Indeed, for the possible dating of the exposure and the epidemiology of this exposure (primary or secondary), our investigational approach should nevertheless be continued. In addition, our approach is valid for bromadiolone, although it will be necessary to repeat the study for the other ARs. It is likely that the results will be similar for all SGARs, considering the similarity of their pharmacokinetic properties. However, as mentioned, the relevance of this matrix for the detection of the FGARs may be limited due to their different pharmacokinetic properties. The results obtained will need to be confirmed in the different animal species given the differences in pharmacokinetics between species. In addition, the stability of AR residues in faeces will need to be further investigated in order to explore the influence of different parameters (light, temperature, humidity, etc).

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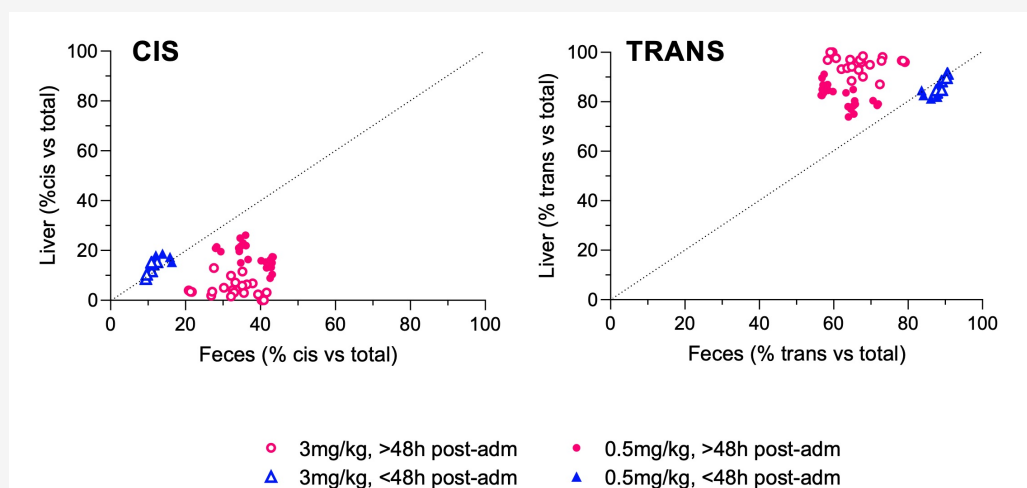


Figure 7. Percentage of cis- or trans-isomers in liver bromadiolone residues as a function of its corresponding percentage of faecal residues (n=60), according to dose and time between bromadiolone administration and sampling.

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5.2.3 Étude sur le risque reprotoxique

5.2.3.1 Article :

Differences in teratogenicity of some vitamin K antagonist substances used as human therapeutic or rodenticide are due to major differences in their fate after an oral administration

Differences in teratogenicity of some vitamin K antagonist substances used as human therapeutic or rodenticide are due to major differences in their fate after an oral administration

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Abstract

All vitamin K antagonist active substances used as rodenticides were reclassified in 2016 by the European authorities as active substances "toxic for reproduction", using a "read-across" alternative method based on warfarin, a human vitamin K antagonist drug. Recent study suggested that all vitamin K antagonist active substances are not all teratogenic. Using a neonatal exposure protocol, warfarin evokes skeletal deformities in rats, while bromadiolone, a widely used second-generation anticoagulant rodenticide, failed to cause such effects. Herein, using a rat model we investigated the mechanisms that may explain teratogenicity differences between warfarin and bromadiolone, despite their similar vitamin K antagonist mechanism of action. This study also included coumatetralyl, a first-generation active substance rodenticide. Pharmacokinetic studies were conducted in rats to evaluate a potential difference in the transfer of vitamin K antagonists from mother to fetus. The data clearly demonstrate that warfarin is highly transferred from the mother to the fetus during gestation or lactation. In contrast, bromadiolone transfer from dam to the fetus is modest (5% compared to warfarin). This difference appears to be associated to almost complete uptake of bromadiolone by mother's liver, resulting in very low exposure in plasma and eventually in other peripheric tissues. This study suggests that the pharmacokinetic properties of vitamin K antagonists are not identical and could challenge the classification of such active substances as "toxic for reproduction".

Keywords

Antivitamin K anticoagulants; warfarin; rodenticide; Bromadiolone; teratogenicity; Fetal warfarin syndrome; pharmacokinetics; risk assessment

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Introduction

In June 2016, the EU Member States, in line with the recommendation of the European Chemicals Agency (ECHA) reclassified all vitamin K antagonist active substances used as anticoagulant rodenticides (AR) as 'toxic to reproduction'. The active substances concerned by this classification are the first-generation anticoagulant rodenticides (FGAR) i.e., warfarin, chlorophacinone, coumatetralyl, and the second-generation anticoagulant rodenticides (SGAR) i.e., brodifacoum, bromadiolone, difenacoum, difethialone and flocoumafen. War-

farin and brodifacoum have been classified as reprotoxic with proven toxicity for embryo development (reprotoxic category 1A), and the other (chlorophacinone, coumatetralyl, difenacoum, flocoumafen, bromadiolone and difethialone) with supposed toxicity for embryo development (reprotoxic category 1B (UE) 2016/1179). All these active substances, except warfarin which is no longer used as rodenticide but used as human therapeutic, are extensively used worldwide by the oral route for rodent management and are almost the only active substances approved for this application [1].

Classification of these ARs as active substances “toxic to reproduction” has major consequences on the use of these active substances in Europe. Any bait with an AR concentration of 30 µg/g or more has been reclassified as ‘toxic to reproduction’. Since March 1, 2018, no product classed this way can be sold to amateurs and professional use products will have to carry the warning symbol and the ‘May harm the unborn child’ wording on their labels.

This classification of all ARs was decided according to a “read-across” approach based on observations obtained for warfarin. The “Read-across” approach is one of the most commonly used alternative approaches for data gap filling in registrations submitted under the REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) regulation in the European Union. It involves the use of relevant information from analogous substance(s) to predict certain physicochemical properties, mammalian toxicity, environmental fate and ecotoxicity for the ‘target’ substance(s) under consideration. This approach consisting to prevent as much as possible any animal experiment leads ineluctably to some misclassification. Warfarin was first marketed in 1948 as a rodenticide. In 1954, warfarin transitioned into clinical use under the trade name “Coumadin” [2–4] and has become the most commonly used human drug to prevent thromboembolic disorders. Since, warfarin has been the most commonly drug used in human medicine to prevent thromboembolic disorders and is now no longer used as a rodenticide. Unfortunately, the prescription of warfarin to pregnant women has been shown to be sometimes responsible for congenital abnormalities known as “fetal warfarin syndrome”, characterized by skeletal malformations, i.e. nasal hypoplasia (hypoplasia of the maxillary bones) and chondrodysplasia punctata [5, 6].

Warfarin is behind the discovery of all the ARs. Indeed, they all have a common structure, i.e. a 4-hydroxycoumarin ring, with warfarin and/or a mechanism of action similar to that of warfarin, i.e. inhibitor of the vitamin K epoxide reductase involved in the recycling of vitamin K by catalyzing the reduction of the vitamin K epoxide to vitamin K (Stafford, 2005). Vitamin K is essential for the carboxylation of many proteins known as vitamin K-dependent proteins - coagulation factors II, VII, IX and X, matrix Gla protein and osteocalcin. These characteristics justified the classification of all ARs as “toxic to reproduction” according to the read-across method.

The teratogenic effect of warfarin was experimentally re-

produced using a rat model, whereas no effect was observed when bromadiolone was used under the same experimental conditions. Indeed, warfarin administered orally daily at high doses [5, 7] or at therapeutic doses [7] to newborn pups leads to a shortening of the growth of facial bones and to a lesser extent long bones. Daily administration of the same dose of bromadiolone under the same conditions does not result in impaired bone growth [7]. These differences challenge the classification of all ARs as “toxic to reproduction” based on the read-across alternative method applied in the absence of data. The aim of this study was to decipher the origin of this observed difference between warfarin, a first-generation vitamin K antagonist, and bromadiolone, a second-generation vitamin K antagonist widely used as rodenticide. Since warfarin is no longer used as rodenticide, this study includes coumatetralyl, a first-generation vitamin K antagonist like warfarin, but still used as rodenticide. As the origin of the difference does not seem to be explained by the pharmacodynamic properties of the active substances, the potential role of pharmacokinetics in explaining the dichotomy was investigated in this study, the differences in pharmacokinetic properties being at the origin of the classification of ARs into two generations, the second generation including highly efficient in a single dose and highly tissue-persistent active substances [8–11]. Herein, we analyzed the differences in distribution among the three active substances during gestation and lactation period.

1. Material and methods

1.1 Chemicals

Warfarin and coumatetralyl were purchased from Sigma-Aldrich (1^{re} Isle d’Abeau, Chesnes, France). Bromadiolone (85% trans/15% cis) were supplied by Liphatech (Agen, France). Dimethylsulfoxide, acetonitrile, methanol, acetone, diethyl ether, and orthophosphoric acid were obtained from VWR International (Fontenay sous bois, France). Vitamin K1 was obtained from TVM (Lempdes, France), and oxytocin 10 UI/mL from Alcyon (Paris, France). High-performance liquid chromatography (HPLC) grade water was prepared using a milli-Q plus system (Millipore, Saint-Quentin en Yvelines, France) and used for the preparation of HPLC eluents. Warfarin, bromadiolone and coumatetralyl were dissolved in 5% of DMSO and 95% of corn oil (Sigma-Aldrich) for per os administration.

1.2 Ethics statement and animals

Animal experiment was reviewed and approved by France government under the European Communities Council Directive of 24 November 1986 (86/609/EEC) and experimental procedures involving animals were performed according to an experimental protocol approved (Authorization n°2017041812474440) from the ethics committee of the Veterinary School of Lyon. Environment, housing and management of rats were in compliance with rat animal welfare and ARRIVE guidelines. Adult female OFA Sprague-Dawley rats (8-weeks old, 175-200g) were obtained from a commercial

breeder (Charles Rivers, L'Arbresle, France) and were acclimated for a minimum of 10 days. They were housed four per cage under a constant photoperiod and ambient temperature. Animals were kept in standard cages, Eurostandard, Type IV (Tecniplast, Limonest, France) and received standard feed (Scientific Animal Food and Engineering, reference A04) and water ad libitum.

1.3 Fetal exposure to vitamin K antagonists

Female OFA Sprague Dawley rats (10 weeks old) were mated with male OFA Sprague Dawley rats. The date of fertilization was determined by eosine/thiazine stained cervical smears (Aerospray, EliTechGroup), with the presence of sperm corresponding to the first day of gestation. From the day D+17 of gestation and for three consecutive days, the rats received daily oral administration of 0.5 mg/kg body weight of bromadiolone, warfarin or coumatetralyl (4 rats per anticoagulant). The dose of 0.5 mg/kg corresponded to the estimated ED50 of bromadiolone (the effective dose capable of inducing a 5-fold increase in prothrombin time in 50% of the animals 24 hours after dosing), so the rats received a cumulative dose for all active substances of 1.5 mg/kg(vehicle volume (corn oil) did not exceed 1 mL/kg of rat body weight). Rats received a co-injection of vitamin K1 (10 mg/kg body weight) to protect them from the hemorrhagic effect of the anticoagulant. At day D+20 of gestation, the rats were euthanized. Fetuses were removed from the placenta. Livers from the dam and fetuses, and the remainder of the fetuses were collected and frozen to determine the concentrations of anticoagulant.

1.4 Exposure during the suckling period

Female OFA Sprague Dawley rats (10 weeks old) were mated with male OFA Sprague Dawley rats. After parturition, from day D+3 of the lactation period and for three consecutive days, suckling Sprague Dawley rats (10-weeks old) received daily oral administration of 0.5 mg/kg of bromadiolone, warfarin or coumatetralyl (7 rats per anticoagulant), so the rats received the same cumulative dose for all anticoagulants (1.5 mg/kg body weight - vehicle volume (corn oil) did not exceed 1 mL/kg of rat body weight.).

Twenty-four hours after administration of the initial AR dose, milk from lactating females was recovered. For this, lactating females were separated from their litters for up to 60 minutes and then received an intramuscular injection of oxytocin (2 IU/animal). Ten minutes after injection, 100 µl of milk was recovered by capillary aspiration under volatile anaesthesia (isoflu vet, Alcyon, France) and frozen for subsequent determination of anticoagulant concentration.

At day D+6 of the lactation period, female rats and their newborns were euthanized. Liver from the dam and the newborns, and the remaining body of the pups were then collected and frozen to determine anticoagulant concentration.

1.5 Pharmacokinetics study

Female OFA Sprague-Dawley rats (10 weeks-old) received under standard feed either a unique per os administration

of warfarin (2.8 mg/kg body weight; n=36 rats, 4 rats per timeframe) or bromadiolone (0.6 mg/kg body weight; n=40 rats, 4 rats per timeframe). Warfarin and bromadiolone were solubilized in corn oil with 5% of dimethyl sulfoxide (DMSO) (vehicle volume was 1 ml/kg body weight). Pharmacokinetics studies were carried out during 5 days for warfarin and 21 days for bromadiolone. Animals were given a daily subcutaneous injection of vitamin K1 (10 mg/kg) to prevent hemorrhaging. One, 2, 4, 6, 824, 48/72, 72/168, 96/336, and 504 hours after warfarin or bromadiolone administration, four rats were anesthetized with isoflurane and blood was taken by cardiac puncture into citrated tubes. Finally, rats were euthanized with CO2 and the liver of each rat was immediately collected and stored at -20°C until analysis.

1.6 Anticoagulant molecule quantification

Anticoagulant residues in plasma, milk, liver or body were quantified as previously described [12, 13]. The limit of quantification (LOQ) of warfarin and bromadiolone was 20 and 0.2 ng/mL for plasma, 1 and 2 ng/g for liver, fetus and newborn body, and 1 ng/mL for milk respectively.

1.7 Statistical analysis

A non-compartmental analysis was carried out using Phoenix®WinNonlin®8.0; Pharsight corporation St Louis MO USA. Linear trapezoidal rule was selected for calculations and the estimation of the terminal slope for extrapolation to infinity was done by regression with the best-fit option of Phoenix. A weighting factor of 1/Y was used to estimate the slopes. Area Under the Curve (AUC) and the Mean Residence Time (MRT) for plasma and liver with and without extrapolation to infinity were computed according to Gibaldi and Perrier [14]. The apparent plasma clearance (Cl/F(0-infinity)) was computed by dividing the administered dose by AUC(0-infinity). The apparent steady-state volume of distribution (Vss/F(0-infinity)) was estimated by the product of the mean Cl/F and the mean MRT(0-inf). Descriptive statistics (arithmetic mean, SD...) were obtained with the statistical tool of Phoenix.

Notwithstanding pharmacokinetic parameters, statistical analysis was done using GraphPad Prism 6 software (CA, USA). A Dunn's multiple comparison test was used with $\alpha < 0.05$ in order to compare statistically the results among groups.

2. Results

2.1 Assessment of the fetal exposure to vitamin K antagonists

After 3 successive oral administrations of warfarin, coumatetralyl or bromadiolone (0.5 mg/kg/day for 3 days) at gestation days D+17, D+18 and D+19, pregnant OFA-Sprague Dawley females showed no signs of hemorrhage until 24 hours after the last administration. Liver weights of pregnant females were between 10 and 15g; number of pups per litter between 11 and 13; average fetal weight between 2.1 and 5.2g depending on

the litter; average fetal liver weight between 0.15 and 0.33g depending on the litter. Anticoagulant concentrations were determined in liver of females and in liver and extrahepatic tissues of fetuses (Figure 1). Bromadiolone concentration was significantly lower in fetal liver or extrahepatic tissues and was significantly higher in female liver compared to warfarin and coumatetralyl. To assess transplacental passage, the total amounts of AR present in the mother's liver and in all fetuses of the litter were calculated by taking into account the number of pups in the litter, the weight of the livers and the bodies of the fetuses. A placental transfer index corresponding to the ratio of the quantities found in the mother to the quantities found in all of these fetuses was calculated for the three active substances. The results are presented in Figure 1D. This index varies from 1.1 to 2.9 for warfarin, from 0.6 to 1.7 for coumatetralyl, and from 0.05 to 0.16 for bromadiolone. At the same initial dose, this placental transfer index is thus twice as high for warfarin compared to coumatetralyl, and 20 times as high for warfarin compared to bromadiolone.

2.2 Assessment of the exposure of newborns to AR by the mother's milk

After three successive oral administrations of warfarin, coumatetralyl or bromadiolone (0.5 mg/kg body weight/day for three days) at days D+3, D+4 and D+5 post-partum, lactating OFA-Sprague Dawley females (7 females per AR) showed no signs of hemorrhage until 24 hours after the last administration. AR exposure of newborns via milk was determined and compared with the exposure of the lactating females. Liver weights of lactating females were between 10 and 15g; number of pups per litter between, 8 and 15; average newborn weight, 6.1g; average newborn liver weight, 0.39g depending on the litter. AR concentrations were determined in liver of females and in liver and extrahepatic tissues of newborns (Figure 2). Bromadiolone concentration was significantly lower in newborns liver or extrahepatic tissues and was significantly higher in female liver compared to warfarin and coumatetralyl. To assess the exposure of newborns through milk, the total amounts of AR present in the mother's liver and in all newborns of the litter were calculated by taking into account the number of newborns in the litter, the weight of the livers and the bodies of the newborns. A milk transfer index corresponding to the ratio of the quantities found in the mother to the quantities found in all of these newborns was calculated for the three active substances. The results are presented in Figure 2D. At the same initial dose, the warfarin milk transfer index is 2.7 times higher than that obtained for coumatetralyl and 31.6 times higher than that obtained for bromadiolone. AR concentrations in milk were also determined. At the same AR dose administered to lactating females (0.5 mg/kg body weight), concentrations of bromadiolone in milk were approximately 8 times lower than that of warfarin 24 hours after administration, with coumatetralyl being intermediate.

2.3 Comparative pharmacokinetics study

To compare the pharmacokinetics of warfarin and bromadiolone in female rats, these active substances were administered orally to female rats. The dose administered corresponded to the estimated ED50, i.e. the dose resulting in a 5-fold increase in prothrombin time in 50% of the animals 24 hours after dosing (estimated ED50 for warfarin and bromadiolone are, respectively, 2.8 mg/kg and 0.6 mg/kg body weight). The change in AR concentrations was then determined in plasma (in blue) and liver (in red) and are presented in Figure 3A for warfarin and Figure 3B and 3B1 for bromadiolone, figure 3B1 corresponding to an enlargement of figure 3B. The concentrations of warfarin and bromadiolone measured in liver and plasma were assessed using a non-compartmental analysis which enabled determination of pharmacokinetic parameters of warfarin and bromadiolone in liver and plasma without performing assumption on the pharmacokinetic model of these compounds (Table 1). The area under the curve for warfarin in liver was 6-times lower than that calculated for bromadiolone. Area under the curve for warfarin in plasma was 60-times higher than that calculated for bromadiolone while the plasmatic MRT of bromadiolone is significantly greater than that of warfarin.

3. Discussion

The fetal warfarin syndrome observed in humans can be reproduced in newborn rats [5] by daily administrations of warfarin for one month at the human therapeutic dose to lactating rats just after birth of their offspring. The same protocol performed with bromadiolone, a SGAR, does not lead to this fetal syndrome [7]. However, pharmacological properties (including pharmacodynamics and pharmacokinetics properties) of ARs are considered equivalent. According to these results, pharmacokinetics properties of ARs are clearly not equivalent. A pharmacodynamic origin leading to this difference in teratogenicity can be excluded, the mechanism of action i.e., the inhibition of VKORC1, being the same [15]. A pharmacokinetic origin leading to a difference in fetal/newborn exposure seems more likely.

In order to explore this hypothesis, we first evaluated the fetal exposure during gestation and the newborn exposure via its mother's milk. This evaluation concerned warfarin (drug at the origin of the reprotoxic classification of ARs), coumatetralyl (first generation AR, moderately toxic and not very persistent in the liver) and bromadiolone (second generation AR, highly toxic and highly persistent in the liver). Exposure of fetuses or newborns was assessed by measuring the amount of AR found in the liver of the exposed mother (in adults, ARs are preferentially located in the liver and are almost absent from other tissues) compared to the amount found in newborns/fetuses. The 3 ARs considered in this study have been detected in fetuses and newborns. These three active substances are thus able to cross the placental barrier and are excreted in milk, as suggested previously [16–18]. However, the transfer from dam to fetus varies dramatically among the

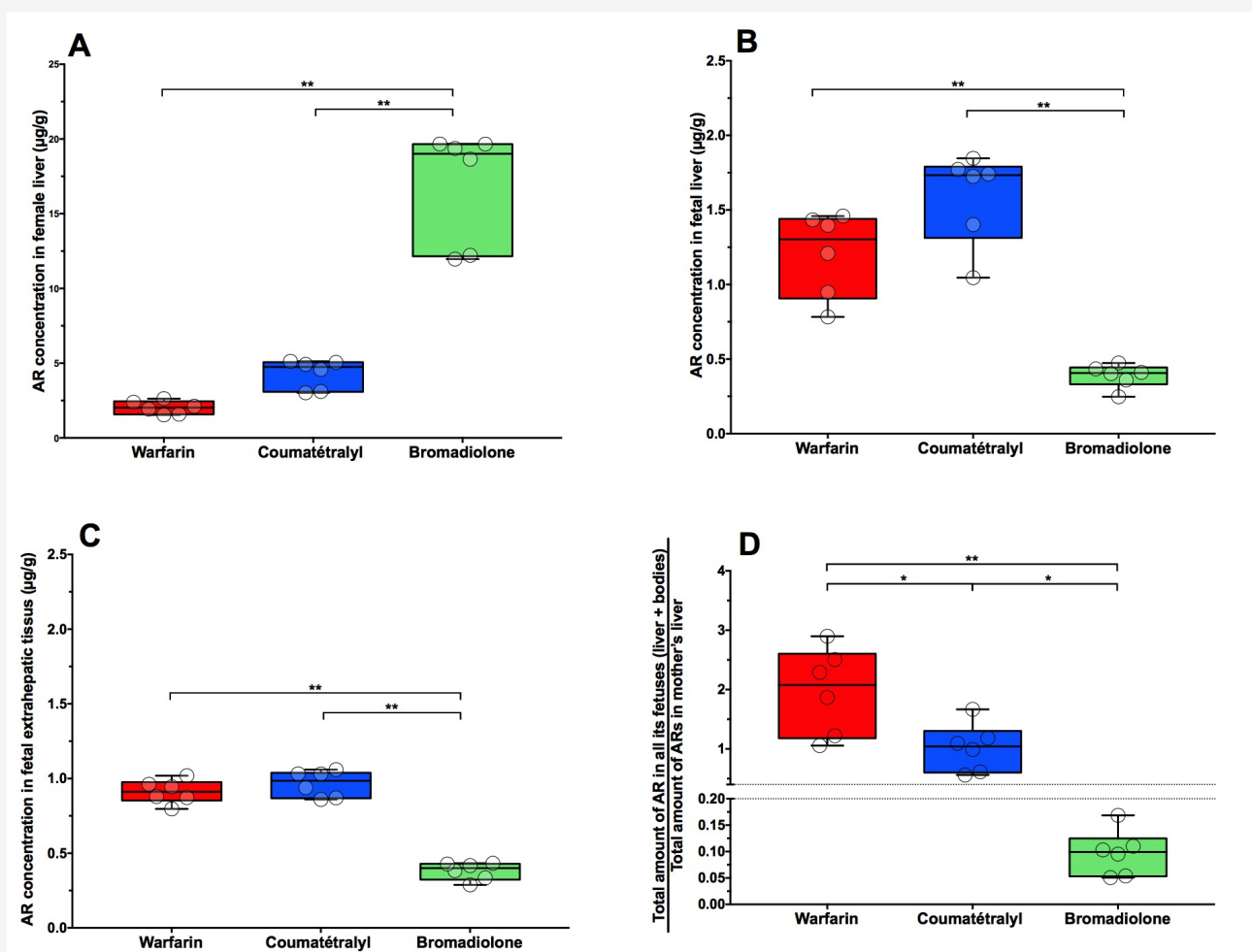


Figure 1. AR distribution between pregnant rats and their fetuses after three days of oral administration (0.5 mg/kg/day) of warfarin, coumatétralyl and bromadiolone to pregnant rat ($n = 4$) at gestation days D+17, D+18 and D+19. A/ AR concentration in mother's livers, B/ AR concentration in fetal livers, C/ AR concentration in fetal bodies, D/ Ratio between AR found in mother's liver and AR distributed in all its fetuses. Individual values and mean \pm standard deviation with 95% confidence interval are presented. * p-value < 0.05 and ** p-value < 0.01.

ARs evaluated in this study. In fact, warfarin and coumatétralyl are roughly half-distributed between the liver of the mother and the fetus while bromadiolone is found almost exclusively in the liver of the mother and in very small quantities in fetus.

Why such a difference among vitamin K antagonists? May liver uptake of bromadiolone prevent its distribution to peripheral tissues? Does the placental barrier prevent the passage of bromadiolone to the fetus? In order to explore these hypotheses, pharmacokinetic studies were performed for warfarin and bromadiolone in female rats, the active substances for which the greatest differences in distribution between mother and fetus are observed. These pharmacokinetic studies were conducted at the 50% effective dose (ED 50) for each compound. Indeed, this ED 50 is the dose that allows an increase by a factor of 5 in the prothrombin time in half of the animals 24 hours after administration. The warfarin and the bromadiolone doses used in this study both evoke the same magnitude

of biological effect.

Warfarin, after oral administration of the estimated ED50 dose, is widely found in the systemic circulation (Figure 4A). At the absorption peak, plasma warfarin represents $\approx 30\%$ of the warfarin administered, i.e., 160 μg out of the 560 μg administered (considering a plasma volume of 8.0 mL for a 200-g female [19–21] with a plasma concentration reaching $\approx 20 \mu\text{g/ml}$ (Figure 4B). Only 16% of the administered amount are found into the liver, the remainder quantities are either distributed to peripheral tissues or metabolized; the hydroxylated metabolites of warfarin [22] have not been quantified in this study. By contrast, bromadiolone, after oral administration of the ED50 dose, is almost exclusively found in the liver (Figure 4A). At the absorption peak, hepatic bromadiolone accounts for more than 80% of the administered bromadiolone. Only 0.65% is found in the plasma, i.e. only 0.8 μg out of the 120 μg administered with a maximum concentration lower than 0.1

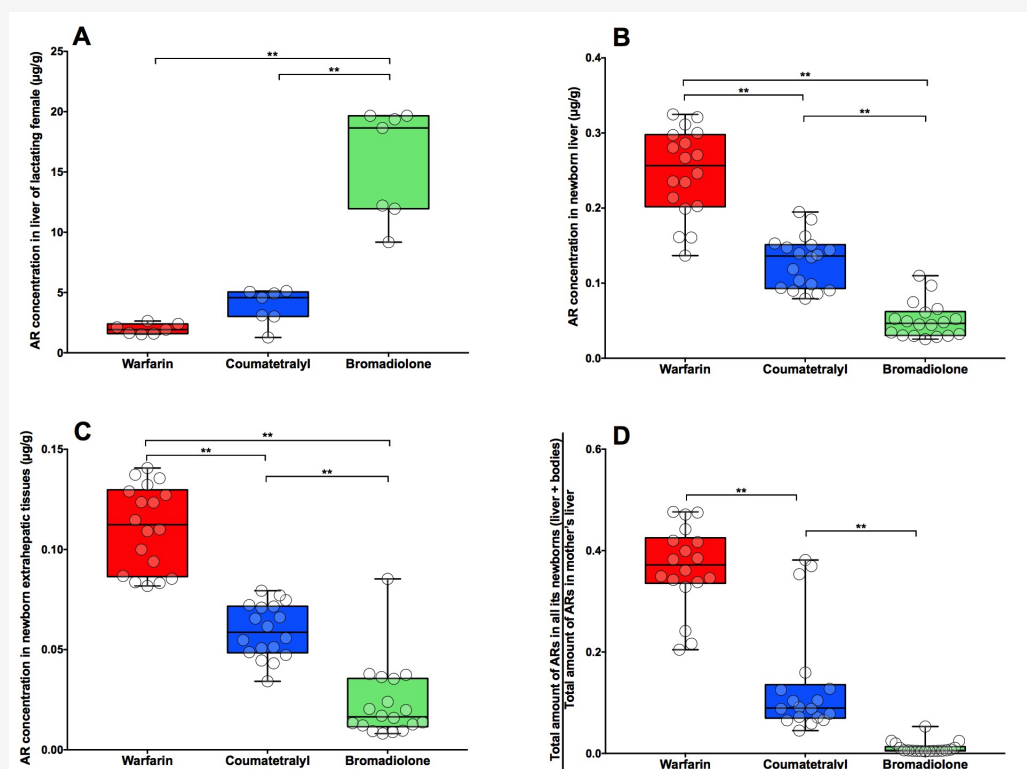


Figure 2. AR distribution between lactating rats and their newborns after three successive oral administration (0.5 mg/kg body weight/day for three days) of warfarin, coumatetralyl or bromadiolone to lactating rat at lactation days D+3, D+4 and D+5. A/ AR concentration in mother's livers, B/ AR concentration in newborn livers, C/ AR concentration in newborn bodies, D/ Ratio between AR found in mother's liver and AR distributed in all its newborns. Individual values and mean \pm standard deviation with 95% confidence interval are presented. * p-value < 0.05 and ** p-value < 0.01.

$\mu\text{g/ml}$ (Figure 4B). In addition, although the plasma MRT of bromadiolone is higher than that of warfarin, its plasma AUC is lower. These findings suggest that bromadiolone may have a greater affinity for hepatic tissue.

Considering the quantity of hepatic bromadiolone at the absorption peak and the available literature on the both ARs [9], it is reasonable to assume that the bioavailability of warfarin and bromadiolone are almost complete ($F \approx 1$). Thus, the apparent clearance (CL/F) and the apparent volume of distribution (V_{ss}/F) are good approximations of the clearance and of the volume of distribution respectively. Volume of distribution of warfarin in rats was found practically equal to one reported in man (140mL/kg) [23], and consistent with previous experiments [24].

A minimal model was used to interpret V_{ss}/F . Equation of the minimal model is: $V_{ss} = V_p + fu_P/fu_T \times V_t$ [25], where V_p is the plasma volume, V_t the extravascular space (tissular volume), fu_P the unbound fraction in plasma and fu_T the unbound fraction in tissue. fu_P and fu_T reflect the fraction of unbound compounds in plasma or tissue respectively. For warfarin, V_{ss} is on the same order of magnitude as the extracellular fluid of rat body, suggesting that warfarin is mainly located in the extracellular space due to its extensive plasma protein binding previously described [10, 26, 27]. The sit-

uation for bromadiolone is the opposite. Here V_{ss} is large and V_p can be ignored in minimal model equation. Thus, the ratio fu_P/fu_T is about 13, which indicate higher affinity of bromadiolone for tissue than for plasma. This difference of affinity does not appear to be due to a lower affinity of bromadiolone for plasma proteins compared to warfarin. Indeed, in vitro studies have highlighted that bromadiolone has a greater affinity for albumin than warfarin [28]. The table 2 present the theoretical distribution of both ARs calculated with their respective V_{ss} , in accordance with the model proposed by Øie and Tozer [29]. This model illustrates bromadiolone's greater affinity for tissue despite its plasma protein binding.

The fraction of AR circulating in the blood is the only fraction available to the fetus or newborn via the placental barrier or via excretion through milk. Because the blood fraction of bromadiolone is very low, fetuses or newborns are not or barely exposed when the dam is administered bromadiolone. Because the blood fraction of warfarin is high, the warfarin available to the fetus or newborn is high. These results are fully consistent with AR quantifications performed in fetal/newborn tissues and would largely explain the differences in teratogenicity observed in vivo [7]. Nevertheless, bromadiolone persists longer in the mother's liver and might cause chronic exposure of the fetus or newborn with regular

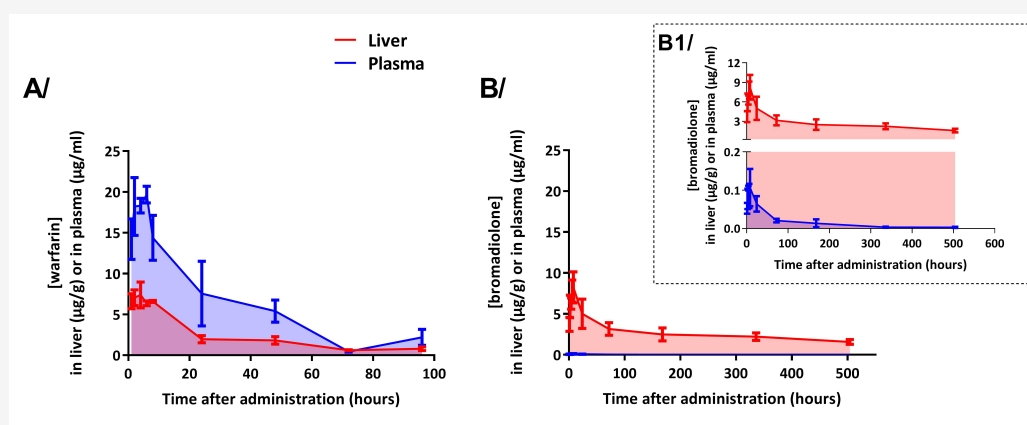


Figure 3. Pharmacokinetic profiles of A/ warfarin (2.8 mg/kg) and B/ bromadiolone (0.6 mg/kg) after single oral administration. Each point represents the mean \pm standard deviation of four individuals. Curves were obtained for plasma (blue line) and liver concentration (red line). B1 is an enlargement of B to observe plasma pharmacokinetic of bromadiolone.

	Warfarin		Bromadiolone	
	Liver	Plasma	Liver	Plasma
Cmax ($\mu\text{g/mL}$ or g)	8.1 ± 0.88	20.1 ± 2.3^b	8.49 ± 1.64	0.127 ± 0.027^b
λ_z (h_{-1})	0.0214 ± 0.0026^a	0.0313 ± 0.0084^b	0.0016 ± 0.0006^a	0.0041 ± 0.0017^b
Terminal half-life calculated with λ_z (h)	32.8 ± 4.1	23.2 ± 5.7	460 ± 198	205 ± 132
AUC 0-last time ($\text{h} \cdot \mu\text{g/mL}$ or g)	212 ± 15.2^a	557 ± 62.2^b	1337 ± 283^a	7.69 ± 2.63^b
AUC 0-infinity ($\text{h} \cdot \mu\text{g/mL}$ or g)	251 ± 22.82	618 ± 63.7	2410 ± 475	8.55 ± 2.90
CL/F (mL/Kg/h)		4.57 ± 0.47^b		77.0 ± 27.9^b
MRT 0-last time (h)	26.8 ± 1.73^a	25.5 ± 3.28^b	202 ± 10.98^a	99.6 ± 17.5
MRT 0-infinity (h)	44.9 ± 7.41	35.7 ± 10.7	648 ± 310	175 ± 52.36
Vss/F 0-last time (mL/kg)		116		845
Vss/F 0-infinity (mL/kg)		163		13502

Table 1. Pharmacokinetic parameters of warfarin and bromadiolone liver and plasma concentrations after a single per os administration of 2.8 mg/kg of warfarin or 0.6 mg/kg of bromadiolone. Values are presented with standard deviation. P-value < 0.05 , a, between warfarin and bromadiolone in liver; b, between warfarin and bromadiolone in plasma. AUC, area under the curve. Considering MRT, AUC and Vss/F, the last time is 96h or 504h for warfarin and bromadiolone, respectively.

	Plasma	Extracellular outside plasma	Intracellular
Warfarin	31%	46%	23%
Bromadiolone	0.4%	0.6%	99%

Table 2. Distribution of drug in the body at the steady state according to the model of Øie and Tozer [29] with a protein binding of 99% for both compounds.

release from the liver to the systemic circulation. The comparison of the plasma AUC of warfarin and bromadiolone demonstrates this phenomenon. Indeed, the AUC can be considered as a reliable index of the plasma fraction over time. The plasma AUC of warfarin is 70-fold higher than that of bromadiolone, with plasma concentrations above $1 \mu\text{g/ml}$ for at least 72 hours after administration. Plasma concentrations of bromadiolone never exceeded $0.1 \mu\text{g/ml}$ and 72 hours after initial administration, they dropped below $0.02 \mu\text{g/ml}$ with a plasma half-life of bromadiolone in the same order of magnitude as that of warfarin.

The high hepatic extraction of bromadiolone could be explained: 1/ by its high partition coefficient ($\log P = 3.8-4.1$, pH 6-7) (European Chem Agency, 2012), higher than that of warfarin ($\log P = 2.7$, pH unspecified) [30] or coumatetralyl ($\log P = 3.46$, pH unspecified), or 2/ by a different active transport among vitamin K antagonists, which has already been suggested by recent studies [31]. Considering the first hypothesis, the higher lipophilicity of bromadiolone could lead to greater association to lipoproteins in the enterocytes. The lipoprotein bound compounds have a greater plasmatic clearance that, in this case, is mainly performed by the liver [32]. Thus, the first pass effect of bromadiolone would be much more important than that of warfarin or coumatetralyl. Notwithstanding the first pass effect that reduce the quantity of bromadiolone reaching the placenta, it would be worthwhile to study the specific abilities and modalities of compounds to cross the placental barrier.

Nevertheless, regardless of the underlying mechanisms, this difference in pharmacokinetics appears to be responsible for the difference in the observed teratogenic effect. The risk

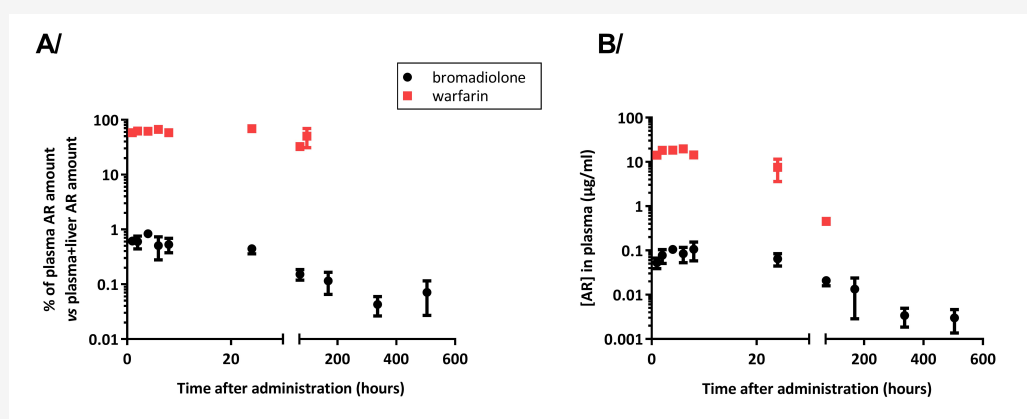


Figure 4. AR concentrations in plasma in female rats after single oral administration of warfarin (2.8 mg/kg) or bromadiolone (0.6 mg/kg). A/ shows the relative AR plasma fraction over time, B/ shows the plasma AR concentration over time.

to the fetus cannot, therefore, be considered identical among vitamin K antagonists. If the mechanism of action of these vitamin K antagonists is identical, the concentration on the site of action, i.e. the fetus, is absolutely not the same among active substances for a comparable exposure of the mother. This assessment should be carried out for each vitamin K antagonist.

The risk assessment in humans is of course based on the assessment of the hazard to the fetus, but also on the assessment of the likelihood of exposure of the mother to vitamin K antagonist. Bromadiolone is an active substance used exclusively in rodent management and formulated in baits (except during the manufacturing of these baits). Moreover, in Europe, these baits have to be used in bait boxes for biocidal use which prevents direct contact between people and baits. Considering warfarin, it is one of the most prescribed drugs in the world and people on such treatment receive it on a daily basis. Moreover, warfarin has been widely prescribed since the 1950s for pregnant women with thromboembolic problems due to its low-cost. The uses of these active substances are therefore different and certainly lead to a different frequency of exposure. In the USA, the incidence of human exposure to second-generation ARs has been reported to be 0.004% per year (315,951 cases of exposure over 25 years, with only 100,000 requiring treatment) according to the annual report of the American Association of Poison Control Centers' National Poison, most of these exposures are unintentional and 90% involve children [33, 34]. In China[35] and Europe, the incidence of this exposure appears to be lower, but still with a predominance of cases in children. In view of these data, the likelihood of a pregnant woman being exposed to ARs via baits appears to be very low. Exposure to warfarin which is used in human medicine seems much more likely.

All pharmacokinetics concerns presented in this work could challenge the read-across approach applied to classify all ARs as toxic to reproduction, which has major consequences on the use of these products in Europe. As no data available for each AR, further work is needed to evaluate

those active substances individually. Indeed, the 30 µg/g-concentration limit defined by the regulations beyond which baits are classified as toxic to reproduction is nowadays resulting in a reduction of the active substance concentration in bait. Could this reduction have consequences on the effectiveness of rodent management? A decrease in concentration at 30 µg/g of brodifacoum would have no consequence on the effectiveness of baits on non-resistant rodent populations [36]. What about the efficacy of low concentrated baits on resistant rodent populations? Moreover, the decrease in concentration of active substance could have serious consequences related to rodent resistance selection in Europe where numerous VKORC1 mutations have been described in rodents [37, 38].

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5.3 Vers de nouveaux outils de gestion des populations

5.3.1 Alimentation et exploitation du coût biologique

5.3.1.1 Article :

Establishment of the Variation of Vitamin K Status According to Vkorc1 Point Mutations Using Rat Models

Article

Establishment of the Variation of Vitamin K Status According to *Vkorc1* Point Mutations Using Rat Models

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Abstract: Vitamin K is crucial for many physiological processes such as coagulation, energy metabolism, and arterial calcification prevention due to its involvement in the activation of several vitamin K-dependent proteins. During this activation, vitamin K is converted into vitamin K epoxide, which must be re-reduced by the VKORC1 enzyme. Various *VKORC1* mutations have been described in humans. While these mutations have been widely associated with anticoagulant resistance, their association with a modification of vitamin K status due to a modification of the enzyme efficiency has never been considered. Using animal models with different *Vkorc1* mutations receiving a standard diet or a menadione-deficient diet, we investigated this association by measuring different markers of the vitamin K status. Each mutation dramatically affected vitamin K recycling efficiency. This decrease in recycling was associated with a significant alteration of the vitamin K status, even when animals were fed a menadione-enriched diet suggesting a loss of vitamin K from the cycle due to the presence of the *Vkorc1* mutation. This change in vitamin K status resulted in clinical modifications in mutated rats only when animals receive a limited vitamin K intake totally consistent with the capacity of each strain to recycle vitamin K.

Keywords: vitamin K epoxide reductase; vitamin K status; mutation; osteocalcin

1. Introduction

K vitamins are a complex family composed of several similarly fat-soluble molecules having a 2-methyl-1,4-naphthoquinone ring in common. The different forms of vitamin K are distinguished according to the length and nature of the carbon chain in position 3 of the naphthoquinone ring [1]. Vitamin K1 or phyloquinone contains a phytyl side chain, which comprises 4 prenyl units. Vitamin K1 is synthesized only by plants, green algae, and some cyanobacteria and is, thus, found mainly in green vegetables. Vitamins K2 or menaquinones (MK) contain an unsaturated aliphatic side chain with a variable number of prenyl units. Depending on the number of prenyl units, vitamins K2 can be divided into different short-chain MK (i.e., mainly menaquinone-4, MK-4) found mainly in meat and fermented foods and long chain MKs mainly of bacterial origin (i.e., MK-7, to MK-14).

Vitamin K is important for many physiological processes as the cofactor of the gamma-glutamyl carboxylase (GGCX) enzyme [2,3]. This enzyme is involved in the activation of many proteins, called vitamin K-dependent proteins (VKDPs), by catalyzing their γ -glutamyl carboxylation. Vitamin K is, thus, crucial for blood coagulation through the activation of hepatic blood clotting factors II, VII, IX, and X. Vitamin K is also essential for energy metabolism and arterial calcification prevention through the activation of osteocalcin [4] and Matrix Gla Protein [5], respectively, which are both

extrahepatic VKDPs. It was also described to be involved in bone metabolism [6,7], cancer progression, inflammatory response [8], oxidative stress [9], shingolipids synthesis [10], and pancreas exocrine activity [11].

Recommended vitamin K intake in a normal healthy adult varies from 50 to 600 µg/day for vitamin K1, and from 5 to 600 µg/day for vitamin K2 [12]. This recommended vitamin K intake is only based on blood coagulation and varies according to the region. Common polymorphisms or mutations in certain key genes involved in vitamin K metabolism might affect nutritional requirements. Nevertheless, this evidence is indirect via effects on warfarin dose requirements and direct demonstrations are missing.

During gamma-carboxylation of VKDP, vitamin K is converted into vitamin K epoxide by GGCX [2]. The latter is then re-reduced first into vitamin K quinone and then into vitamin K hydroquinone by the vitamin K epoxide reductase (VKORC1) enzyme to be reusable for a new activation of VKDP. It is estimated that one molecule of vitamin K can be used up to 500 times by GGCX [13]. Inhibition of the VKORC1 by vitamin K antagonists such as warfarin leads to inhibition of recycling, which results in a hemorrhage due to a deficiency of activated clotting factors II, VII, IX, and X. This is indicative of the central role played by VKORC1 in the low vitamin K requirement, and any change in this activity may lead to an increased or decreased vitamin K requirement [14].

Various polymorphisms and mutations of the *VKORC1* gene have been described in humans. Five common non-coding polymorphisms of the *VKORC1* gene located in the promoter region, in the first and second intron, and in the 3' downstream region described in patients requiring increased doses of vitamin K antagonists are associated with reduced expression of VKORC1 [15]. More than 30 mutations in the coding region of *VKORC1* have also been described in patients requiring increased doses of anticoagulants [16–20]. While these mutations have been widely suspected of causing anticoagulant resistance, their association with an increase in the vitamin K requirement due to a modification of the enzyme efficiency has rarely been considered [21].

Vkorc1 mutations are also widespread in other animal species, particularly rats and mice whose populations are managed with vitamin K antagonists [22]. Recently, characterization of an animal model of rats carrying a *Vkorc1* mutation in the coding region leading to the Y139C mutation has demonstrated that the latter fed with a vitamin K3 deficient diet, which is a synthetic vitamin K normally widely present in rodent diets, presented arterial media calcification [23]. These calcifications were shown to be associated with a gamma-carboxylation defect of Matrix Gla Protein (MGP) involved in the vascular calcification prevention. This gamma-carboxylation defect could be due to a decrease in the efficiency of VKORC1 activity. This work aims to compare the vitamin K status in different rats carrying different mutations of the *Vkorc1* gene in order to evaluate the impact of such mutations and the influence of the nature of the mutations on the vitamin K status and, therefore, on the vitamin K requirement.

2. Materials and Methods

2.1. Animals

Rats homozygous for Y139C-*Vkorc1*, rats homozygous for Y139F-*Vkorc1*, and rats homozygous for L120Q-*Vkorc1* were derived from back-crossings and inter-crossings between laboratory Sprague-Dawley rats (designed as recipient strain) (Charles River, St Germain sur l'Arbresles, France) and wild rats homozygous for Y139C-*Vkorc1*, Y139F-*Vkorc1*, or L120Q-*Vkorc1* (designed as a donor strain for the *Vkorc1* mutation). Wild rat strain, carrying the Y139C mutation in *Vkorc1* at the homozygous state, was a generous gift from Julius Kuhn Institute [23]. A wild rat strain carrying the Y139F mutation in *Vkorc1* at the homozygous state was initially trapped on French farms in the 1980s and have since been maintained at the Lyon College of Veterinary Medicine, France [24]. Berkshire rats homozygous for Q120 in the *Vkorc1* were a generous gift from U.K.-based Sorex Ltd., Widnes, Great Britain. The founder animals of this strain were initially trapped in an English farm in the Berkshire countryside [25,26].

To form congenic strains, male wild rats homozygous for Y139C-*Vkorc1*, Y139F-*Vkorc1*, or L120Q-*Vkorc1* were crossed with Sprague-Dawley females to create the F1 hybrid generation, respectively. The F1 heterozygous for the Y139C-*Vkorc1*, Y139F-*Vkorc1*, or L120Q-*Vkorc1* mutation males were backcrossed to Sprague-Dawley females to give the F2 generation. The genotype of F2 young rats was determined by the allele-specific PCR method, as described by References [23,24,26]. Heterozygous females were back-crossed to the recipient Sprague-Dawley strain for 18 additional generations yielding F20 generation. Lastly, an F20 intercross of males heterozygous for Y139C-*Vkorc1*, Y139F-*Vkorc1*, or L120Q-*Vkorc1* with females heterozygous for Y139C-*Vkorc1*, Y139F-*Vkorc1*, or L120Q-*Vkorc1*, respectively, was carried out to obtain F20-introgressed rats homozygous for Y139C-*Vkorc1*, Y139F-*Vkorc1*, or L120Q-*Vkorc1* [23,24,26].

Animals were kept in standard cages (Eurostandard, Type IV, Tecniplast, Limonest, France), and received standard feed (Scientific Animal Food and Engineering, reference A04) and water *ad libitum*.

2.2. Animal Treatment

Experimental research on the rats was performed according to an experimental protocol following international guidelines and with approval from the ethic committee of the Veterinary School of Lyon (authorization n°2017041910166230).

During vitamin K deficiency experiments, eight-week-old male rats received for a maximum of 12 days a diet deficient in vitamin K₃ (menadiolone) without barley (Scientific Animal Food and Engineering, SAFE A04 v231) (designed in this study as diet-K3) *ad libitum*. This diet was composed of corn starch 36.1% (instead of barley rich in vitamin K₁), wheat 25%, corn 15%, wheat bran 5%, peanut oil 3%, soybean meal 8%, fish hydrolyzate 4%, and minerals/vitamins mix 3.9% (without vitamin K₃). Concentrations of vitamin K₁ and K₃ were determined by the supplier and were under the limit of quantification.

Rats were killed 0, 4, 8, or 12 days after the beginning of the feeding period with the special diet -K3 with CO₂. The organs (liver, kidney, lung, heart, and testis) of each rat were immediately collected and stored at −20 °C.

2.3. Preparation of Liver Microsomes

Microsomes were prepared from fresh organs by differential centrifugation, as described by Reference [27]. Livers, lungs, kidneys, and testis of rats fed with a standard diet were resuspended in 50 mM phosphate buffer (pH 7.4) containing 1.15% (*w/v*) of KCl. Organ cells were broken and homogenized in buffer by using a motor driven Potter glass homogenizer and further submitted to differential centrifugation at 4 °C. The 100,000 g pellet corresponding to the membrane fraction was resuspended by Potter homogenization in HEPES glycerol buffer (50 mM HEPES, 20% glycerol, pH 7.4). Protein concentrations were evaluated by the method of Bradford [28] using bovine serum albumin as standard microsomes were frozen at −80 °C and used for kinetic analysis and ELISA kits.

2.4. Vitamin K Epoxide Reductase Activity (VKOR) Assays and Kinetics

Microsomal vitamin K epoxide reductase (VKOR) activity was assayed as described previously [29]. Standard reactions were performed in 200 mM Hepes buffer (pH 7.4) containing 150 mM KCl, 1 mM dithiothreitol, and 1 g.L⁻¹ of total proteins. The reaction was started by the addition of vit K1 > O solution in 1% Triton X-100 and incubated at 37 °C for 30 min. In these conditions, the reaction was linear, according to the time of incubation and the quantity of incubated proteins. After incubation at 37 °C for 30 min, the reaction was stopped by adding 2 mL of isopropanol. After centrifugation at 3000× g for 10 min in order to precipitate proteins, 2 mL of hexane was added. After centrifugation at 3000× g for 10 min, the hexane layer was removed and dried under nitrogen. The dry residue was immediately dissolved in 0.2 mL of methanol and the reaction product was analyzed by liquid chromatography-mass spectrometry [29].

2.5. VKORC1 and VKORC1L1 ELISA Quantitative Assays

The VKORC1 protein was quantified from liver, testis, kidney, and lung microsomes using the “Rat Vitamin K epoxide reductase complex subunit 1” ELISA kit (Cliniscience, Nanterre, France), according to the manufacturer’s recommendations. The VKORC1L1 protein was quantified from liver, testis, kidney, and lung microsomes using the “Rat Vitamin K epoxide reductase complex Subunit 1-Like Protein 1” ELISA kit (Cliniscience, Nanterre, France), according to the manufacturer’s recommendations.

2.6. Measurement of Vitamin K Concentrations

Furthermore, 0.5 g of tissue was homogenized with ethanol 33% using an Ultra Turrax tissue disperser from IKA Labortechnik® (VWR International, Strasbourg, France) and then extracted with 4 mL of hexane. After centrifugation at 3000 rpm for 5 min at 4 °C, the supernatant was collected and extracted again with 4 mL of hexane. After centrifugation at 3000 rpm for 5 min at 4 °C, the supernatant was collected and evaporated at 37 °C to dryness under a gentle stream of nitrogen. The final dry extract was dissolved in 500 µL of methanol and vitamins K (i.e., Menaquinone 4-MK4-, Menaquinone 4 epoxide-epoxide -K1OX-) were analyzed by HPLC on reverse phase C18 column (4.6 × 100 MK4OX-, phylloquinone -K1-, phylloquinone mm, 2.7 µm, VWR, Fontenay-sous-bois, France). A post-column reactor (2.1 × 50 mm steel column packed with zinc powder) was connected in series between the analytical column and detector to ensure the reduction of K vitamins and allow the detection of reduced K vitamins by fluorescence with excitation and emission wavelengths of 246 and 430 nm, respectively. A gradient elution system was used with a flow rate of 1 mL/min as follows. After 24 min of elution with 99% methanol/1% water (acidified with 1% acetic acid containing 1.1 g.L⁻¹ of zinc acetate), the mobile phase was quickly changed from 99% methanol/1% water (acidified with 1% acetic acid containing 1.1 g.L⁻¹ of zinc acetate) to 50% methanol/49% dichloromethane with 1% acetic acid and 1.1 g.L⁻¹ of zinc acetate in 0.1 min. Under these HPLC conditions, MK4OX, MK4, K1OX, and K1 were eluted at 7.5 min, 10.3 min, 12.5 min, and 18.8 min, respectively.

2.7. Undercarboxylated Osteocalcin (ucOC) and Undercarboxylated Matrix Gla Protein (ucMGP) ELISA Quantitative Assays

The ucOC concentration was quantified in plasma by ELISA using the “Rat Glu-osteocalcin High Sensitive EIA” kit (Ozyme, Saint Cyr l’école, France), according to the manufacturer’s recommendations.

2.8. Tissue Calcium Measurement

Tissues were placed in an oven at 50 °C for 72 h and then reduced to a powder using a glass mortar. Calcium was extracted from 10 to 50 mg of tissue powder (depending on the organs) in 200 µL to 1 mL of a 10% formic acid solution for at least 2 h. The supernatant was recovered after centrifugation at 3000× g for 10 min and then evaporated at 110 °C. Residues were resuspended with mili-Q water corresponding to half of the volume of the formic acid solution. Calcium was assayed using the calcium colorimetric assay kit (Sigma-Aldrich, l’Isle d’Abeau, Chesnes, France), according to the manufacturer’s recommendations.

2.9. Data Analysis

K_m and V_{max} values were obtained from at least four separate experiments performed on four different batches of protein. The estimation of K_m and V_{max} values was achieved by the incubation of at least nine different concentrations of vit K > O (from 0.003 to 0.2 mM) to the standard reaction. Incubations were performed in duplicate. Data were fitted by nonlinear regression to the Michaelis-Menten model using the R-fit program.

Data are presented as the mean ± SD. Statistical analysis was done by using the Tukey’s multiple comparisons test or the Dunn’s multiple comparisons tests, and using GraphPad Prism 6 software (GraphPad, San Diego, CA, USA). $p < 0.05$ was the accepted level of significance.

3. Results

3.1. Tissue VKOR Activity in Rats According to *Vkorc1* Genotype

3.1.1. Kinetics Parameters of VKOR Activity

To evaluate the influence of the *Vkorc1* genotype on the ability to recycle vitamin K epoxide in vitamin K quinone between rat tissues, liver, kidney, testis, and lung microsomes from male rats with a different *Vkorc1* genotype (i.e., homozygous for wild type, Y139C, Y139F, or L120Q) were prepared. Catalytic properties of the VKOR activity measured in these microsomes were determined. *Km* and *Vmax* results are presented in Table 1. For all strains, VKOR activity was higher in liver, then in testis, then in kidneys, and then in lungs. Statistical differences were found between strains only concerning the *Vmax* value. The *Vmax* value of VKOR activity in liver of rats belonging to the wild type (WT) strain was, respectively, 4.1, 2.8, and 2.8-fold higher than that of rats belonging to L120Q, Y139F, and Y139C strains. The *Vmax* value of VKOR activity in the testis of rats belonging to the WT strain was, respectively, 6.0, 6.0, and 3.1-fold higher than that of rats belonging to L120Q, Y139F, and Y139C strains. The *Vmax* value of VKOR activity in the kidney of rats belonging to the WT strain was, respectively, 4.2, 4.0, and 2.3-fold higher than that of rats belonging to L120Q, Y139F, and Y139C strains. In lungs, VKOR activity was similar between strains.

Table 1. Kinetics parameters of VKOR (Vitamin K epoxide reductase) activity and ratio between VKORC1 and VKORC1L1 proteins in different rat tissues according to the *Vkorc1* genotype. Results are the mean of four independent determinations with its 95% confidence interval.

Tissue	Strain	<i>Km</i> (μM)	<i>Vmax</i> ($\text{pmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$)	<i>Vmax/Km</i> ($\mu\text{L}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$)
Liver	WT	29.58 [18.96–40.21]	95.62 ^{a,b,c} [84.13–107.1]	3.2 ^{a,b,c} [2.2–4.2]
	L120Q	37.98 [20.45–55.52]	22.18 ^{a,d,e} [18.45–25.91]	0.6 ^a [0.4–0.8]
	Y139F	22.52 ^a [14.49–30.56]	34.50 ^{b,e} [30.62–38.39]	1.6 ^b [1.3–1.9]
Testis	Y139C	46.89 ^a [30.64–53.13]	34.52 ^{c,d} [29.97–39.07]	0.8 ^c [0.5–1.1]
	WT	9.92 [4.00–15.84]	53.26 ^{a,b,c} [45.03–61.49]	5.5 ^{a,b,c} [5.3–5.7]
	L120Q	16.70 [11.18–22.22]	8.94 ^{a,d} [8.08–9.79]	0.5 ^a [0.4–0.6]
	Y139F	5.43 [2.31–8.55]	8.86 ^{b,e} [6.53–11.19]	1.2 ^b [0.6–1.8]
	Y139C	12.35 [3.55–21.15]	17.05 ^{c,d,e} [12.11–21.99]	1.4 ^c [1.0–1.8]
Kidney	WT	12.12 [3.79–20.45]	20.39 ^{a,b,c} [15.42–25.36]	1.7 ^{a,b} [1.0–2.4]
	L120Q	23.32 [19.80–26.84]	4.82 ^{a,d} [4.59–5.05]	0.2 ^a [0.1–0.3]
	Y139F	12.25 [1.48–23.03]	5.11 ^{b,e} [3.89–6.32]	0.5 [0.1–0.9]
	Y139C	23.24 [4.98–41.5]	8.67 ^{c,d,e} [6.06–11.28]	0.4 ^b [0.2–0.6]
Lung	WT	13.16 [6.017–20.29]	6.53 ^{a,b} [5.56–7.51]	0.5 [0.2–0.8]
	L120Q	19.24 [14.84–23.63]	3.94 ^{a,c} [3.67–4.21]	0.20 [0.1–0.3]
	Y139F	10.03 [3.4–16.66]	4.11 ^b [2.87–5.35]	0.4 [0.2–0.6]
	Y139C	15.78 [3.06–28.50]	6.43 ^c [4.59–8.26]	0.4 [0.2–0.6]

$p < 0.05$ was the accepted level of significance. a, b, c, d, e statistical difference between the two groups. WT: wild type.

3.1.2. Ratio Between VKORC1 and VKORC1L1 Enzymes

Because two different proteins, VKORC1 and VKORC1L1, may be involved in the reduction of vitamin K epoxide in vitamin K quinone, concentrations of both enzymes in liver, testis, kidneys, and lungs were determined by ELISA (Enzyme-linked immunoabsorbent assay) quantitative assays. The results are presented in Table 2. No statistical difference for the ration between VKORC1 and VKORC1L1 was observed between strains. In liver, whatever the strain considered, the concentration of the VKORC1 protein was always much higher than that of the VKORC1L1 protein with no statistical difference between strains except for the Y139F strain for which VKORC1 was 1.5-fold higher than that of the WT strain. In other tissues, concentrations of VKORC1 and VKORC1L1 proteins were comparable with the VKORC1 protein, which is slightly more present than the VKORC1L1 protein

in testis and lung. This is the opposite in the kidney. In the lung, VKORC1L1 expression was 1.5 to 1.9-fold greater in mutant strains when compared to the WT strain.

Table 2. VKORC1 and VKORC1L1 concentrations and ratio between VKORC1 and VKORC1L1 proteins in different rat tissues according to the *Vkorc1* genotype. Results are the mean of 4 independent determinations with its standard deviation.

Tissue	Strain	VKORC1 ($\mu\text{g/g}$ of Tissue)	VKORC1L1 ($\mu\text{g/g}$ of Tissue)	[VKORC1 Protein]
				[VKORC1L1 Protein]
Liver	WT	4.44 \pm 0.97	0.27 \pm 0.04	16.2 \pm 1.7
	L120Q	2.66 \pm 0.38	0.19 \pm 0.03	14.0 \pm 1.4
	Y139F	7.13 \pm 2.30 *	0.43 \pm 0.13	17.6 \pm 5.7
	Y139C	4.31 \pm 0.80	0.30 \pm 0.05	14.4 \pm 2.2
Testis	WT	0.25 \pm 0.07	0.19 \pm 0.04	1.4 \pm 0.5
	L120Q	0.35 \pm 0.08	0.21 \pm 0.09	1.6 \pm 0.5
	Y139F	0.24 \pm 0.10	0.22 \pm 0.02	1.1 \pm 0.5
	Y139C	0.27 \pm 0.06	0.21 \pm 0.04	1.3 \pm 0.3
Kidney	WT	0.10 \pm 0.03	0.18 \pm 0.02	0.54 \pm 0.14
	L120Q	0.14 \pm 0.02	0.17 \pm 0.01	0.82 \pm 0.13
	Y139F	0.08 \pm 0.04	0.23 \pm 0.02	0.33 \pm 0.19
	Y139C	0.12 \pm 0.01	0.18 \pm 0.01	0.67 \pm 0.06
Lung	WT	1.25 \pm 0.12	0.70 \pm 0.06	1.8 \pm 0.2
	L120Q	1.25 \pm 0.22	1.35 \pm 0.22 *	0.9 \pm 0.2
	Y139F	1.83 \pm 0.54	1.22 \pm 0.11 *	1.5 \pm 0.2
	Y139C	1.23 \pm 0.19	1.10 \pm 0.04 *	1.1 \pm 0.2

* $p < 0.05$ was the accepted level of significance compared to the WT strain.

3.2. Vitamin K Status According to the *Vkorc1* Genotype in Rats fed with a Standard Rodent Diet

3.2.1. Vitamin K Concentrations

To evaluate the influence of the *Vkorc1* genotype on the vitamin K status, concentrations of vitamin K1, K1OX, MK4, and MK4OX were determined in liver, testis, kidneys, and lungs of rats homozygous for wild type, Y139C, Y139F, or L120Q fed with a standard rodent diet. Vitamin K1 and K1OX were almost undetectable in all the considered tissues, whatever the genotype of rats. Only MK4 and MK4OX were, therefore, quantified. Results are presented in Figure 1. Statistic differences were found between strains only concerning the concentration of MK4.

Whatever the tissues considered, MK4 concentration was systematically higher in rats homozygous for wild type *Vkorc1* compared with rats homozygous for Y139F-*KORC1* or Y139C-*KORC1*. In liver, it was 1.5-fold and 2.4-fold higher. In testis, it was 1.7-fold and 2.1-fold higher and, in kidneys, it was 1.9-fold and 2.6-fold higher. In the lung, it was 2.7-fold and 2.8-fold higher when compared with rats homozygous for Y139F-*Vkorc1* or Y139C-*Vkorc1*, respectively.

In liver and testis, MK4 concentration was similar between wild type rats and rats homozygous for L120Q, while it was 1.9-fold higher in lungs and kidneys of wild type rats compared with rats homozygous for L120Q.

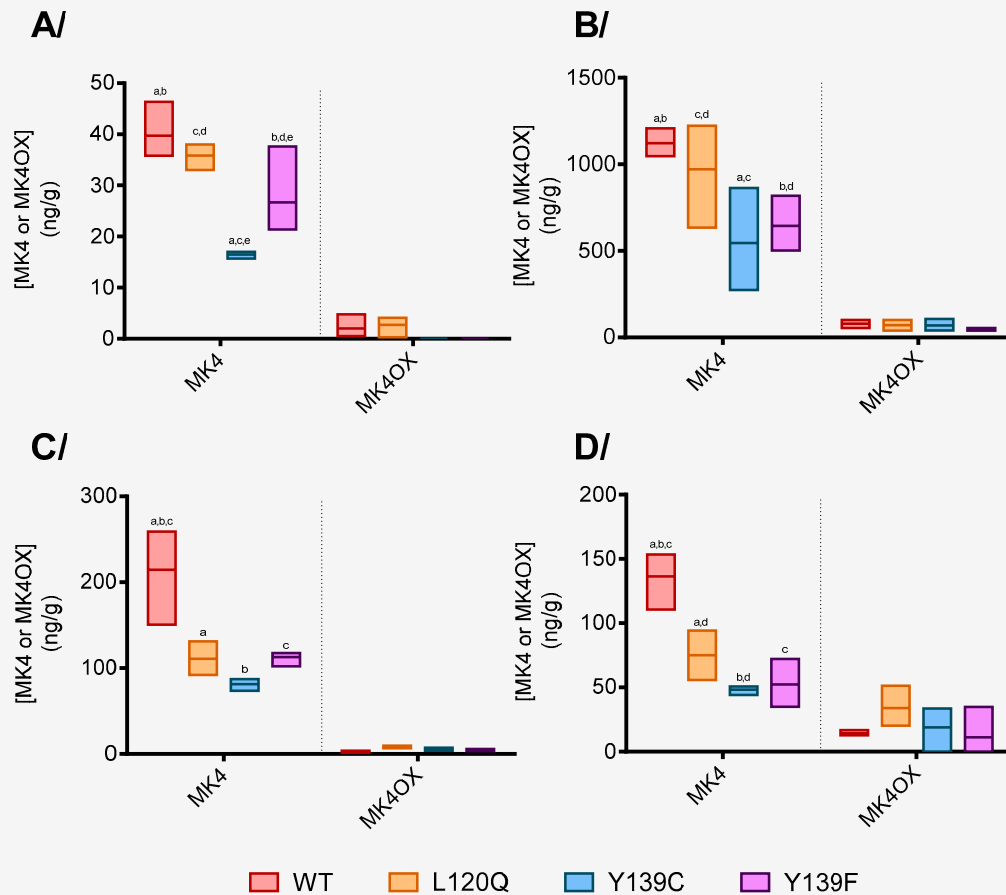


Figure 1. Vitamins K concentrations in (A)/liver, (B)/testis, (C)/kidney, and (D)/lung of rats homozygous for WT-, L120Q-, Y139C-, or Y139F-*Vkorc1* fed with a standard rodent diet. The box extends from the 25th to 75th percentiles and the line corresponds to the mean. Statistical analyses were performed using a Tukey's multiple comparisons test. $p < 0.05$ was the accepted level of significance. a,b,c,d,e, statistical difference between 2 groups.

3.2.2. Plasma ucOC Concentration

Under-gamma carboxylated osteocalcin (ucOC) and matrix Gla protein (ucMGP) concentration was measured in plasma of rats homozygous for wild type, Y139C, Y139F, or L120Q fed with a standard rodent diet. Results for ucOC are presented in Figure 2. Statistical differences were found only for ucOC concentration between wild type rats and rats homozygous for Y139C and Y139F. ucOC concentration was 6.1-fold and 7.0-fold higher in the plasma of rats homozygous for Y139F- and Y139C-*Vkorc1*, respectively, compared with wild type rats. A difference between wild type rats and rats homozygous for L120Q was not statistically significant.

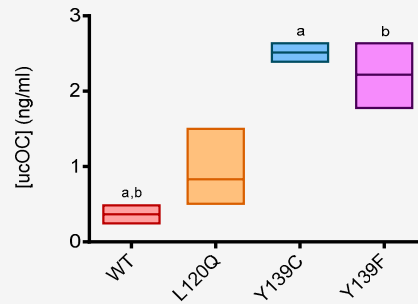


Figure 2. ucOC concentrations in the plasma of rats homozygous for WT-, L120Q-, Y139C-, or Y139F-*Vkorc1* fed with a standard rodent diet. The box extends from the 25th to 75th percentiles and the line corresponds to the mean. Statistical analyses were performed using a Dunn's multiple comparisons test. $p < 0.05$ was the accepted level of significance. a,b, statistical difference between two groups.

3.2.3. Tissue Calcium Concentration

Calcium concentration was measured in testis, kidneys, lungs, and hearts of rats homozygous for wild type, Y139C, Y139F, or L120Q fed with a standard rodent diet. Results are presented in Figure 3. No difference was observed between strains.

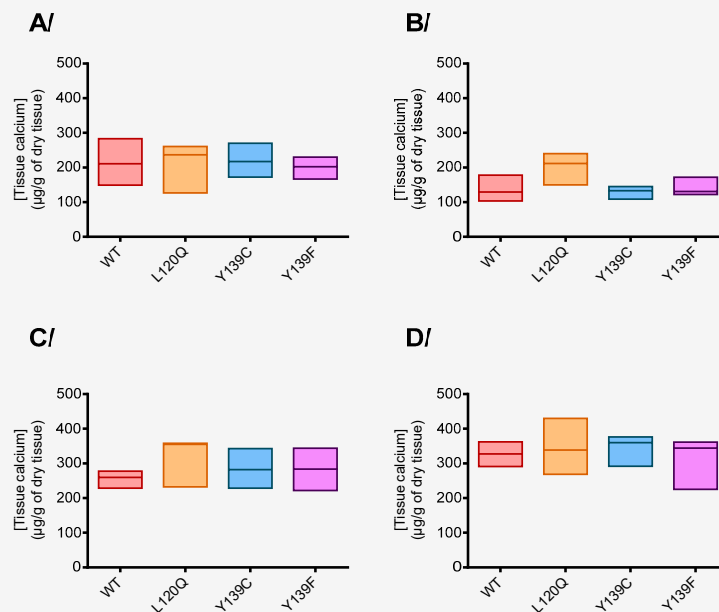


Figure 3. Tissue calcium content in (A) testis, (B) kidney, (C) lung, and (D) heart of rats homozygous for WT-, L120Q, Y139C or Y139F-*VKORC1* fed with a standard rodent diet. The box extends from the 25th to 75th percentiles and the line corresponds to the mean. Statistical analyses were performed using a Dunn's multiple comparisons test.

3.3. Evolution of the Vitamin K Status According to the *Vkorc1* Genotype in Rats Receiving a Specific Diet-K3 for 12 Days

3.3.1. Vitamin K Concentrations

The evolution of MK4 concentrations in different tissues (i.e., testis, kidneys, and lungs) was determined in rats receiving a vitamin K3 deficient diet for a short period of time from 0 to 12 days. The results are presented in Figure 4. This evolution could not be determined in the liver since this concentration was too low even before the beginning of the deficiency.

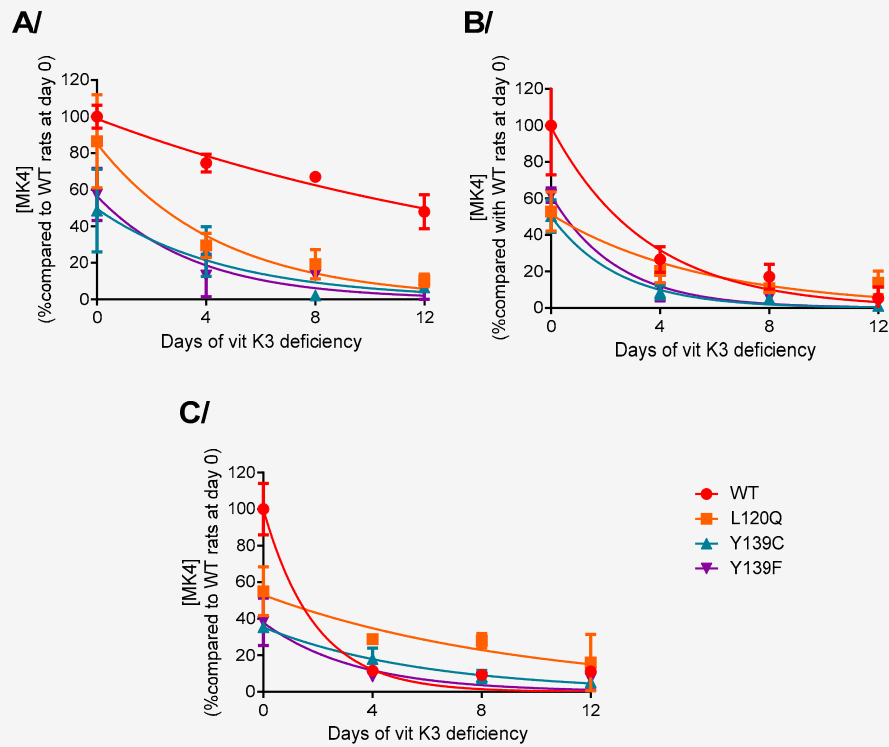


Figure 4. Vitamins K concentrations evolution in (A) testis, (B) kidney, and (C) lung of rats homozygous for WT-, L120Q-, Y139C-, or Y139F-*Vkorc1* receiving a specific diet -K3 for 12 days. Results are expressed as the mean of the percentage of four animals compared to wild type rats at day 0 ± standard deviation.

Half-lives of MK4 in the different tissues of the different rat strains were calculated using a non-linear regression model using equation $C = (C_0)^{-kel \cdot t}$. Results are presented in Table 3. Half-life of MK4 was four-fold longer in testis of the wild type when compared to mutated rats. In kidney, half-lives were similar for all strains. In lung, half-life of MK4 was lower in wild type rats when compared to rats homozygous for L120Q or Y139F.

Table 3. Kinetic parameters of VKOR activity and the ratio between VKORC1 and VKORC1L1 proteins in different rat tissues according to the *Vkorc1* genotype. Results are the mean of four independent determinations with its 95% confidence interval.

Half-Life of MK4	WT (in Days)	L120Q (in Days)	Y139F (in Days)	Y139C (in Days)
In testis	12.09 ^{a,b,c} [10.03–13.82]	3.10 ^a [2.22–5.11]	3.31 ^b [2.07–8.21]	2.50 ^c [1.69–4.74]
In kidney	2.43 [1.78–3.84]	3.85 [2.79–6.27]	1.71 [1.31–2.47]	1.68 [1.34–2.28]
In lung	1.31 ^{a,b} [0.91–2.39]	6.60 ^a [4.41–13.20]	4.08 ^b [3.37–5.18]	2.37 [1.37–8.93]

p < 0.05 was the accepted level of significance. a, b, and c: statistical difference between two groups.

3.3.2. Prothrombin Time

Consequences of the vitamin K3 deficiency were assessed by the determination of the prothrombin time at day 0, 4, 8, and 12 after the beginning of the vitamin K3 deficiency. Results are presented in Figure 5. Prothrombin time was not modified in rats homozygous for the wild type, Y139C, or Y139F fed with a standard rodent diet. Prothrombin time increased in rats homozygous for L120Q from the eighth day after the beginning of the vitamin K3 deficiency.

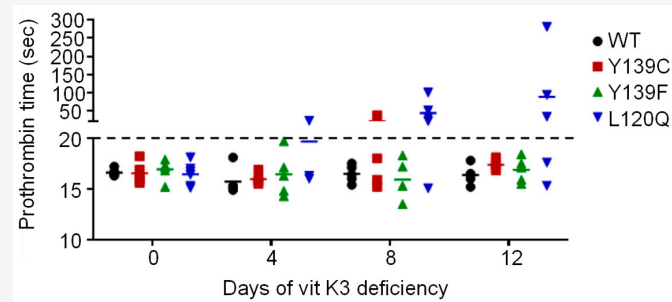


Figure 5. Prothrombin time evolution of rats homozygous for WT-, L120Q-, Y139C-, or Y139F-*Vkor1* receiving a specific diet -K3 for 12 days. Results are the mean of 3 or 5 animals, with each point representing each individual.

3.3.3. Plasma ucOC Concentration

Consequences of the vitamin K3 deficiency were also assessed by determining the plasma ucOC concentration at day 0, 4, 8, and 12 after the beginning of the vitamin K3 deficiency. Results are presented in Figure 6. The ucOC concentration remained unchanged during the 12 days of vitamin K3 deficiency in wild type rats, while, in the other strains, ucOC concentration increased rapidly. ucOC concentration reached its maximum 4 days after the beginning of the vitamin K3 deficiency and increased two-fold in rats homozygous for Y139F and Y139C and four-fold in rats homozygous for L120Q.

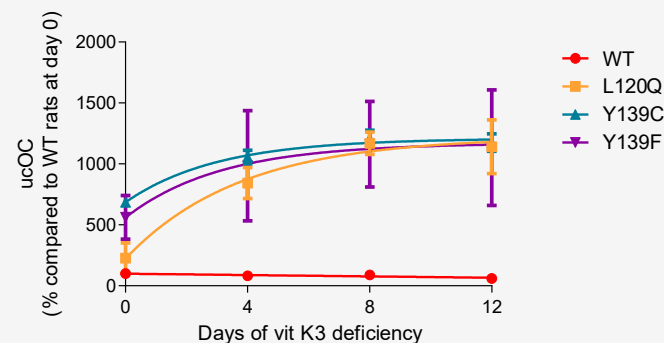


Figure 6. ucOC concentrations evolution in plasma rats homozygous for WT-, L120Q-, Y139C-, or Y139F-*Vkor1* receiving a specific diet -K3 for 12 days. Results are expressed as the mean of the percentage of 4 animals compared to wild type rats at day 0 \pm standard deviation.

4. Discussion

Anticoagulant-resistant rats due to *Vkor1* gene mutations are good models for mimicking the consequences of human *VKORC1* mutations. In humans, mutations of the *VKORC1* promoter would decrease *VKORC1* expression and some *VKORC1* coding mutations would affect its *VKOR* activity [30]. The consequences of these mutations may, therefore, be different, in terms of activity and/or the consequences on vitamin K status. Using the rat as a model, we have considered these possible differences depending on the nature of the mutation. For this, we used four different rat strains, the OFA-Sprague Dawley rats, and three strains of introgressed rats homozygous for mutations L120Q, Y139F, or Y139C, with the amino acid 139 being located near the catalytic site, and the amino acid 120 being further away from the latter [31]. These strains were derived from the OFA-Sprague Dawley rat in which the mutated *Vkor1* gene was introduced with less than 0.0001% of the initial genome of the wild brown rat. We can, thus, legitimately affect the phenotypic modifications to the *Vkor1* mutation.

We first assessed the impact of the mutation on the ability of each tissue to recycle vitamin K. For this, we measured VKOR activity from liver, kidney, lung, and testicular microsomes. VKORC1 is localized in the membrane of the endoplasmic reticulum [2]. The liver is responsible for the activation of factors II, VII, IX, and X [2]. The kidney, lung, and testis contain high amounts of vitamin K [32] and calcify strongly in the absence of vitamin K [23]. Regardless of the tissue, microsomal VKOR activity is always greatly decreased when VKORC1 is mutated, regardless of the position (i.e., amino acid 120 or amino acid 139) and nature (whether amino acid 139 is replaced by phenylalanine or cysteine) of the mutation. Affinity for vitamin K does not seem to be modified (K_m unchanged), but velocity of VKOR activity is systematically greatly reduced (by a factor of 3 to 6 depending on the tissue and the mutation). This loss of velocity may be due to a malfunction of VKORC1 due to mutations and/or a decrease in the VKORC1 expression level because of instability of the mutated proteins. The malfunction is certain. The characterization of the corresponding recombinant proteins has demonstrated this loss of velocity [33]. On the other hand, this study shows that the VKORC1 expression level remains unchanged despite the presence of mutations L120Q, Y139F, or Y139C regardless of the tissue, except in the liver of the Y139F strain where VKORC1 is slightly expressed. Expression of VKORC1L1, which is another enzyme that is able to catalyze VKOR activity [34] and, thus, compensate for loss of VKORC1 activity, also remains unchanged except in lungs where its expression is slightly increased to compensate for the loss of activity of VKORC1 in the lung.

The presence of these mutations in rats that can be considered laboratory rats allowed us to appreciate the consequences of a decrease in VKOR activity on the “vitamin K” status (Table 4). This status was evaluated by determining tissue vitamin K concentrations, measurement of plasma ucOC concentration, and determination of tissue calcium levels. Among the K vitamins, only MK4 was measured because the rats used received, from the age of 3 weeks up to 8 weeks, a diet supplemented with menadione as their sole source of vitamin K (the amounts of vitamin K1 in the standard feed being limited and provided by the cereals incorporated). The latter received virtually no vitamin K1 and, since there is no endogenous synthesis of vitamin K1, vitamin K1 was almost undetectable in rat tissues. Osteocalcin is a vitamin K-dependent protein produced mostly by osteoblasts [35]. In the absence of vitamin K, the concentration of ucOC increases rapidly in plasma [36] and is, therefore, considered a good marker of overall vitamin K status. Tissue calcium concentrations have also been shown to be negatively correlated with the vitamin K status. In the absence of vitamin K, MGP, which is another vitamin K-dependent protein, cannot be activated and, therefore, cannot inhibit tissue calcification [23,37,38].

Surprisingly, while all rats have always received the same standard diet supplemented with menadione, the vitamin K status of rats homozygous for Y139C and Y139F mutations is strongly altered with a sharp decrease (by a factor of two to four) in MK4 concentrations in all tissues considered in this study and a significant increase in plasma ucOC concentration (by a factor of almost 7). The “vitamin K” status of rats homozygous for the L120Q mutation can be considered an intermediate between the wild type rat and the rats homozygous for the Y139C or Y139F mutations (Table 4). It is surprising to observe such a difference in vitamin K status in rats receiving the same diet supplemented with menadione, even if they have *Vkorc1* mutations. This decrease is even more surprising in the liver, which is the first organ to capture vitamin K from food, where the intake is the same for all strains of rats. Decreasing the recycling efficiency of vitamin K should eventually lead to a decrease in MK4 concentration, but an increase in MK4OX concentration. This is not observed. These results suggest a loss of vitamin K induced by the mutation. The metabolism of vitamin K is still largely unknown. Metabolism of vitamin K by ω -hydroxylation [39,40] with involvement of cytochromes 4F2 and 4F11 in humans has been described. The substrates of cytochromes 4F2 and 4F11 have not been characterized. In rats, cytochromes metabolizing vitamin K have not been identified. Metabolism of vitamin K in its epoxide form by cytochromes might be more efficient than in its quinone form. Since recycling of vitamin K epoxide is less efficient in mutated rats, the elimination of vitamin K could be, thus, increased via greater cytochrome P450 dependent metabolism of vitamin K epoxide. Nevertheless, the difference

in status observed between rats homozygous for the L120Q mutation and rats homozygous for the Y139F and Y139C mutations is surprising, whereas the recycling efficiency of the different tissues is similar, or even lower, in rats homozygous for the L120Q mutation (the efficiency that corresponds to V_{max}/K_m is systematically the lowest). The “vitamin K” status of rats homozygous for the Y139F or Y139C mutations could be aggravated by a modification of the catalysis mediated by VKORC1 due to the mutation. It has been shown that the replacement of tyrosine 139 by cysteine, and, to a lesser extent by phenylalanine, allowed VKORC1 to catalyze a 3-hydroxylation activity, in addition to its activity of reduction [33,41]. The production of 3-hydroxyvitamin K constitutes an additional pathway of elimination of vitamin K and, thus, precludes the recycling of the vitamin by increasing the vitamin K requirements in these rats. Wild type VKORC1 and VKORC1 mutated at position 120 do not produce this 3-hydroxyvitamin K [33].

Table 4. Summary of the results obtained for each strain compared to the WT strain; ↘, decrease compared to WT strain; ↗, increase for WT strain; =, similar with WT strain.

	L120Q	Y139F	Y139C
VKOR ACTIVITY			
In liver	↘↘	↘	↘
In testis	↘↘	↘↘	↘
In kidney	↘↘	↘↘	↘
In lung	↘	↘	=
VKORC1 CONCENTRATION			
In liver	=	↗	=
In testis	=	=	=
In kidney	=	=	=
In lung	=	=	=
VKORC1L1 CONCENTRATION			
In liver	=	=	=
In testis	=	=	=
In kidney	=	=	=
In lung	↗	↗	↗
MK4 CONCENTRATION			
In liver	=	↘	↘↘
In testis	=	↘	↘↘
In kidney	↘↘	↘↘	↘↘
In lung	↘	↘	↘↘
PLASMA ucOC CONCENTRATION	=to ↗	↗	↗
TISSUE CALCIUM CONCENTRATION			
In liver	=	=	=
In testis	=	=	=
In kidney	=	=	=
In lung	=	=	=

The modification of the vitamin K status in the mutated rat strains does not appear to have apparent clinical consequences when animals receive a diet supplemented with menadione. In fact, the rats do not show any changes in coagulation (the quick times are comparable between strains) and do not seem to have calcification problems (tissue calcium concentrations are also comparable between strains). On the other hand, it has been shown that rats homozygous for the Y139C mutation receiving a synthetic diet (cereals replaced by starch) not supplemented with menadione for three months presented very strong vascular calcifications in testes, lungs, and kidneys [23]. This is why we evaluated the ability of different rat strains to resist a limited vitamin K intake by feeding them a

synthetic diet without menadione (diet-K3) for a period of 12 days. To evaluate their resistance, we determined tissue MK4 concentrations (except those in liver because of the extra low concentrations), plasma ucOC concentrations, and prothrombin time, throughout the period of limited intake of vitamin K. Menadione deficiency results very rapidly into lower tissue levels of MK4 in all tissues, regardless of strains. After only four days of menadione deficiency, tissue MK4 concentrations are no longer different between strains (except in testis of wild type rats). In the absence of menadione intake, synthesis of MK4 by the enzyme UBIA1 is stopped [42,43] and tissue MK4 levels decrease to reach the same concentration in all rats. The residual quantities are potentially synthesized by the intestinal microflora [44], with the animals being all housed under the same breeding conditions, in the same rooms, and receiving all the same food. It was discovered that the presence of MK4 in the testis of wild type rats persisted longer. This persistence remains unexplained. The rapid decrease in testis of mutated strains might be due to the needs of vitamin K in other tissues to maintain the pool of activated VKDP.

This rapid decrease in MK4 concentrations is associated with a rapid increase in ucOC concentration in all mutated rats. This increase is even more rapid in the homozygous rats for the L120Q mutation with a concentration of ucOC becoming similar to that of rats homozygous for mutations Y139C and Y139F from the fourth day after the beginning of the deficiency, whereas this concentration was initially lower than that of rats homozygous for mutations Y139C and Y139F. The ucOC concentration remains unchanged in wild type rats. The same findings had already been reported for MGP in the Y139C rat with an increase in tissue ucMGP concentrations [23]. These evolutions are completely consistent with the capacity of each strain to recycle vitamin K. Since dietary vitamin K intake is limited, the gamma-carboxylation of vitamin K dependent proteins is dependent on the recycling of residual concentrations of vitamin K. The wild type rat has optimal recycling and is virtually independent of vitamin K dietary intakes. Mutated rats with limited recycling have high dietary vitamin K requirements and, when vitamin K intake is reduced, activation of vitamin K-dependent proteins becomes insufficient due to a lack of vitamin K quinone. Nevertheless, activation of hepatic vitamin K-dependent proteins (i.e., coagulation factor II, VII IX, and X) appears to be relatively preserved when compared to those of extrahepatic vitamin K dependent proteins (i.e., OC). The prothrombin times remain unchanged throughout the 12 days of deficiency, (with the exception of those of rats homozygous for the L120Q mutation which increase after 8 days of deficiency), whereas the ucOC concentration increases from the fourth day of deficiency. Since the liver is the first organ to capture dietary vitamin K, it is possible that vitamin K1 present in reduced amounts in food may maintain activated clotting factor concentrations at levels consistent with a normal prothrombin time [45]. In rats homozygous for the L120Q mutation, the recycling efficiency being the lowest (3 and 1.5 times lower than the homozygous rats for the Y139F and Y139C mutation, respectively), the low dietary intake is no longer sufficient to maintain the pools of activated clotting factors at sufficient levels.

In this study, we clearly showed that coding mutations of the *Vkorc1* gene could affect the vitamin K status due to failing vitamin K recycling. This change in the vitamin K status has no apparent clinical consequences if vitamin K dietary intake is sufficient. In case of insufficient dietary vitamin K intake, these mutations can lead to insufficient activation of extrahepatic vitamin K-dependent proteins that can lead to vascular calcification due to the absence of MGP activation [23,46] changes in glucose homeostasis due to the increase in plasma ucOC described as an osteoblast-derived hormone [47], or even coagulation problems in the case of mutations severely affecting the efficiency of vitamin K recycling. Nevertheless, during this study, only three mutations were characterized. The three mutated amino acids are all localized in the fourth transmembrane domain containing the catalytic site [31], whereas, in humans, mutations have been detected in the four transmembrane domains and in the cytosolic loop and do not always seem to affect VKORC1 activity [30]. Further studies will be needed to evaluate the impact of each mutation on the vitamin K status. Nevertheless, the prevalence of these mutations in patients with vascular calcifications could be informative with regard to their impact on vitamin K status.

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5.3.1.2 Article :

Seasonal diet-based resistance to anticoagulant rodenticides in the fossorial water vole (*Arvicola amphibius*)

Seasonal diet-based resistance to anticoagulant rodenticides in the fossorial water vole (*Arvicola amphibius*)

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Abstract

Anticoagulant rodenticides (AR) resistance has been defined as “a major loss of efficacy due to the presence of a strain of rodent with a heritable and commensurately reduced sensitivity to the anticoagulant”. The mechanism that supports this resistance has been identified as based on mutations in the *Vkorc1* gene leading to severe resistance in rats and mice. This study evaluates the validity of this definition in the fossorial water vole and explores the possibility of a non-genetic diet-based resistance in a strict herbivorous rodent species.

Genetic support was explored by sequencing the *Vkorc1* gene and the diet-based resistance was explored by the dosing of vitamins K in liver of voles according to seasons.

From a sample of 300 voles, only 2 coding mutations, G71R and S149I, were detected in the *Vkorc1* gene in the heterozygous state with low allele frequencies (0.5–1%). These mutations did not modify the sensitivity to AR, suggesting an absence of genetic *Vkorc1*-based resistance in the water vole. On the contrary, vitamin K1 was shown to be 5 times more abundant in the liver of the water vole compared to rats. This liver concentration was shown to seasonally vary, with a trough in late winter and a peak in late spring/early summer related to the growth profile of grass. This increase in concentration might be responsible for the increased resistance of water voles to AR.

This study highlights a non-genetic, diet-related resistance mechanism in rodents to AR. This diet-based resistance might explain the different evolution of the *Vkorc1* gene in the fossorial water vole compared to rats and mice.

Keywords

Water vole; Anticoagulant rodenticides; VKORC1; Vitamin K; Diet-based resistance

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Introduction

Rodents are responsible for major food losses as well as crop damage and electrical damages. They have negative impacts on the ecology and survival of some wildlife species and are considered as reservoir of various zoonotic pathogens [1–3]. Their populations are therefore managed in areas at risk. This management is based on sanitary, agricultural, architectural and physical measures which are very often complemented by the use of chemicals. Since the 1950s, anticoagulant rodenticides (ARs) have been heavily used worldwide. These compounds are classified into two generations according to their toxicity and dangerousness [4]. ARs are all molecules related to warfarin and are all derivatives of the 4-hydroxycoumarin or 1,3-indanedione ring. These molecules by their common structure, present the same mechanism of action, they are inhibitors of the VKORC1 enzyme. The latter is involved in the regeneration cycle of vitamin K, an essential cofactor in the activation of certain coagulation factors [5, 6]. In the presence of an AR, the vitamin K cycle is stopped, the vitamin K-dependent coagulation factors are insufficiently activated, and haemorrhages are triggered causing the death of the intoxicated animal. This effect is reversed by the administration of vitamin K1, an efficient antidote used in AR intoxications [7].

The lack of other effective chemical strategy has led to a global (over)use of ARs to manage populations of brown and black rats, mice, voles, etc. This intense use has resulted in the selection of resistant rodents. This resistance was first identified in England in 1960 [8, 9] and then described worldwide [10–16]. Different mechanisms have been demonstrated or suggested. The most widely described mechanism is based on mutations in the *Vkorc1* gene. In brown rats, mice and even black rats, mutations in this gene have been demonstrated to cause severe resistance to ARs [17–19]. Due to the structural similarity between ARs, this resistance is cross-linked and generally affects all first-generation and sometimes even second generation ARs. Another assumed mechanism is based on an increase in the metabolism of AR by an overexpression of certain metabolic enzymes, especially some cytochromes P450 [20–22]. Based on the genetically-based mechanisms described above, AR resistance has been defined in many studies as “a major loss of efficacy of ARs in practical conditions where the AR has been applied correctly, the loss of efficacy being due to the presence of a strain of rodent with a heritable and commensurately reduced sensitivity to the anticoagulant” [23] However, to what extent this definition is applicable to all phenomena leading to inefficacy of ARs in wild rodents remains under question. Indeed, in humans, antivitamin K anticoagulants are also widely used in the treatment and prevention of thromboembolic diseases, and their use was the primary cause of iatrogenic accidents until the develop-

ment of direct oral anticoagulants. This was partly due to the high variability in the dosage required to achieve a stable anticoagulation state. Thirty to 50% of this variability was explained by polymorphisms of the cytochromes P450 [24] and *Vkorc1* [25] genes. Other non-genetic factors could also support this variability [26, 27]. The existence of non-genetic factors (food-drug interactions, etc.) in rodent resistance to ARs has not been explored to date.

Rats and mice, for which genetic resistance mechanisms have been widely described, are opportunistic omnivorous rodents. Fossorial water voles *Arvicola amphibius* (previously referred as *Arvicola terrestris* or *Arvicola Sherman*) are strict herbivores found mainly in meadow areas in mid mountains. The latter are subject to periodic proliferation occurring approximately every five years, favored by agricultural practices (i.e., grass monoculture) leading to a homogenization of the environment. For many years, voles' populations have been managed using ARs [28] and have therefore been subjected to a selection pressure comparable to that of rats and mice. Reports of non-efficacy of AR treatments have been reported [29]. Nevertheless, an underlying genetic mechanism could not be demonstrated and the origin of resistance in fossorial water voles remains to be identified.

1. Material and methods

1.1 Ethics

Protocol and procedures were ethically reviewed and approved by ethics committees on animal testing of Marie Skłodowska-Curie, by the institutional ethics and animal welfare committees, by the French Ministry of Research (projects n°660718 and n°12047) and by the Austria authority (project GZ 68.205/0196-WF/V/3b/2016), conforming to Directive 2010/63/EU.

1.2 Animals and trapping sites

Water voles were trapped in two French regions of mid-mountain areas; i) on the second Plateau of the Jura Mountains in Franche-Comté (Eastern France) from 9 different sites- and ii) in the Massif Central Mountains from twenty one different sites i.e., eight sites in Puy-de-Dôme department and thirteen sites in Cantal department (thirteen sites) (Fig. 1). Water voles (n = 100) trapped in Franche-Comté (see [30]) were only used for *Vkorc1* sequencing. These animals were obtained in 2016 from sites where bromadiolone selection pressure was either absent (Site 1, 2, 4, 5, 9), medium (Site 7 and 8) or high (Site 3 and 6) [30]. Water voles (n = 372) trapped in Massif Central were used for *Vkorc1* sequencing and/or vitamin K measurement. In Puy-de-Dôme, bromadiolone selection pressure was either absent or low. In Cantal, information about bromadiolone use was not available.

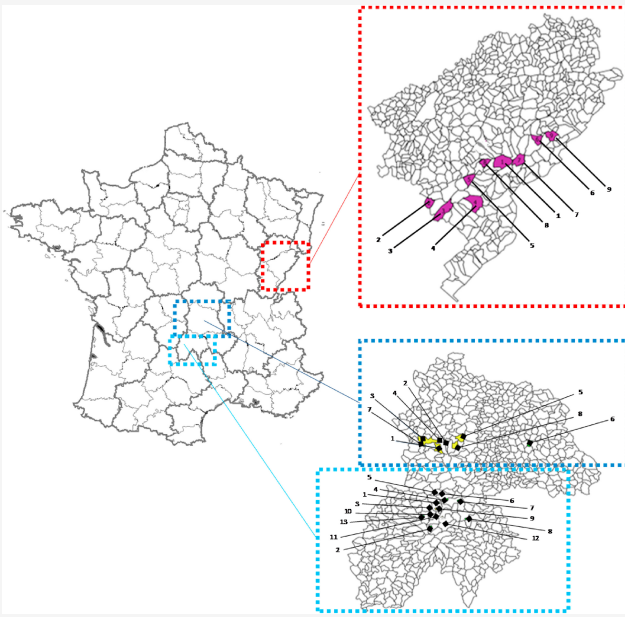


Figure 1. French regions where water voles were trapped. In red, the 9 sites of trapping in Jura Mountain; in dark blue, the 8 sites of trapping in Puy-de-Dôme in Massif central mountain; in light blue, the 13 sites of trapping in Cantal in Massif central mountain. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Urban brown rats were trapped between 2016 and 2017 in the city center of Vienna, Austria (see [31]). Only animals with hepatic concentrations lower than 100 ng/g of ARs were used for vitamin K measurements, i.e. 65 animals.

Species identification by cytochrome b sequencing
Species of trapped rodents were first identified morphologically and then confirmed by sequencing a portion of the mtDNA cytochrome b gene, as described by [32] and [2, 3].

1.3 *Vkorc1* sequencing

Genomic DNA was extracted from tail sample using the Macherey-Nagel Nucleo-spin Tissue extraction kit (Hoerd, France). Genomic DNA was amplified using specific primers of the *Vkorc1* gene. In order to sequence the totality of the *Vkorc1* gene, two sets of primers were systematically used for voles (*Arvicola amphibius*). The first primer set ArvVKas (5'-TCCTGGTGGTCTGGACTGTC-3') and ArvVKAas (5'-TAATCCAGCACTTGGAAGG-3') was used to amplify ground vole *Vkorc1* gene from nucleotide 5 to nucleotide 1416. The second primer set ArvVKbs (5'-GCCCCGCTTCAGTTTTTAAT-3') and ArvVKbas (5'-AGGGGATTGGAATGTAAGG-3') was used to amplify the vole's *Vkorc1* gene from nucleotide 1767 to nucleotide 2608. Ground vole *Vkorc1* gene amplifications were performed using 10 pmol of each primer of one primers set, Accuprime polymerase (1 unit, Invitrogen) in a 25 μ l reaction volume containing 2 μ l DNA, 2.5 μ l 10X Accuprime buffer and 200 μ M of each deoxynucleotide triphosphate. The amplification

was performed at 94 °C for 3 min followed by 40 cycles at 94 °C for 20 s, 61 °C for 20 s, 68 °C for 50 s, and a final extension step at 68 °C for 10 min. The amplified products were sequenced on both strands and compared to the reference sequences (Genbank n° FJ986204.1) using the CLC Sequence viewer7@software.

1.4 Heterologous expression of wild type and mutated *Arvicola amphibius* VKORC1

The coding sequence corresponding to the *Arvicola amphibius* VKORC1 fused with a c-myc tag via a flexible (GGG)₃ in its 3'-extremity was optimized for heterologous expression in yeast and synthesized by GenScript (Piscataway, NJ, USA). The synthesized nucleotide sequence included EcoRI and XbaI restriction sites at its 5'- and 3'- extremities, respectively. This nucleotide sequence was subcloned into pPICZ-B (Invitrogen, Cergy Pontoise, France) and sequenced on both strands.

Construction of mutants was carried out using pPICZ-VKORC1 as template with the Quick change site directed mutagenesis kit (Stratagene) according to the manufacturer's recommendations. Each mutant was checked by sequencing, and thus expressed in *P. pastoris* as previously described [33, 34].

Yeats microsomes containing wild type or mutated VKORC1 were prepared from thawed yeast cells by differential centrifugation. Briefly, yeast cells were resuspended in 50 mM Phosphate Buffer (pH 7.4) containing 1.15% (w/v) of KCl. Yeast cells were broken with Zircon beads using Dispermat LC30 (VMA-GETZMANN, Germany) (15 min–3500 rpm) continuously at 4 °C and further submitted to differential centrifugation as previously described [33, 34]. The 100,000g pellet corresponding to the membrane fraction was resuspended by Potter homogenization in HEPES glycerol buffer (50 mM Hepes, 20% glycerol, pH 7.4). Protein concentrations were evaluated by the method of Bradford [35] using bovine serum albumin as a standard. Microsomes were frozen at -80 °C and used for kinetic analysis and immunoblot analysis.

1.5 VKOR activity and determination of inhibition constants

Microsomal vitamin K epoxide reductase (VKOR) activity was assayed according to the protocol described by Goulois et al. [14]. The inhibiting effect of various ARs was evaluated by the determination of K_i after the addition of various concentrations of the anticoagulant to the standard reaction in the presence of increasing amounts of vitamin K epoxide (from 0.001 to 0.2 mM) using AR concentrations from approximately 0.05 to 20 \times K_i . Three replications were performed for each AR.

1.6 Vitamin K concentration measurement

0.5 g of liver was homogenized with ethanol 33% using an Ultra Turrax tissue disperser from IKA Labor Technik® (VWR International, Strasbourg, France) and then extracted with 4 mL of hexane. After centrifugation at 3000 rpm for 5 min at 4

°C, the supernatant was collected and extracted again with 4 mL of hexane. After centrifugation at 3000 rpm for 5 min at 4 °C, the supernatant was collected and evaporated at 37 °C to dryness under a gentle stream of nitrogen. The final dry extract was dissolved in 500 µL of methanol and vitamins K (i.e., Menaquinone 4-MK4-, Menaquinone 4 epoxide- MK4OX-, phylloquinone -K1-, phylloquinone epoxide -K1OX-) were analyzed by HPLC on reverse phase C18 column (4.6 × 100 mm, 2.7 µm, VWR, Fontenay-sous-bois, France). A post-column reactor (2.1 × 50 mm steel column packed with zinc powder) was connected in series between the analytical column and detector to ensure the reduction of K vitamins and allow the detection of reduced vitamins K by fluorescence with excitation and emission wavelengths of 246 and 430 nm, respectively. A gradient elution system was used with a flow rate of 1 mL/min as follows. After 24 min of elution with 99% methanol/1% water (acidified with 1% acetic acid containing 1.1 g.L⁻¹ of zinc acetate), the mobile phase was quickly changed from 99% methanol/1% water (acidified with 1% acetic acid containing 1.1 g l⁻¹ of zinc acetate) to 50% methanol/49% dichloromethane with 1% acetic acid and 1.1 g l⁻¹ of zinc acetate in 0.1 min. Under these HPLC conditions, MK4 and K1 were eluted at 10.3 min and 18.8 min, respectively.

1.7 Data analysis

Statistical analyses were performed in R v. 3.1.2 and Graph Pad. Results were expressed as mean values ± SD or confidence interval. Constant of inhibitions were compared using ANOVA then, if relevant, a Tukey's multiple comparisons test was performed. Concentrations of vitamins K in the liver of rats and voles were compared using an unpaired t-tests. For seasonal variation, Dunn's multiple comparison tests were used for pairwise comparison of months within the same sampling year. $P < 0.05$ was the accepted level of significance.

2. Results

2.1 *Vkorc1* mutations in *Arvicola amphibius*

A total of nine haplotypes were detected in the 300 *Arvicola amphibius* samples sequenced for the coding sequence of *Vkorc1* (Table 1). All samples were molecularly confirmed to be *A. amphibius*. Nucleotide sequence variations of *Vkorc1* leading to amino acid change compared to the reference sequence were found in 11 out of 300 *A. amphibius* analyzed (Table 1). Eight animals carried an arginine mutation for glycine at VKORC1 amino acid 71 at the homozygous state. Two were trapped from the Franche-Comté sampling area (one in site 3 and one in site 9), six were trapped from the Puy de Dôme sampling area (five in site 2 and one in site 4) (Fig. 1). Three animals carried in the homozygous state an isoleucine mutation for serine at VKORC1 amino acid 149. All were trapped from the Franche-Comté sampling area (one in site 7 and two in site 8) (Fig. 1).

The allelic frequency of G71R was 1.35% on the total sampling. It was 2% in Franche-Comté, 2.5% in Puy de Dôme,

and 0% in Cantal. The frequency of the G71R mutation on site 3 in Franche-Comté in which bromadiolone has been highly used in previous years and the year of trapping was 3.7%, i.e. identical to that found on site 9 in Franche-Comté for which treatments have been reported absent, and below that of site 2 in Puy de Dôme in which treatments have been rare in previous years.

The allelic frequency of S149I was 0.5% on the total sampling. It was 1.5% in Franche-Comté and 0% in Puy de Dôme and Cantal. This mutation was detected only on sites 7 and 8 where the treatment with bromadiolone is considered as medium, but never detected on sites where bromadiolone has been highly used for many years.

Consequences of the detected mutations on the ability of bromadiolone (composed of 85% Trans-isomers and 15% Cis-isomers) and cis-bromadiolone (composed of 4% Trans-isomers and 96% Cis-isomers) to inhibit VKORC1 was determined by overexpressing recombinant VKORC1 variants in *Pichia pastoris* microsomes and determination of inhibition constants. Values are represented in Fig. 2. No significant difference in sensitivity to bromadiolone or cis-bromadiolone was observed between mutants and the wild type VKORC1 (with bromadiolone, p -value = 0.7143 for G71R and 0.1418 for S149I; with cis-bromadiolone, p -value = 0.9910 for G71R and 0.6389 for S149I).

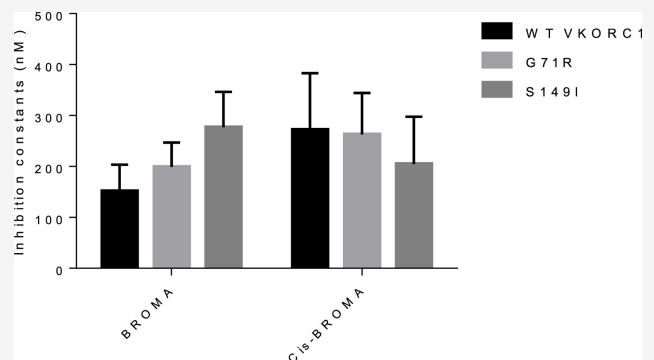


Figure 2. Constant of inhibition of wild type VKORC1 or its mutants G71R or S149I towards bromadiolone (composed of 85% Trans- and 15% Cis-isomers) or Cis-bromadiolone (composed of 4% Trans- and 96% Cis-isomers). Values are presented as mean ± SD. ANOVA analysis was used to compare results. $P < 0.05$ was the accepted level of significance.

2.2 Vitamin K concentrations in liver in *Arvicola amphibius* compared to *Rattus norvegicus*

Vitamins K concentrations in liver - i.e., phylloquinone or vitamin K1 (K1) and menaquinone 4 or vitamin K2 (MK4), and their quinone or epoxide forms-, were determined in fossorial water voles, but also in urban brown rats as references. Sixty-five urban rats - 30 males and 35 females - captured in Vienna, and seventy-three voles - 38 males and 35 females - captured in Puy-de-Dôme (45° 42' 47" N, 2° 46' 12" E, alt. 887 m, from November 2018 to October 2019) on sites not treated

Table 1. Location, sample size (shown as 2N) and frequency of each SNP. Reference allele published in Genbank is noted left of the slash and the detected new allele on the right. Wild type is haplotype number 4. Nucleotide position refers to the positions in the *Arvicola amphibius* *Vkorc1* sequence deposited in GenBank (Accession numbers FJ986204.1). Amino acid positions (AA) refer to residue positions of the complete *VKORC1* protein.

	2N	SNP in Exon1	SNP in Exon2		SNP in Exon 3				
		1/2	1/3	1/4	1/5	1/6	1/7	1/8	1/9
Haplotype numbers									
Nucleotide position		162	1001	1006	2333	2395	2457	2470	2479
Nucleotide polymorphism		C/T	G/A	T/C	C/T	C/T	G/T	A/T	C/T
Resulting AA mutation		D44D	G71R	A72A	L108L	L128L	S149I	A153A	H156H
Total	600	599/1	592/8	60/540	595/5	567/33	597/3	590/10	599/1
Franche-Comté	200	199/1	198/2	60/140	200/0	200/0	197/3	200/0	199/1
Site 1	28	28/0	28/0	0/28	28/0	28/0	28/0	28/0	28/0
Site 2	28	28/0	28/0	16/12	28/0	28/0	28/0	28/0	28/0
Site 3	28	28/0	27/1	10/18	28/0	28/0	28/0	28/0	28/0
Site 4	8	8/0	8/0	4/4	8/0	8/0	8/0	8/0	8/0
Site 5	34	34/0	34/0	18/16	34/0	34/0	34/0	34/0	34/0
Site 6	8	8/0	8/0	0/8	8/0	8/0	8/0	8/0	7/1
Site 7	28	28/0	28/0	4/24	28/0	28/0	27/1	28/0	28/0
Site 8	12	12/0	12/0	0/12	12/0	12/0	10/2	12/0	12/0
Site 9	26	25/1	25/1	8/18	26/0	26/0	26/0	26/0	26/0
Puy de Dôme	240	240/0	234/6	0/240	235/5	212/28	240/0	235/5	240/0
Site 1	18	18/0	18/0	0/18	18/0	14/4	18/0	18/0	18/0
Site 2	50	50/0	45/5	0/50	45/5	46/4	50/0	49/1	50/0
Site 3	32	32/0	32/0	0/32	32/0	21/11	32/0	32/0	32/0
Site 4	26	25/0	24/1	0/25	25/0	23/2	25/0	23/2	25/0
Site 5	14	14/0	14/0	0/14	14/0	9/5	14/0	12/2	14/0
Site 6	38	38/0	38/0	0/38	38/0	38/0	38/0	38/0	38/0
Site 7	16	16/0	16/0	0/16	16/0	14/2	16/0	16/0	16/0
Site 8	46	46/0	46/0	0/46	46/0	46/0	46/0	46/0	46/0
Cantal	160	160/0	160/0	0/160	160/0	155/5	160/0	155/5	160/0
Site 1	10	10/0	10/0	0/10	10/0	10/0	10/0	10/0	10/0
Site 2	20	20/0	20/0	0/20	20/0	15/5	20/0	20/0	20/0
Site 3	10	10/0	10/0	0/10	10/0	10/0	10/0	10/0	10/0
Site 4	10	10/0	10/0	0/10	10/0	10/0	10/0	10/0	10/0
Site 5	20	20/0	20/0	0/20	20/0	20/0	20/0	20/0	20/0
Site 6	10	10/0	10/0	0/10	10/0	10/0	10/0	10/0	10/0
Site 7	20	20/0	20/0	0/20	20/0	20/0	20/0	20/0	20/0
Site 8	20	20/0	20/0	0/20	20/0	20/0	20/0	15/5	20/0
Site 9	8	8/0	8/0	0/8	8/0	8/0	8/0	8/0	8/0
Site 10	12	12/0	12/0	0/12	12/0	12/0	12/0	12/0	12/0
Site 11	10	10/0	10/0	0/10	10/0	10/0	10/0	10/0	10/0
Site 12	10	10/0	10/0	0/10	10/0	10/0	10/0	10/0	10/0

with bromadiolone were used. All these animals presented hepatic concentrations of antivitamin K anticoagulants below 100 ng/g.

The results are presented in Fig. 3. Vitamin K1 (in quinone or epoxide form) is the major form present in the liver of the water vole (95% of vitamin K) and the rat (62% of vitamin K). Vitamin K1 (in quinone or epoxide form) is globally 5 times more abundant in the liver of the water vole compared to the rat. This difference is not compensated by vitamin K2 which is only 2 times more abundant in the rat compared to the water vole. No sex difference was detected in voles or rats for vitamins K in any form (data not shown).

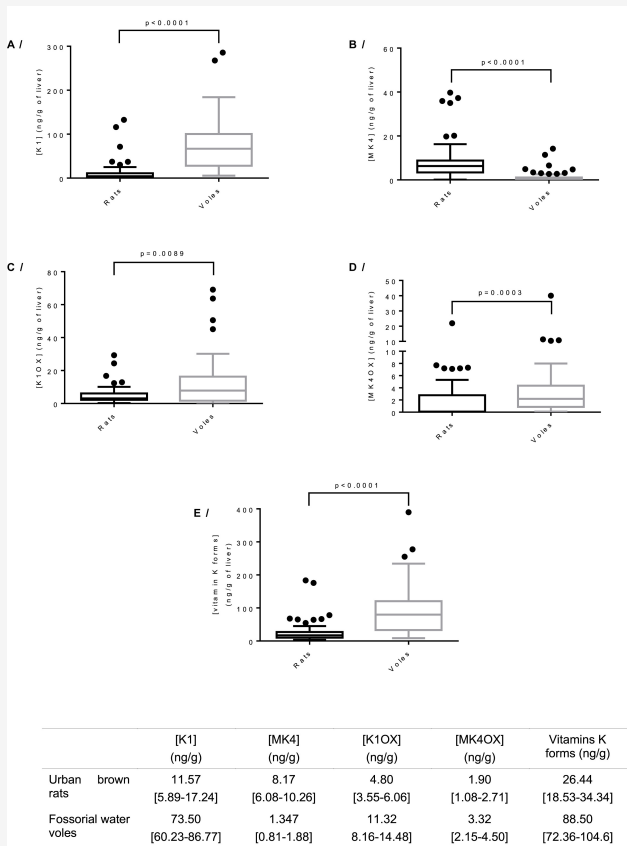


Figure 3. Concentrations of vitamins K in the liver of rats and fossorial water voles. In A/Vitamin K1 in its quinone form, in B/Vitamin K2 in its quinone form, in C/Vitamin K1 in its epoxide form, in D/Vitamin K2 in its epoxide form, in E/Total of the different forms. Boxplot presents median, quartiles, minimum and maximum and outliers. In table are presented the means and their 95% confidence interval. Statistical analysis was done using unpaired t-tests. $P < 0.05$ was the accepted level of significance.

2.3 Seasonal variation in hepatic vitamins K concentrations in *Arvicola amphibius*

Seasonal variations of vitamins K concentrations in liver - i.e., phylloquinone or vitamin K1 (K1) and menaquinone 4 or

vitamin K2 (MK4), in their quinone or epoxide forms-, were determined in fossorial water voles.

73 voles - 38 males and 35 females - captured from November 2018 to October 2019 and 99 voles - 45 males and 54 females-captured from November 2019 to October 2020 were used for this comparative analysis. These voles were all captured around Angle-bas (45° 42' 47" N, 2° 46' 12" E, alt. 887 m). All these animals presented hepatic concentrations of antivitamin K anticoagulants below 100 ng/g.

Seasonality of vitamin K concentrations was observed with variations from one to six with respect to the rat mean value, with two high periods in July and October/November and two low periods in February and August (Fig. 4 A, Table 2). Peak concentrations were observed in July. This seasonality was observed during the two years of monitoring. Vitamin K seasonality was associated with vitamin K1 seasonality in its quinone form (Fig.4 B, Table 2) with variations of two to fifteen between February and July and a vitamin K1 seasonality in its epoxide form (Fig. 4 B, Table 2). Unlike vitamin K1, menaquinone-4 in the quinone or epoxide form did not show seasonal variations. Whatever the month of sampling, vitamin K1 in its quinone form was consistently the major form present in the liver; its proportion ranged from 67% in winter to 87% in summer as a proportion of total vitamins K. Vitamins K seasonality was observed for males and females.

3. Discussion

This study highlights a new mechanism of resistance to antivitamin K anticoagulants in rodents. Until now, only genetic mechanisms have been reported. The mechanism described in this article involves a non-genetic support in a rodent species, the water vole (*Arvicola amphibius*), for which resistance studies are scarce. This species was targeted because of the similarity of the management methods used with those used to control rats or mice. Indeed, the proliferation of voles has been managed for many years by the use of first or second-generation ARs like rats or mice. This intensive use having resulted in the selection of resistant strains in rats and mice, the same phenomenon could be anticipated in voles. However, since the ground vole has a different habitat, proximity to humans and behavior than rats or mice, this hypothesis could be challenged.

Of course, before considering non-genetic support to ARs resistance such a hypothesis, the exclusion of the main genetic mechanism described in rodents was the first step in this work. In the studied regions, water vole populations have been managed with antivitamin K anticoagulants for many years, as in rats and mice. The same consequences could therefore be suspected: the selection of individuals carrying *Vkorc1* mutations conferring them a strong resistance to these molecules. Indeed, in brown rats, more than 15 different mutations have been described worldwide [13, 15–18], including some associated with severe resistance (Y139F, Y139C and L120Q) that can reach very high allelic frequency up to 100% locally. In mice, the same observations were made [14]. All these

Table 2. Concentration of vitamins K in liver of water voles at different periods of trapping. Results are presented as medians and 95% confidence intervals. Statistical analysis was done using Dunn's (multiple comparisons tests. $P < 0.05$ was the accepted level of significance ^{a, b, c, d, e}, statistical difference between two groups of the same row.

	NOVEMBER	FEBRUARY	JULY	AUGUST
Vitamins K (ng/g of liver)				
	92.8 ^a	22.0 ^{a,b}	140.4 ^{b,c}	27.7 ^c
Year 1	[70.0–131.3] n = 10	[10.8–47.7] n = 10	[99.1–237.5] n = 9	[23.7–46.7] n = 9
	122.6 ^a	48.7 ^b	118.7 ^{b,c}	38.5 ^{a,c}
Year 2	[82.0–145.0] n = 10	[24.5–86.5] n = 10	[83.1–256.2] n = 8	[24.3–69.3] n = 10
	114.7 ^{a,b}	31.1 ^{a,c}	139.7 ^{a,c,d}	32.8 ^{b,d}
Year 1 + 2	[86.8–126.7] n = 20	[24.9–59.8] n = 20	[120.8–217] n = 17	[29.2–53.4] n = 19
Phylloquinone(ng/g of liver)				
	90.0 ^a	16.2 ^{a,b}	136.1 ^{b,c}	30.7 ^c
Year 1	[68.1–128.1] n = 10	[7.4–40.2] n = 10	[97.8–219.1] n = 9	[21.6–44.9] n = 9
	116.9 ^{a,b}	37.4 ^{a,c}	116.4 ^{a,c,d}	36.9 ^{b,d}
Year 2	[77.4–138.4] n = 10	[19–68.4] n = 10	[81.2–254.3] n = 8	[22.9–67.5] n = 10
	102.5 ^{a,b}	25.8 ^{a,c}	132.2 ^{c,d}	30.7 ^{b,d}
Year 1 + 2	[83.4–122.1] n = 20	[19.6–47.9] n = 20	[117.3–208.3] n = 17	[27.4–52.4] n = 19
Menaquinone-4(ng/g of liver)				
	2.2	3.4	4.5	1.4
Year 1	[1.7–3.4] n = 10	[1.3–9.6] n = 10	[0.7–18.9] n = 9	[0.3–5.9] n = 9
	2.3	10.5 ^{a,b}	1.9 ^a	1.6 ^b
Year 2	[0.3–10.3] n = 10	[5.0–18.7] n = 10	[1.1–2.6] n = 8	[1.0–2.2] n = 10
	2.3	3.8 ^a	3.3	1.5 ^a
Year 1 + 2	[1.5–6.3] n = 20	[4.7–12.6] n = 20	[1.3–10.9] n = 17	[1.0–3.6] n = 19

Vitamin K = Vitamin K1 in its quinone and epoxide forms + Menaquinone-4 in its quinone and epoxide forms; Phylloquinone = Vitamin K1 in its quinone and epoxide forms; Menaquinone-4 = Menaquinone-4 in its quinone and epoxide forms.

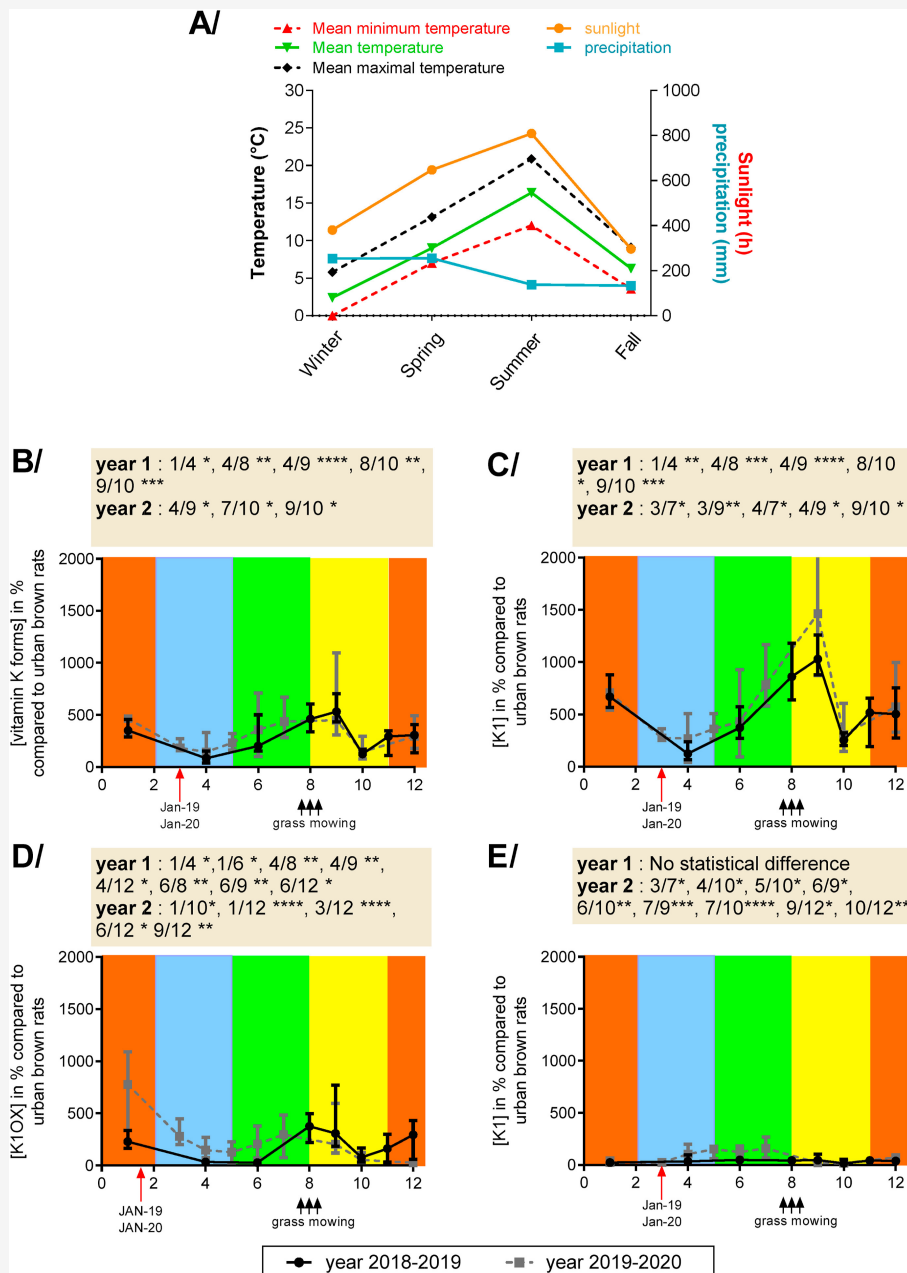


Figure 4. Seasonal variation of concentrations of vitamins K in the liver of voles. In A/Meteorological parameters for the four seasons, in B/Total of the different 1forms, in C/Vitamin K1 in its quinone form, in D/Vitamin K1 in its epoxide form, in E/Menaquinone-4 in its quinone + epoxide forms. Results are presented as percentage compared to the mean value measured in Vienne urban rats trapped from September 2016 to June 2017. The black circles correspond to the sampling points of year 1 (from November 2018 to October 2019), the grey squares correspond to the sampling points of year 2 (from November 2019 to October 2020). Values are presented as median and interquartile range. Orange area is fall; blue area is winter; green area is spring; yellow area is summer. Month # 3 corresponds to January. Statistical analysis was done using Dunn's multiple comparisons tests by comparing the months of the same sampling year in a pairwise comparison. $P < 0.05$ was the accepted level of significance. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$, ****, $p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

mutations, in view of what has been reported in humans -for whom more than 30 *Vkorc1* mutations have been described [25, 34], certainly existed in rats and mice prior to the use of anticoagulants. The selection pressure exerted with the use of ARs only resulted in an increase in their frequency. While a similar selection pressure has been exerted for many years for the control of water voles, a different evolution of the *Vkorc1* gene is revealed by this study. Indeed, this study reports only two coding mutations, G71R and S149I, out of 300 water voles trapped from different regions and sites. Amino acid 71 has already been described as susceptible to be mutated in humans [25]. This G71R mutation was detected in water voles with an allelic frequency of 1.3%, which is low compared to the allelic frequencies of the mutations found in mice or rats [14, 18]. This mutation was found only in the heterozygous state, unlike the *Vkorc1* mutations in rats or mice for which the selection pressure resulted in the massive selection of homozygous individuals. The *Vkorc1* mutations have so far been described as codominant, homozygosity providing a significant advantage over heterozygosity in these species. [22, 36]. Voles carrying this mutation have been captured from four sites from different regions, of which, only one was heavily treated with bromadiolone, the others being little or not treated at all - which is not in favor of a positive correlation between treatment intensity and the presence of this mutation. Consistent with this distribution, enzymatic characterization revealed that the presence of this mutation does not lead to resistance to bromadiolone, a second-generation anticoagulant, nor to *cis*-bromadiolone. This latter can be assimilated by its short persistence to a first generation anticoagulant and may constitute an eco-compatible alternative to the current use of bromadiolone [37–39]. The S149I mutation was found at a lower allelic frequency (0.5%) and systematically in the heterozygous state. It was detected on sites undergoing medium selection pressure and was not detected on plots with high selection pressure. Enzymatic characterization did not indicate any association between this mutation and resistance to antivitamin K anticoagulants.

Genetic resistance associated with mutations in *Vkorc1* does not appear to be present in the water vole despite important selection pressure. However, resistance phenomena have been described in this species [29]. A metabolic resistance of genetic origin could be considered as the one described in black [20, 21] or brown rats [22]. Nevertheless, since the persistence of bromadiolone is long-lasting in the fossorial water vole [39], a simple overexpression of metabolic enzymes capable of conferring metabolic resistance is doubtful. Other genetic mechanisms supported by other genes have been proposed in humans [40], but their involvement has not been proven. In contrast, non-genetic factors that cause variability in clinical response are involved in humans [26]. However, this aspect has never been considered in rodents. These non-genetic factors include individual factors such as age and sex, that cannot explain resistance at the population level if only one age-class and/or gender is concerned. Environmental fac-

tors have also been described. For example, diet may provide exogenous vitamin K that can counteract the effect of antivitamin K anticoagulants [41]. The predominant form of vitamin K presents in the diet is vitamin K1 (i.e., phylloquinone). The latter is mainly provided by plants [42], menaquinones being synthesized by bacterial microflora or provided by fermented foods [43]. The water vole is an herbivorous rodent and its diet is therefore compatible with a high exogenous intake of vitamin K1. To support this hypothesis, we compared the concentrations of vitamin K1 and menaquinone-4 present in the vole's liver with those found in rats. Only the short forms of vitamin K were considered in this study, the long forms being considered rather as storage forms and not useable by the *VKORC1* enzyme [44]. Mean liver concentrations of total vitamin K, i.e., the sum of vitamin K1 in quinone and epoxide form plus menaquinone-4 in quinone and epoxide form, are about 4 times higher in the vole than in rats. This difference is mainly due to the highest concentration of phylloquinone (about six times higher) in the vole compared to the rat. We postulate that this increase in concentration is responsible for the increased resistance of water voles to antivitamin K anticoagulants compared to rats. Indeed, vitamin K1 is used as an antidote in antivitamin K anticoagulant intoxications [7]. On the other hand, in human medicine, an increased dietary intake of vitamin K due to the over-consumption of certain plant-based foods can cause a change in a patient's response to anticoagulant treatment although this treatment was stabilized [41].

The water vole's diet remains poorly characterized and it is therefore challenging to elucidate the origin of its diet-based resistance. The different studies on water vole's diet are indirect field studies based on the analysis of plants in the reserves, of the stomach contents or comparison of the floristic composition in relation to vole occupation and abundances. Alas, the resulting interpretations are difficult to use because of the methodological challenges related to the burrowing behavior of the water vole and the plant diversity. Few studies on food preference are available. It is therefore difficult to determine the plant species that potentially induces this diet-based resistance, especially because data available on the vitamin K content of plants are related to human food [45–47]. Their identification would therefore enable to optimize water vole management efforts. In the current state, this management should be optimized by considering the seasonality of the fossorial water vole's diet-based resistance. Indeed, the presence and abundance of plants in grasslands vary seasonally and their vitamin K content is certainly dependent on their stage of maturation [48]. We therefore evaluated the average hepatic concentrations of vitamin K in the vole according to the season. To certify the results and to avoid bias due to unidentified one-off phenomena, the follow-up was carried out over two consecutive years. The results obtained are remarkably reproducible between year 1 and year 2 with two vitamin K1 peaks in June/July and October/November and two troughs in January/February and August. This seasonality

of vitamin K1 is closely related to the growth pattern of grass in Auvergne with a regular growth from March to June of plants known to be rich in vitamin K1 (dandelions, clovers...) as a result of favorable climatic conditions (Fig. 4 A) leading to an accumulation of vitamin K1 in the liver of voles feeding on these plants. Grass mowing in June/July [49] associated with the increase in temperature and summer dryness leads to a decrease in food resources (Fig. 4 A), leading to a depletion of vitamin K1 reserves in the water vole liver. In autumn, grass grows again, however in a more limited extent than in spring, leading again to an accumulation of vitamin K1 in the liver of the ground vole before winter. In view of these results, it appears that management efforts to control water vole outbreaks could be concentrated at the end of the winter period while considering the feasibility of such treatments (snow cover, frozen soil) and the risk of their side effects on wildlife. A modification of the vegetation cover could also be considered to decrease the intake of vitamin K1 and improved efficacy of management methods. Nevertheless, further studies will be necessary to evaluate the influence of altitude, climate, soil type and intensity of fertilization and grazing on vitamin K1 seasonality, because voles abound on semi-natural meadows where vegetation grows spontaneously as a result of environmental conditions and agricultural practices.

4. Conclusion

In summary, this study highlights a non-genetic, diet-related resistance mechanism in rodents. This diet-based resistance might explain the different evolution of the *Vkorc1* gene in the fossorial water vole compared to rats and mice, where mutations associated with anticoagulant resistance have not been selected another non-genetic resistance mechanism was in place. This diet-based resistance might explain the inter-species variability in susceptibility to antivitamin K anticoagulants and the resistance of some non-target species, particularly herbivorous species, to antivitamin K intoxications.

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5.3.2 Révolution des anticoagulants par les stéréoisomères

5.3.2.1 Article :

**Management of Rodent Populations by Anticoagulant Rodenticides :
Toward Third-Generation Anticoagulant Rodenticides**

Management of Rodent Populations by Anticoagulant Rodenticides: Toward Third-Generation Anticoagulant Rodenticides

Marlène Damin-Pernik^{1,2}, Bernadette Espana¹, Sébastien Lefebvre¹, Isabelle Fourel¹, Hervé Caruel², Etienne Benoit¹, Virginie Lattard¹

Abstract

Second-generation anticoagulant rodenticides (SGARs) have been used since the 1980s for pest management. They are highly efficient even in warfarin-resistant rodents. Nevertheless, because of their tissue persistence, nontarget poisoning by SGARs is commonly described in wildlife. Due to this major problem, a new generation of anticoagulants must be developed to limit this risk. This study proposes a method of developing a new generation of anticoagulant rodenticides by revisiting the old SGARs based on the concept of stereochemistry. Each current SGAR is a mixture of diastereomers. Diastereomers of each compound were purified, and their biologic properties were compared by determining their ability to inhibit vitamin K epoxide reductase (VKOR) activity involved in the activation of vitamin K-dependent clotting factors and their toxicokinetic properties. Systematically, for each SGAR, both diastereomers are as effective in inhibiting VKOR activity. However, their toxicokinetic properties are very different, with one of the two diastereomers always more rapidly cleared than the other one. For all SGARs except flocoumafen, the less persistent diastereomer is always the less predominant isomer present in the current mixture. Therefore, the development of baits containing only the less persistent diastereomer would avoid the ecotoxicological risk associated with their use without decreasing their efficacy.

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Introduction

Managing populations of commensal rodents is essential. By sharing our environment, they can transmit to humans, directly or indirectly, more than 40 zoonotic pathogens, such as *Yersinia pestis*, Hantavirus, and *Leptospira* sp. [1]. They can cause the destruction or degradation of cereals, accounting for nearly 10% of the grain crops in the world, with considerable variation depending on the country [1, 2]. Rodents also damage substructures and electronic networks.

The management of rodent populations should be based on sanitary and architectural approaches. However, these approaches are often widely associated with the use of rodenticides. The sophisticated social organization of the populations of rats and neophobic behavior of these animals make the action of toxicity with an immediate effect completely ineffective. The only really effective molecules that can be used to manage such populations are anticoagulant rodenticides (ARs). Their delayed action, with mortality occurring several days after consumption, makes them effective in controlling neophobic species. Indeed, ARs, by inhibiting the VKORC1-dependent vitamin K epoxide reductase activity,

lead to the progressive reduction of the pool of vitamin K necessary for the activation of clotting factors II, VII, IX, and X [3, 4]. Therefore, prolonged or repeated exposure to ARs leads to rodent death by hemorrhage within 3 to 7 days. ARs are usually classified into two generations. The first generation of ARs includes diphacinone, warfarin, coumatetralyl, and chlorophacinone. The second generation of ARs includes bromadiolone, difenacoum, brodifacoum, flocoumafen, and difethialone. All ARs are 4-hydroxycoumarin derivatives except chlorophacinone and diphacinone, which is a derivative of indane-1,3-dione (Hadler and Buckle 1992)[5].

First-generation ARs (FGARs) generally require several days to cause rodent death. The activity of these FGARs is now largely limited by the genetic resistance phenomenon. The first case of resistance to FGARs in brown rats was observed in 1958 in the United Kingdom [6]. In the following years, resistance was reported everywhere in rats and mice in Europe [7], the United States [8], Canada [9], Australia [10], and Japan. The most common resistance mechanism is a result of single-nucleotide polymorphisms (SNPs) in the *Vkorc1* gene, leading to an enzyme that is less sensitive to the action of ARs [11, 12].

The development of second-generation ARs (SGARs) resulted from the emergence of resistance to FGARs in rodent populations. SGARs have been registered from 1975 to 1984, with difenacoum being used first, followed by brodifacoum, bromadiolone, difethialone, and flocoumafen. They are more potent and active after only one feeding due to their longer half-life [13]. Nevertheless, due to their increased tissue persistence, nontarget poisoning by SGARs is commonly described in wildlife. Different species are affected by this exposition, such as stoats, weasels [14], red foxes [15, 16], European minks [17], bobcats [18], various raptors such as red kite, buzzards, kestrels, peregrine falcons and eagle owls [19–23]. ARs were thus identified by the European Union as candidates for future comparative risk assessment and substitution. However, in the absence of an alternative, AR molecules were included in Annex 1 of the EU Biocidal Products Directive 98/8/EC, and their use is still tolerated until a more appropriate solution is found. This study proposes a method of developing a new generation of ARs by improving the old SGARs based on the concept of stereochemistry. A previous study [24] demonstrated that difenacoum, the first developed SGAR, is a mixture of two diastereomeric forms, namely, 43% of the composition is trans-isomers, with a short half-life and 57% of the composition is cis-isomers, with a long half-life. The production of a new difenacoum containing exclusively trans-isomers could significantly reduce its tissue persistency and thus avoid secondary poisoning associated with its use. This method of producing an ecofriendly difenacoum may be applicable to all SGARs. Indeed, all SGARs have two asymmetric carbons [1, 25]. Therefore, all SGARs are a mixture of two diastereomeric forms that could have different biological properties. This study aimed to explore the differences in the biological properties of the diastereomers of SGARs and to

propose the best diastereomer of each SGAR mixture to use as a rodenticide.

1. Material and methods

1.1 Chemicals

Bromadiolone (3-[3-[4-(4-bromophenyl)phenyl]-3-hydroxy-1-phenylpropyl]-4-hydroxychromen-2-one) and difethialone (3-[3-[4-(4-bromophenyl)phenyl]-1,2,3,4-tetrahydronaphthalen-1-yl]-2-hydroxythiochromen-4-one) were supplied by Liphatech (Agen, France). Difenacoum (2-hydroxy-3-[3-(4-phenylphenyl)-1,2,3,4-tetrahydronaphthalen-1-yl]chromen-4-one), brodifacoum (3-[3-[4-(4-bromophenyl)phenyl]-1,2,3,4-tetrahydronaphthalen-1-yl]-4-hydroxychromen-2-one), and flocoumafen (4-hydroxy-3-[3-[4-[[4-(trifluoromethyl)phenyl]methoxy]phenyl]-1,2,3,4-tetrahydronaphthalen-1-yl]chromen-2-one) were purchased from Hangzhou Ich Biofarm Co. (China).

(1R,3R)(1S,3S)-isomers (called trans-isomers) and (1R,3S)(1S,3R)-isomers (called cis-isomers) of difenacoum, difethialone, flocoumafen, and brodifacoum were separated and purified in a silica gel column in our laboratory and assigned according to information available in published articles [24], regulatory documents (European Parliament, 2007, 2009) and patents [26]. (1R,3S)(1S,3R)-isomers (called trans-isomers) and (1R,3R)(1S,3S)-isomers (called cis-isomers) of bromadiolone were also separated and purified in a silica gel column in our laboratory. Dimethyl sulfoxide (DMSO), acetonitrile, methanol, acetone, diethyl ether, hexane, and orthophosphoric acid were obtained from VWR International (Fontenay sous bois, France), and Vetflurane® and vitamin K1 were obtained from Alcyon (Miribel, France). Vitamin K1 was converted to vitamin K epoxide according to the method described by Tishler et al. [27]. Purity was estimated by LC/MS and was higher than 99%. High-performance liquid chromatography (HPLC)-grade water, which was prepared using a milli-Q plus system (Millipore, Saint-Quentin en Yvelines, France), was used for the preparation of the HPLC eluents.

1.2 Animals

Eight-week-old male OFA Sprague-Dawley rats (each weighing 175–200 g) were obtained from a commercial breeder (Charles Rivers, l'Arbresle, France) and were acclimated for a minimum period of 5 days. The rats were housed four per cage under a constant photoperiod and ambient temperature. The animals were kept in standard cages (Eurostandard, Type IV, Tecniplast, Limonest, France) and received standard feed (Scientific Animal Food and Engineering, reference A04) and water ad libitum. Experimental research on the rats was performed according to an experimental protocol following international guidelines and with approval from the ethics committee of the Veterinary School of Lyon.

1.3 Pharmacokinetic study

Male OFA Sprague-Dawley rats received, by per os administration, cis- or trans-isomers of difenacoum, bromadiolone, flocoumafen, brodifacoum, and difethialone. Anticoagulants were dissolved in 10% DMSO and 90% vegetable oil and were administered by force feeding. The animals were given a daily subcutaneous injection of vitamin K1 (5 mg/kg). Finally, the rats were anesthetized with Vetflurane®. Blood samples were obtained via terminal blood collection by cardiac puncture in citrated tubes. The rats were euthanized with CO₂, and the livers were then collected and stored at -20°C until analysis.

1.4 No-choice feeding test with cis- or trans-isomers of bromadiolone and difethialone

Before testing, all of the animals were maintained on diet A04 (Scientific Animal Food and Engineering) and water ad libitum. Individuals were weighed before the start of the test. Nochoice feeding tests were performed on groups of 10 rats. The rats were fed bait containing 0.005% cis- or trans-isomers of bromadiolone or 0.0015% cis- or trans-isomers of difethialone. Cis- and trans-isomers of bromadiolone and of difethialone are pure at 93, 98, 99, and 94%, respectively (the remaining part is the other stereoisomer). Feeding periods of 4 days were used. The contents of food pots were weighed daily, and the intake of bait was recorded. The mortality of rats was also observed daily. At the end of the feeding period with toxic baits, the animals were given diet A04 again and were observed for an additional period of 17 days.

1.5 Determination of AR concentration in the liver

Liver tissue (1 g) was extracted with acetone using an Ultra Turrax tissue disperser from IKA Labor Technik® (VWR International, Strasbourg, France) according to the method previously published by Damin-Pernik et al [24] and validated according to the guideline on Bioanalytical Method Validation published by the European Medicines Agency¹. The extract was centrifuged at 3000 rpm for 10 min, and the supernatant was evaporated at 60°C under a gentle nitrogen flow. Dry extract was resuspended in acetonitrile/hexane (50%/50%). The hexane layer was eliminated, and the remainder was dried at 60°C under a gentle nitrogen flow. The final dry extract was dissolved in methanol, and the AR concentrations were analyzed by HPLC on a reverse phase C-18 column (4.6x150 mm, 5 μm; Waters, Milford, MA, USA) at detection wavelengths of 258, 260, 265, 267, and 272 nm for difenacoum, difethialone, bromadiolone, brodifacoum, and flocoumafen, respectively. The C-18 column was heated at 40°C for difenacoum, bromadiolone, flocoumafen, and brodifacoum and at 30°C for difethialone. A gradient elution system was used with a flow rate of 1 mL/min as follows: from 30%

methanol/70% water (acidified with 0.2% H₃PO₄) to 70% methanol/30% water at 10 min for difenacoum; from 30% acetonitrile/70% water (acidified with 0.1% H₃PO₄) to 90% acetonitrile/10% water at 14 min for brodifacoum; from 60% methanol/40% water (acidified with 0.2% H₃PO₄) to 80% methanol/20% water at 20 min for bromadiolone; from 60% methanol/40% water (acidified with 0.2% H₃PO₄) to 90% methanol/10% water at 22 min for flocoumafen; and from 60% acetonitrile/40% water (acidified with 0.2% H₃PO₄) to 75% acetonitrile/25% water at 10 min for difethialone. The recovery rate of diastereomers of SGAR from tissues were between 75 to 95 % in liver and the CV% values for precision of the different ARs were less than 15%.

1.6 VKOR activity assay and kinetics

Liver microsomes were prepared from fresh livers by differential centrifugation according to the protocol described by Hodroge et al.[28]. Microsomal vitamin K epoxide reductase (VKOR) activity was assayed according to the protocol described by Hodroge et al.[28, 29]. The inhibiting effect of the silica gel column-purified cis- or trans-isomers of ARs was evaluated by the determination of K_i after the addition of various concentrations of the anticoagulant to the standard reaction in the presence of increasing amounts of vitamin K epoxide (from 0.001 to 0.2 mM) using anticoagulant concentrations from approximately 0.05 to 20 x K_i.

1.7 Data analysis

Pharmacokinetic calculations were performed using the non-compartmental approach on the mean results per group. The elimination half-life ($t_{1/2(el)}$) was calculated using linear regression in GraphPad Prism 6 software (California, USA). The total area under the curve (AUC) was calculated using the linear trapezoidal method and adding the estimated terminal portion of the curve (AUC 0→∞). Statistical analysis of pharmacokinetic parameters were done using GraphPad Prism 6 software (CA, USA). A Mann-Withney test was used with $\alpha < 0.05$ in order to compare statistically the results between the two groups.

For kinetic analysis of the VKOR activity, data were fitted by non-linear regression to the non-competitive or competitive inhibition model using GraphPad Prism 6. The choice of the best model was based on the *Corrected Akaike Information Criterion*.

2. Results

2.1 Separation of the diastereoisomers of SGARs

The analysis of SGARs by HPLC on a C18 reverse phase column as described in the experimental procedure showed, for each compound, two different peaks corresponding to the cis- and trans-diastereomer forms. The structures of the cis- and trans-isomers of flocoumafen, difethialone, brodifacoum, bromadiolone, and difenacoum are shown in Figs. 1A, 2A, 3A, 4A, and 5A, respectively. The cis- and trans-isomers are shown in only one alternative orientation of substituents.

¹Guideline on bioanalytical method validation, 21 July 2011 EMEA/CHMP/EWP/192217/2009 Rev.1 Corr. Committee for Medicinal Products for Human Use (CHMP) http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf

Both peaks of each compound presented the same absorption spectrum, with maximum absorbances at 258, 265, 272, 267, and 260 nm for difenacoum, bromadiolone, flocoumafen, brodifacoum, and difethialone, respectively. The retention times, respectively, were 11.3 and 12.5 min for the cis- and trans-isomers of difenacoum, 15.0 and 16.2 min for the cis- and trans-isomers of brodifacoum, 20.1 and 20.7 min for the cis- and trans-isomers of flocoumafen, 17.1 and 17.9 min for the cis- and trans-isomers of bromadiolone, and 20.2 and 21.3 min for the cis- and trans-isomers of difethialone.

2.2 Comparative analysis of the tissue persistence of the trans- and cis- isomers of the five SGARs in the liver

To study the hepatic persistence of the trans- and cis-isomers of SGARs, male OFA Sprague- Dawley rats were divided into groups of 4 rats that received, by per os administration, 3.0 mg.kg⁻¹ of trans- or cis-isomers of flocoumafen, difethialone, brodifacoum, bromadiolone, or difenacoum. The rats were killed 24, 72, 168, 336, or 504 hours after the administration, and the concentrations of the trans- and cis-isomers of SGARs were determined in the liver for each rat. The time-dependent evolution of the cis- and trans-isomers in the liver is presented in Fig. 1B for flocoumafen, Fig. 2B for difethialone, Fig. 3B for brodifacoum, Fig. 4B for bromadiolone, and Fig.5B for difenacoum.

The profile of elimination was the same for the 5 molecules studied; specifically, one isomer was eliminated more quickly than the other, and the decrease followed a clear biphasic profile. For difenacoum, difethialone, and brodifacoum, the cis-isomer was eliminated less rapidly than the trans-isomer. However, for bromadiolone and flocoumafen, the cis-isomer is eliminated more quickly. The half-lives and areas under the curve (AUCs) of the trans- and cis-isomers in the liver were calculated using a linear regression model and are presented in Table 1.

2.3 Analysis of the inhibiting effect of the trans- and cis-isomers of SGARs on vitamin K epoxide reductase activity

The ability of the cis- and trans-isomers of each SGAR to inhibit the VKOR activity catalyzed by warfarin-susceptible rat liver microsomes was evaluated by determining Ki constants. The results are presented in Table 2. The cis- and trans-isomers of each SGAR were all able to inhibit VKOR activity. For each SGAR, the Ki constants were similar between the diastereomers.

2.4 No-choice feeding test with the cis- or trans-isomers of bromadiolone or difethialone

A no-choice feeding test was performed to confirm the efficiency of baits enriched with the less persistent pair of diastereomers and to determine the benefits in term of liver residues. Assays were performed with bait containing mainly one isomer of bromadiolone or difethialone at the concentrations of 50 and 15 ppm for bromadiolone and difethialone,

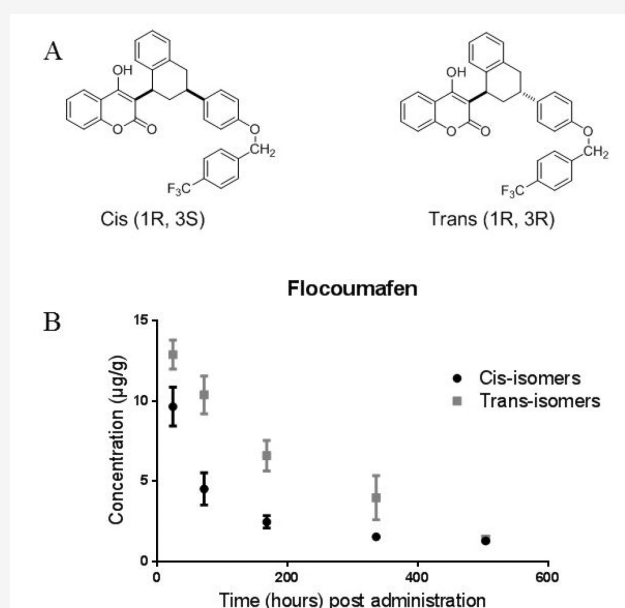


Figure 1. Structures of the cis- and trans-isomers of flocoumafen (A). The cis- and trans-isomers referenced in the text arise from the alternative orientations of the substituents on the tetralin ring system. Time-dependent concentrations of the cis- and trans-isomers of flocoumafen after the per os administration of, respectively, 3 mg.kg⁻¹ of silica gel column-purified cis- or trans-isomers of flocoumafen to susceptible rats. The results are the mean \pm SD of four rats. The time points are 24, 72, 168, 336, and 504 hours, which correspond, respectively, to 1, 3, 7, 14, and 21 days (B).

respectively (Table 3). Bromadiolone was chosen because it is the SGAR with the greatest difference of half-life between cis- and trans-isomers. Difethialone was chosen because it is the SGAR with the smallest difference of half-life between cis- and trans- isomers. The baits were composed of 93% cis-isomers with 7% trans-isomers or 2% cis-isomers with 98% trans-isomers with 1% trans-isomers for difethialone. The daily consumption of baits was similar for the first 3 days between treatments, with an important decrease on day four. A 100% mortality rate was observed for all of the baits used. Regardless of the bait used, the rats died in an average of 5 days, with mortality observed between days 3 and 9. The concentrations of the AR residues were determined after the death of the rats. The residues of bromadiolone in the liver following the death of the rats were 4-fold reduced using the bait containing principally the cis-isomers compared with the bait containing the trans-isomers of bromadiolone. The residues of difethialone in the liver were only 1.5-fold reduced using the trans-isomers compared with the cis-isomers.

3. Discussion

ARs are crucial for controlling rodent populations. Indeed, trapping can be effective if there is a low density of rodents; however, in the presence of a large population,

Table 1. Pharmacokinetic parameters in the liver after the single oral administration of the cis- or trans-isomers of anticoagulant rodenticides in susceptible rats at a dose of 3 mg.kg⁻¹.

Molecule	AUC (μg.h.g ⁻¹)	t _{1/2} (h) [95% CI]	p-value
DFC cis-isomers	709,4 ± 206,10*	78.33 [64.66-99.33]	p=0.0044
DFC trans-isomers	236,8 ± 87,37*	24.18 [14.67-68.56]	
BDF cis-isomers	4827 ± 830,40*	120.82 [110.74-732.89]	p<0.0001
BDF trans-isomers	1665 ± 242,80*	68.73 [57.28-85.84]	
FLO cis-isomers	1247 ± 176,00	76.73 [60.48-104.91]	p<0.0001
FLO trans-isomers	2713 ± 446,00	177.45 [145.13-228.31]	
BDL cis-isomers	822,7 ± 131,40*	26.89 [19.65-42.55]	p<0.0001
BDL trans-isomers	2733 ± 379,90*	75.62 [64.72-90.96]	
DFT cis-isomers	2410 ± 308,00*	71.60 [44.52-61.78]	p=0.0310
DFT trans-isomers	1894 ± 459,00*	52.93 [46.36-61.67]	

The asterisk (*) denotes a significant difference between two groups (AUC of cis- or trans-isomers in isolated administration) with $\alpha < 0.05$ by using a Mann-Whitney test.

Table 2. Ki values of the VKOR activity catalyzed by warfarin-susceptible rat liver microsomes for the cis- and trans-isomers of each SGAR.

Molecule	Ki of VKOR activity toward cis-isomers (nM)	Ki of VKOR activity toward trans-isomers (nM)
Brodifacoum	18 ± 2	22 ± 1
Bromadiolone	31 ± 2	26 ± 2
Difenacoum	17 ± 1	21 ± 2
Difethialone	27 ± 1	22 ± 1
Flocoumafen	47 ± 2	55 ± 5

To determine the VKOR activity, standard reactions were performed in 200 mM Hepes buffer (pH 7.4) containing 150 mM KCl and 0.5 g.L⁻¹ of microsomal proteins. Each data point represents the mean ± SD of three individual analyses. * Comparison between two groups was performed using the Mann-Whitney test with p<0.05

trapping is timeconsuming and ineffective) [1]. Efficient repellents/attractants and ultrasound seem limited because of the habituation of rodents) [30]. Other chemical products (alphachloralose, cholecalciferol, zinc phosphide, and bromethalin) present major drawbacks, making their use or their approval difficult [31]. Nevertheless, despite the importance of ARs in the management of rodent populations, their use must take into account different issues: the resistance problem associated with the use of FGARs [5, 32] and the secondary poisoning of wildlife essentially associated with the use of SGARs [33]. Therefore, third-generation ARs are urgently needed. The stereochemistry-based solution proposed recently to produce a new and ecofriendly difenacoum [24] seems to be a method of rapidly generating this third-generation of ARs at a reduced cost. Indeed, the 5 commercially available SGARs are a mixture of diastereomeric forms [25, 34–36], which are generally easy to separate and purify. To rapidly generate the TGARs, it is necessary to revisit the SGARs to identify the diastereomer of each SGAR with the smallest half-life and increase its amount in each mixture.

The data found in the scientific literature, in the regulatory documents, allowed the systematic identification of the major and minor diastereomers in the commercial preparation of each SGAR. Flocoumafen, difenacoum, and brodifacoum

are a mixture of 50 to 80% cis-isomers and 20 to 50% trans-isomers [24, 25, 37, 38]; bromadiolone is a mixture of more than 70 of trans-isomers and less than 30% of cis-isomers; and difethialone is a mixture of more than 70 of cis-isomers and less than 30% of trans-isomers [26]. The original reasons for the development of SGARs with such ratios between diastereomers have never been mentioned previously in the literature. We could assume that such ratios resulted from the need to kill the rodent after a single administration when these SGAR were developed [5] by enriching the mix with either the most potent inhibitor or the most persistent pair of diastereomer. Another possibility is that the SGAR synthesis process does not give a racemate but a mixture of isomers enriched in one of them.

To characterize the inhibiting properties, ability to inhibit VKOR activity of cis- or trans-isomers of SGAR was determined. Ki results obtained in this study demonstrated that cis- and trans-isomers of the same SGAR could similarly inhibit VKOR activity (Table 2 and Fig. 6). Therefore, at identical hepatic concentrations, diastereomers of the same SGAR have the same anticoagulant properties when used in warfarin-susceptible rats.

To characterize the tissue persistence, toxicokinetic analyses were done. These toxicokinetic studies were conducted in rats after a single oral administration of the AR. This administration was performed by force feeding to better control the dose and time of ingestion. The same dose was used between SGARs regardless of the LD50 to be able to compare the molecules. Only the concentrations of ARs in the liver could be followed for 21 days because plasma and other tissue concentrations rapidly decrease below the LOQ. Two toxicokinetic parameters were calculated in this study, the AUC and half-life (Table 1 and Fig. 6). The AUC is the integrated area under the hepatic AR concentration versus time curve. The more the AUC is elevated, the greater the importance of the risk of secondary poisoning of wild predators or scavengers. The results obtained herein demonstrated that, in each mixture of SGAR, there is systematically one diastereomeric form with a lower AUC. The ratios of the AUCs between

Table 3. No-choice feeding test with the cis- or trans-isomers of bromadiolone and difethialone.

Bait containing	Number of death	Day of death	Bait (g)	Consumption of		Residues in liver ($\mu\text{g/g}$)		
				cis-isomers (μg)	trans-isomers (μg)	cis-isomers	trans-isomers	% residues / total AR ingested
BDL, 50 ppm 93% cis / 7% trans	6/6	6	44	2025	152	25	32	57
BDL, 50 ppm 2% cis / 98% trans	6/6	5	47	47	2292	236	0	236
DFT, 15 ppm 94% cis / 6% trans	5/5	5	49	44	691	43	4	47
DFT, 15 ppm 1% cis / 99% trans	5/5	5	47	695	7	0	71	71

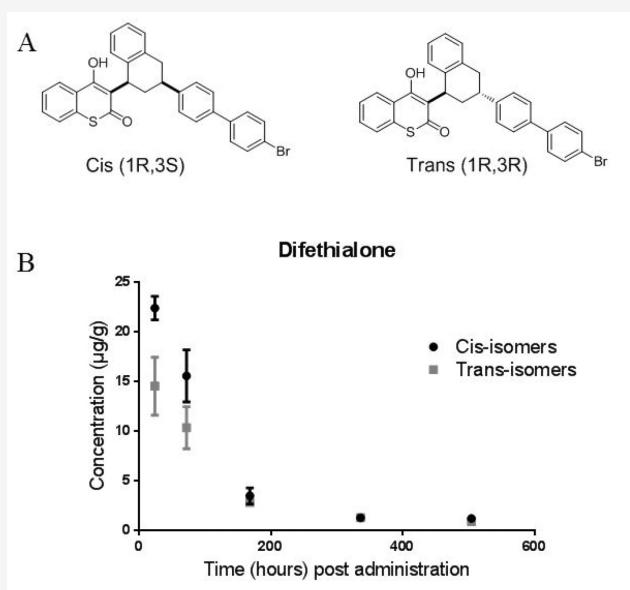


Figure 2. Structures of the cis- and trans-isomers of difethialone (A). The cis- and trans-isomers referenced in the text arise from the alternative orientations of the substituents on the tetralin ring system. Time-dependent concentrations of cis- and trans-isomers of difethialone after the per os administration of, respectively, $3 \text{ mg}\cdot\text{kg}^{-1}$ of purified cis- or trans-isomers of difethialone to susceptible rats. The results are the mean \pm SD of four rats. The time points are 24, 72, 168, 336, and 504 hours, which correspond, respectively, to 1, 3, 7, 14, and 21 days (B).

diastereomers of the same SGAR were 1.5 for difethialone and 3-3.5 for difenacoum, bromadiolone, and brodifacoum. The lowest AUC for all of them was systematically observed for the minor diastereoisomer, except for flocoumafen, for which the lowest AUC was observed for the major diastereomer of the current commercial preparation. Because AUCs could be affected by absorption or clearance, the half-life of diastereomers in the liver was also calculated. Interestingly, the diastereomer with the lowest AUC systematically corresponded to the diastereomer with the lowest half-life in the liver, suggesting a faster elimination of this diastereomer than

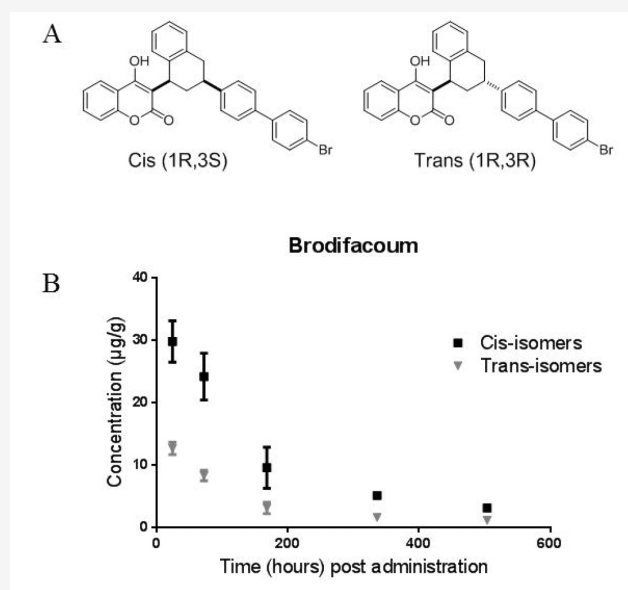


Figure 3. Structures of the cis- and trans-isomers of brodifacoum (A). The cis- and trans-isomers referenced in the text arise from the alternative orientations of the substituents on the tetralin ring system. Time-dependent concentrations of cis- and trans-isomers of brodifacoum after the per os administration of, respectively, $3 \text{ mg}\cdot\text{kg}^{-1}$ of silica gel column-purified cis- or trans-isomers of brodifacoum to susceptible rats. The results are the mean \pm SD of four rats. The time points are 24, 72, 168, 336, and 504 hours, which correspond, respectively, to 1, 3, 7, 14, and 21 days (B).

of the other one. This result was already demonstrated for difenacoum[24].

These AUC and half-life differences between diastereomers of the same SGAR could result in major practical consequences. The composition of each SGAR could thus be systematically modified to enrich the mixture with the less persistent diastereomer to obtain a new AR with the same anticoagulant properties but with decreased tissue persistence. Thus, a new bromadiolone containing more than 70% cis-isomers and a new brodifacoum, difenacoum, or difethialone containing more than 70% trans-isomers may be developed to

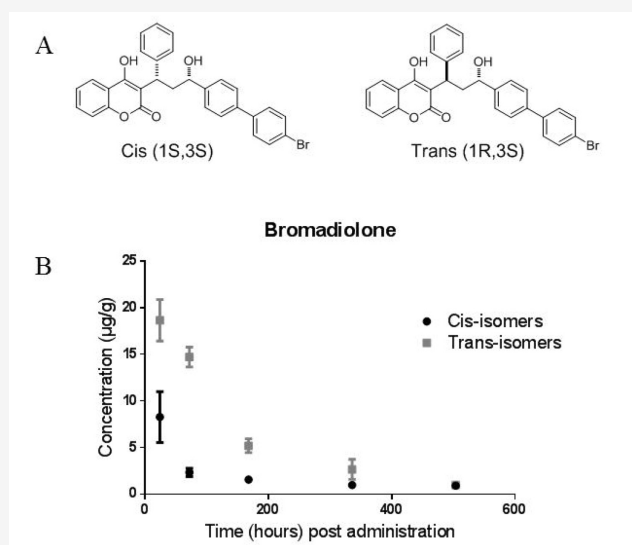


Figure 4. Structures of the cis- and trans-isomers of bromadiolone (A). The cis- and trans-isomers referenced in the text arise from the alternative orientations of the substituents on the tetralin ring system. Time-dependent concentrations of cis- and trans-isomers of bromadiolone after the per os administration of, respectively, 3 mg.kg⁻¹ of purified cis- or trans-isomers of bromadiolone to susceptible rats. The results are the mean ± SD of four rats. The time points are 24, 72, 168, 336, and 504 hours, which correspond, respectively, to 1, 3, 7, 14, and 21 days (B).

create the new generation of anticoagulants.

The benefits will be greater for the current ARs i) containing essentially the most persistent diastereomer and ii) with the most significant differences in half-life between the diastereoisomers. Bromadiolone and difethialone were chosen because bromadiolone is the most favorable example to illustrate the concept (highest difference in AUC and half-life and high percentage of the persistent diastereomer in the current mixture), while difethialone is the most unfavorable example (almost no difference in the AUC between the diastereomers). To illustrate this benefit, the efficiency and persistence of 4 different types of baits were evaluated: a bait containing the current bromadiolone mixture was compared with a bait containing a new bromadiolone mixture containing 93% cis-isomers, and a bait containing the current difethialone mixture was compared with a bait containing a new difethialone mixture containing 94% trans-isomers. The baits were all effective, with 100% mortality obtained after 4 days of exposure at the current commercial concentration for bromadiolone baits or even below for difethialone baits (concentrations of commercial baits containing bromadiolone or difethialone is currently 50 ppm and 25 ppm, respectively). However, the baits enriched with the lowest persistent diastereomer systematically showed a decrease in the quantity of residues in the liver at the time of death of the animals. The reduction of the residues in the liver was greater for bromadiolone enriched

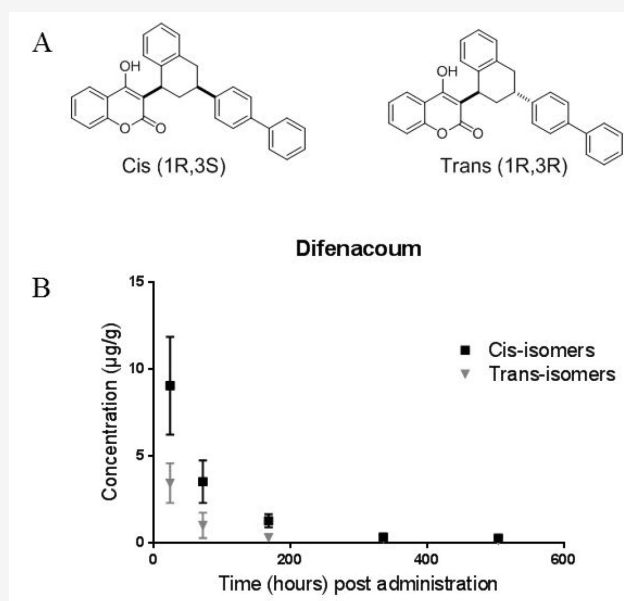


Figure 5. Structures of the cis- and trans-isomers of difenacoum (A). The cis- and trans-isomers referenced in the text arise from the alternative orientations of the substituents on the tetralin ring system. The time-dependent concentrations of the cis- and trans-isomers of difenacoum after the per os administration of, respectively, 3 mg.kg⁻¹ of purified cis- or trans-isomers of difenacoum to susceptible rats. The results are the mean ± SD of four rats. The time points are 24, 72, 168, 336, and 504 hours, which correspond, respectively, to 1, 3, 7, 14, and 21 days (B).

in cis-isomers with a 4-fold reduction of the residues compared with the current bromadiolone. For difethialone, the use of trans-isomers reduced the liver residues by a factor of approximately 2 compared with the current difethialone.

The development of such baits enriched in the less persistent diastereomer could avoid the ecotoxicological risk associated with their use, especially regarding plant protection. These baits would reduce the amount of residues at the time of death of the rodents. Nevertheless, if these dying rodents with reduced residues are eaten by scavengers or predators, the elimination of such an AR could also be accelerated in the liver of scavengers or predators. Therefore, the ecotoxicological gain could be at both levels, and these TGARs would be a good solution to avoid environmental problems associated with pest management.

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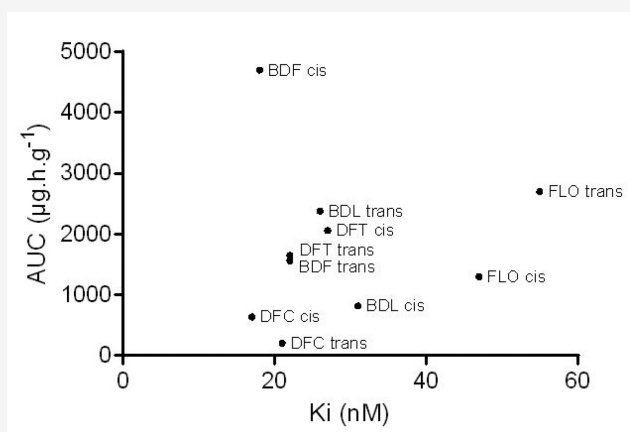


Figure 6. 2D-representation of the biological properties of cis- and trans-of SGAR.

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5.3.2.2 Article :

Comparative biological properties of the four stereoisomers of difethialone, a second-generation anticoagulant rodenticide, in rats : development of a model allowing to choose the appropriate stereoisomeric ratio

Pharmacokinetic and inhibition potency of the Four stereoisomers of difethialone in rats–Development of modeling allowing the choice of appropriate stereoisomers ratio

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Abstract

The current management of rodent pest populations is based on second-generation anticoagulant rodenticides (SGAR). These molecules, of which difethialone is part, are much more efficient than the first generation. Nevertheless, this efficiency comes with a major drawback, SGARs are tissue-persistent that increases the exposure of rodent predators to them. According to its chemical structure, difethialone has four stereoisomers, whose specific inhibition potency and pharmacokinetic have never been described and might be useful to design new eco-friendly rodenticides. The study aimed to investigate the ability to inhibit anticoagulant target enzyme (VKORC1) and the pharmacokinetics in rats of the four difethialone stereoisomers in rats. We show that stereoisomers are all highly efficient to inhibit VKORC1 activity, but they have distinct initial half-life with 6.0h, 25.4h, 69.3h and 82.3h for respectively E4-trans, E2-cis, E1-trans and E3-cis stereoisomer. These results open the way of the development of eco-friendly and efficient rodenticide by mixing some of these stereoisomers. Preferential incorporation of the E4-trans stereoisomer (high inhibitory VKORC1 potency, relatively shorter liver half-life) into difethialone rodenticides baits might produce a more eco-friendly product than current commercially-available difethialone formulations. In addition, we put forward modeling to help design bait according to the circumstance of use (presence of non-target species, food competition, etc) by modulating the theoretical AUC and the theoretical concentration of the product at the death of the rodent pest. Thus, this modeling might allow to diminish the use of laboratory animal in assay.

Keywords

Modeling — Difethialone — Rodents

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Introduction

The use of second generation anticoagulant rodenticides (SGAR) is the cornerstone of the management of the rodent pest population. Indeed, as the first-generation anticoagulant rodenticides (FGARs), SGARs have a delayed action avoiding food aversion due to the association by rodents of bait consumption and death of other rodents, if this occurs quickly after bait consumption. In addition, SGARs like FGARs have an efficient and safe antidote, vitamin K1. A considerable advantage over FGAR, SGAR molecules are also efficient on rodents carrying *Vkorc1* mutations leading to resistance to

vitamin K anticoagulant, this is the main reason for the development of these molecules. However, this advantage comes with a dark side, SGARs are tissue-persistent. This last point is a major concern today, considering the ecotoxicology of the SGAR. Indeed, many studies have pinpointed an exposure of rodent predators to SGAR [1–6]

In the two last years, significant progress has been made in the development of new AR. It is based on the stereochemistry of SGAR and more specifically on the modification of cis/trans diastereoisomers proportions. Indeed, for the same AR molecule, both diastereoisomers exhibit always different half-life compared to the other one while keeping their inhibitory potency [7, 8]. Moreover, a recent ecological study found that only trans-bromadiolone is involved in the poisoning of non-targeted species whereas the rodent bait contained both diastereoisomers [9, 10]. However, considering difethialone, the diastereoisomers approach failed. The initial half-lives of both diastereoisomers are not sufficiently different. To go through the diastereoisomers concept and to improve again the efficiency and the eco-compatibility of rodenticides, we have separately studied properties of the two enantiomers of each diastereomer of difethialone.

This study opens an innovative way to improve difethialone by producing a new mix of the four stereoisomers instead of the mix currently used in the different formulations containing difethialone, the latter being composed of more than 90% of cis-isomers. This new approach implies to study the pharmacokinetics of the four molecules of difethialone to describe their respective elimination kinetic and inquire about the possibility for improved ecotoxicology. Then, their ability to inhibit VKORC1 activity has to be confirmed as equivalent to actual formulation to validate their efficacy.

The context of rodent pest management can widely differ according to the prevalence of resistant phenotype among rodent populations, the availability of palatable foods that can reduce the interest for the baits or even the presence of non-target species or predators. Hence, it may be interesting to increase or decrease the persistence of formulated products to achieve the best compromise between safety and efficiency appropriate to each ecosystem taking into account resistance, non-target species presence, a food competition, and the environment. Nevertheless, how the choice the mixes to test for each situation among the wide range of mixes that can be produced with four stereoisomers? It is fundamental to rationalize the choice of mix composition by mathematic modeling in order to reduce laboratory animal use in new rodenticide research and homologation tests. Thus, the end of this study proposes a modeling approach to optimize rodenticide formulation before animal testing.

1. Material and methods

1.1 Chemicals

Difethialone with the diastereoisomers ratio of 45% of cis-isomers (corresponding to the racemic mixture of 1S,3R and 1R,3S isomers) and 55% of trans-isomers (corresponding to

the racemic mixture of 1S,3S and 1R,3R isomers) and purity above 98% was provided by Liphatech (Pont de Casse, France). The ratio was confirmed by reversed-phase LC-MS/MS [11]. Individual stereoisomers of difethialone were provided by Liphatech by separation of the stereoisomers contained in difethialone mixture by the chiral chromatography method described below.

HPLC-grade acetonitrile and methanol, acetone, dichloromethane and hexane for analysis were supplied by Merck (Darmstadt, Germany). The cartridges used for solid-phase extraction were Oasis® HLB (Hydrophilic-Lipophilic-Balanced) (1mL) purchased from Waters (Milford, Massachusetts, USA). Isoflurane® and vitamin K1 were from Alcyon, (Miribel, France). Vitamin K1 was converted to vitamin K epoxide according to the method described by Tishler et al. [12]. Purity was estimated by LC/MS using an analytical standard (Cayman Chemical, MI, USA) and was higher than 99%. HPLC grade water was prepared using a Milli-Q plus system, (Millipore, Saint-Quentin en Yvelines, France) and used for preparation of HPLC eluents.

1.2 Animals

Eight-week-old male OFA-Sprague Dawley rats (each weighing 175-200 g) were obtained from a commercial breeder (Charles Rivers, l'Arbresle, France) and were acclimated for a minimum period of 7 days. The rats were housed three per cage under a constant photoperiod (12h of light per day) and ambient temperature ($20^{\circ}\text{C} \pm 2$). Animals were kept in standard cages (Eurostandard, Type IV, Tecniplast, Limonest, France), and received standard feed (Scientific Animal Food and Engineering, reference A04) and water ad libitum.

1.3 Pharmacokinetic study

Experimental research on the rats was performed according to an experimental protocol following international guidelines and with approval from the ethics committee of the Veterinary School of Lyon (authorization n°201704190941578).

Male OFA-Sprague Dawley rats received through per os administration of 3.4 mg/kg of difethialone composed of 45% of cis- and 55% of trans-isomers dissolved in 10% DMSO and 90% vegetable oil. Rats were maintained in life by daily subcutaneous administration of vitamin K1 (5 mg.kg⁻¹). 4, 9, 24, 48, 120, 168 and 216 hours after difethialone administration, 3 rats were anesthetized with isoflurane and blood was taken by cardiac puncture into citrated tubes. Finally, rats were euthanized with CO₂ and the liver of each rat was immediately collected and stored at -20°C until analysis.

1.4 VKOR activity essay and kinetics

Liver microsomes were prepared from fresh livers of Sprague Dawley rats by differential centrifugation according to the protocol described by Hodroge et al.[13]. Then, the microsomes are pooled from five individuals. Microsomal vitamin K epoxide reductase (VKOR) activity was assayed according to the protocol described by Hodroge et al. [13, 14]. The inhibiting effect of the silica gel column-purified cis- or trans-isomers

of difethialone was evaluated by the determination of K_i after the addition of various concentrations of the anticoagulant to the standard reaction in the presence of increasing amounts of vitamin K epoxide (from 0.001 to 0.2 mM) using anticoagulant concentrations from approximately 0.05 to 20 K_i . Three replications were performed for each stereoisomer.

1.5 Dosages

Difethialone stereoisomers are dosed as described in Fouré et al. [15]

1.6 Data analysis

The VKA elimination pharmacokinetics are composed of two distinct phases, with two different half-lives, an initial phase during a few days with a half-life of the order of some hours [8] and a terminal-phase may last several months with a half-life of the order of some days considering molecule [16]. Currently, the underlying mechanism is not understood. Considering ecotoxicological and efficacy studies, only the initial half-life is relevant as rodents die between five to ten days. Thus, the initial half-life determines part of the molecule's effectiveness in killing rodents and the remnant concentration that can be consumed by a predator. The terminal half-life is only observed when animals survive (often experimentally by co-administration of vitamin K). Thus, in this study, we only consider the initial half-life of the difethialone stereoisomers. Half-lives are calculated for each stereoisomer with all values from the time to peak to 216h.

Statistical analyses were performed with R v. 3.1.2. Results were expressed as mean values \pm SD or fitted value and confidence interval. Constant of inhibitions or regression curve parameters were compared with Fisher's test. Areas under the curves are compared with a Student's test corrected by Bonferroni correction.

2. Results

2.1 Inhibiting activity of the four individual stereoisomers of difethialone

Prior to our study, the constant of inhibition (K_i) of each stereoisomer was evaluated in vitro on rat liver microsomes expressing wild type VKORC1 (Sprague Dawley rats), in order to confirm their action on vitamin K regeneration cycle. Values are represented in figure 1. Even though, the K_i of E2-cis stereoisomer was significantly greater than the three others ($P < 0.05$) with a K_i about twice as high as the others, all K_i were in range between 22.37 and 51.98 nM comparable to those of effective molecules used in the field. As an example, the K_i value of warfarin, which is ineffective in the field, is 500 nM.

2.2 Determination of the pharmacokinetics of the four individual stereoisomers of difethialone

The first step of our study of the four stereoisomers of difethialone was to compare their respective pharmacokinetic parameters in rats. At $t=0$ h, 3.4 mg/kg of difethialone has been

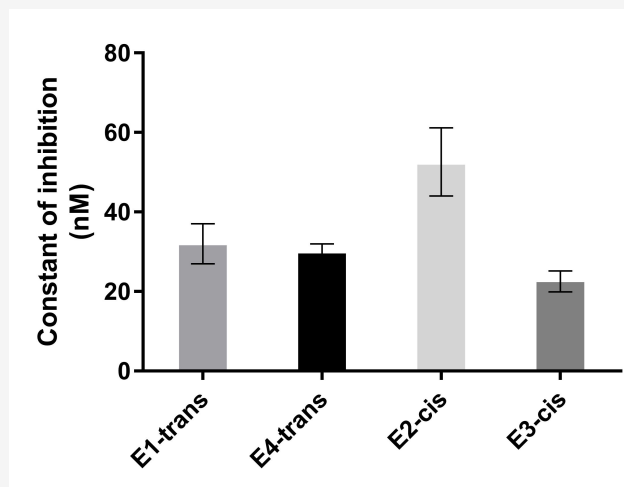


Figure 1. Constant of inhibition of each difethialone stereoisomer for wild Vkorc1 from liver microsomes of laboratory rats. Values are presented with 95% confidence interval. E2-cis K_i is significantly higher than those of three other difethialone stereoisomer ($P < 0.05$).

administered to each rat, then at 4, 9, 24, 48, 120, 168 and 216 hours a group of three rats was euthanized and their liver was collected. The hepatic difethialone concentrations of each stereoisomer versus time are shown in Figure 2 A, B, C and D for respectively the trans isomers (E1-trans and E4-trans) and the cis-isomers (E2-cis and E3-cis). The pharmacokinetic parameters of each stereoisomer were reported in Table 1. To calculate the initial half-life of each stereoisomer concentration we used only values after the peak concentration time in an exponential decay model. The peak concentration time occurred at 9h for E1-trans, E3-cis and E2-cis stereoisomers and at 4h for E4-trans stereoisomer.

Half-life of the E4-trans stereoisomer (6.0 h) is significantly lower than the one of the three others ($P < 0.0001$). Likewise, E2-cis half-life is significantly lower compared to E1-trans and E3-cis stereoisomers ($P < 0.05$). Considering the area under the curve, stereoisomer AUCs are significantly different between them ($P < 0.001$).

2.3 Development of a pharmacokinetic model able to predict the half-life of a mixture

Finally, we tested the ability of a simple model made with elimination constants previously estimated to predict the difethialone liver concentration versus time after administration of a stereoisomers mix. In order to build this model, we assume that (i) the absorption constant of each of the four stereoisomers is common (ii) after 24h post oral administration, the variation of liver difethialone concentration only depends on the elimination rate (iii) the elimination rate of each stereoisomer is not influenced by the presence of the other stereoisomer. Thus, the equation of the difethialone concentration versus time can be defined as in equation 1. In this equation, f_1 to f_4 are the fractions of each stereoisomer

Stereoisomer	Elimination coefficient (h ⁻¹)	Initial Half-life (h)	R ²	Area under the curve (h x μg/g of liver)
E1-trans	0.009 [0.006-0.014]	69.3 [48.2-123.4]	0.793	458.1 [381.1-535.2]
E4-trans	0.114 [0.083-0.146]	6.0 [4.8-8.3]	0.959	54.5 [43.5-65.6]
E2-cis	0.027 [0.015-0.039]	25.4 [17.5-46.1]	0.871	226.9 [166.1-287.6]
E3-cis	0.008 [0.004-0.012]	82.3 [57.7-143.8]	0.777	952.9 [806.2-1,099.8]

Table 1. Pharmacokinetic parameters of each difethialone stereoisomer liver concentration after a single per os administration of 3.4 mg/kg of difethialone composed of 45% of cis- and 55% of trans-isomer. Values are presented with 95% confidence interval.

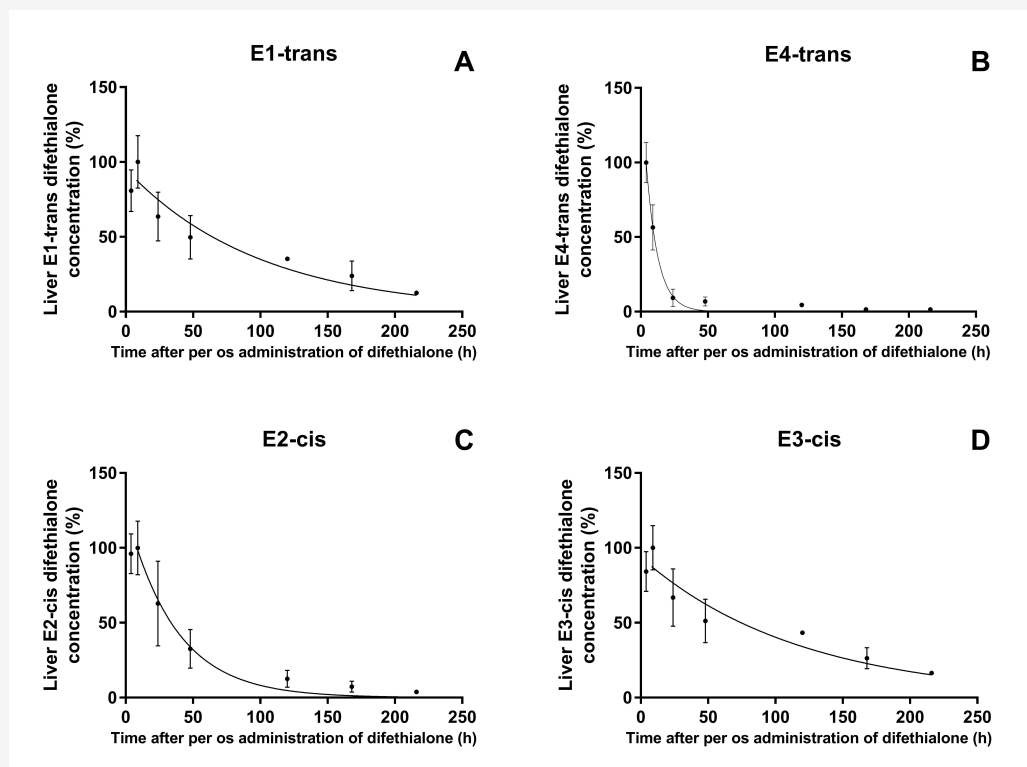


Figure 2. Difethialone stereoisomer liver concentration over time after a single per os administration of 3.4 mg/kg of difethialone composed of 45% of cis- and 55% of trans-isomers. Values are represented as mean of concentrations ± standard deviation, 100% is set as the max mean of each stereoisomer. Parameters of fitting curves are presented in table 1.

in the product administered per os, and C₀ is the theoretical concentration if the mix would have been administered by intravenous. To verify this model's ability for liver concentration prediction of stereoisomers mix, we used two pharmacokinetic data published in [8]. One of these mixes mainly contains trans stereoisomers: 47% of E4-trans and 47% of E1-trans with 3% of each two other stereoisomers, and a cis mix that is a racemic composition of E3-cis and E2-cis stereoisomers. In this validation, only the 24h concentration is used to fit the C₀ value. Then, fitting indicators were calculating only with other point concentrations (figure 3). Models have R_p² of 0.9184 for trans mix and of 0.9100 for cis mix.

$$C(t) = C_0 * (f_4 * e^{-0.1147*t} + f_1 * e^{-0.009998*t} + f_3 * e^{-0.008419*t} + f_2 * e^{-0.0273*t})$$

1. Equation predicting the concentration of difethialone in rat liver as a function of the time. The constants f₁, f₂, f₃ and f₄ are respectively the fraction of E1-trans, E2-cis, E3-cis and E4-trans in the product.).

3. Discussion

The management of rodent populations is based on the use of anticoagulant rodenticides. Indeed, physical methods as

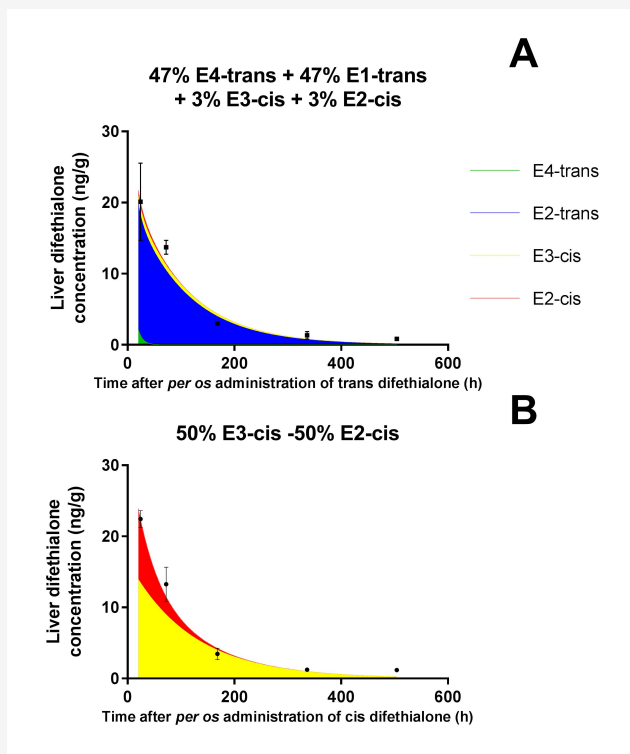


Figure 3. Liver concentration of difethialone over time after administration of two difethialone mixes: (A) trans mix with 47% of E4-trans, 47% of E1-trans, 3% of E3-cis and 3% of E2-cis and (B) cis mix with 50% of E3-cis and 50% of E2-cis. Points represent mean of experimental values \pm standard deviation. Green, blue, yellow and red areas represent respectively the proportion of E4-Trans, E1-trans, E3-cis and E4-trans stereoisomers liver concentrations obtained with the model. The R^2 of the model are 0.9184 for trans mix and 0.9100 for cis mix.

trapping are limited to small invasions of rodents [17]. In addition, there are many problems with the effectiveness of other chemical rodenticides. For example, due to their rapid action, their use results in an association between the bait and its toxicity, leading for the rodent to an aversion to the consumption of the bait [18]. Furthermore, in the case of human or animal intoxication, other chemical rodenticides have no antidote, unlike anticoagulant rodenticides [18]. The FGARs are few remnant, but also hardly efficient when many rodent populations with a resistant mutation of *Vkorc1* have emerged in the pas [19]. Conversely, since they were discovered, the SGARs have been categorized as highly tissue persistent and effective in a single administration at lower concentrations compared to FGARs, including against rodents with resistant mutation [13]. These characteristics have established the renown of second-generation molecules in the management of rodent populations. Nevertheless, their reluctance is both an advantage for their efficacy and a drawback for their ecotoxicology. The new approach proposed recently by

Damin-Pernik et al. [7, 8], based on the diastereomers of existing SGARs to reduce their ecotoxicology, is here expanded to the four stereoisomers of difethialone.

The capacity of each difethialone stereoisomer to inhibit the wild rat *Vkorc1* enzyme activity has been determined in vitro on a fresh rat liver microsome. Each stereoisomer inhibits the enzyme with a K_i within a range of 20 to 60 nM. These constant of inhibition are similar to those of the SGARs and chlorophacinone which are used in the field. Indeed, K_i of difenacoum, chlorophacinone and bromadiolone are respectively of 30, 40 and 70 nM [13].

Our results show that each stereoisomer has its own initial half-life, which is included between 6.0h and 82.3h. Thus, difethialone E4-trans stereoisomer has a comparable half-life to warfarin enantiomers. Indeed, E4-trans half-life is 6.0h compared to 7.6h and 11h half-life of both warfarin enantiomers [20]. However, the four difethialone stereoisomers reveal a lower K_i than warfarin (500 nM)[13]. Regarding E2-cis and E3-cis, which compose the commercial difethialone currently used, E3-cis half-life is three times greater than E2-cis half-life. This important difference may explain the biphasic elimination described when commercial difethialone liver elimination was studied [16] E2-cis would be early eliminated, constituting the first elimination stage, followed by E3-cis late efflux leading to the second elimination step. These results push us toward questioning the assessment of composed products half-life. Indeed, the half-life of the stereoisomers mix depends on the chosen range of measurement. Thus, for the first time, the global half-life of a mix is linked to the ratio and the half-life of the less remnant stereoisomer. Consequently, as the global half-life of a stereoisomer mix is increasing over time, the mix should not only be assessed by its final half-life. Indeed, with this unique indicator, it is not possible to estimate what is the amount of product remaining in the rodent when it dies and being at risk for the environment. Moreover, if the only aim is to reduce the persistence of the product, the choice of E4-trans stereoisomer is obvious since it is the less remnant difethialone stereoisomer (Figure 2 and Table 1). However, the area under the curve of such products would be so low that its efficiency would be marred.

In our opinion, two new pharmacokinetic indicators have to be designed in order to assess both the presumptive efficacy and the presumptive persistence of an anticoagulant. First, to assess the presumptive efficacy, we propose to consider the relative AUC between 24h and 120h. The goal of this indicator is to confirm that, if rodents eat the target concentration of bait, the AUC within 5 days after the ingestion is sufficient to conduct to its death. The second one is the percent of the 24 h concentration remaining at 120h after administration. Indeed, the death of rodents occurs most of the time from this fifth day after administration. Consequently, this indicator should be minimized and the AUC should be maximized.

We created a model based on the half-lives of each stereoisomer (equation 1). This model has been validated with experimental data of pharmacokinetic of difethialone

products with different stereoisomers ratios. Thus, we were able to simulate the elimination pharmacokinetics of different hypothetical difethialone mixes. These simulations allowed us to determine the best theoretical mix composition. While such simulations do not replace pharmacokinetic or efficacy studies, they can help to reduce the number of tests on animals, in accordance with the 3R principle. Once the constraints of efficacy and ecotoxicity have been defined, which is equivalent to defining a minimum AUC (efficacy) and a maximum anticoagulant concentration at the time the rodent pest begins to be symptomatic and die (ecotoxicity). The model makes it possible to target the best stereoisomer mixtures to be tested, which allows sparing laboratory animals by avoiding to test inefficient bait or bait with an ecotoxicity not adapted to the intended use.

To preselect some mixes with our model, we determined the best stereoisomers composition to maximize the AUC, according to a fixed persistence of difethialone at 120h. The results of these simulations are presented in Figure 4. According to this diagram, it is possible to design rodenticides with a wide range of AUC and persistence. The diagram pinpoints the importance of E2-cis stereoisomer in the design of new products. Indeed, with combinations of E2-cis and one of the trans stereoisomers (E1-trans or E4-trans), it is possible to design a large range of products adapted to different situations. In a vulnerable environment with few food competition (rodents eat the bait several times), a rodent population with moderate or no resistance might be controlled with a moderate persistence bait (E2-cis and E1-trans). Conversely, to control resistant populations or in environments where a one-shot bait is mandatory (important food competition), the use of more persistent baits would be more relevant to reach or maintain sufficient hepatic concentrations (E4-trans fraction and E2-cis fraction, but still without E3-cis which is really too persistent and even almost not eliminable). Obviously, the level of actions to prevent non-target poisoning has to be in accordance with the persistence of the used product.

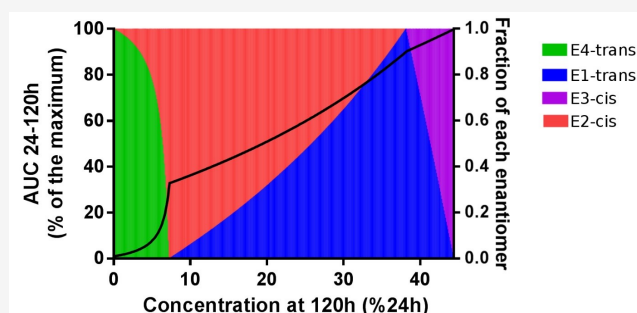


Figure 4. Evolution of difethialone AUC between 24 and 120h (curve and left axis) simulated with the best bait mix of the four difethialone stereoisomers (color area and right axis) for a fixed residual concentration of difethialone at 120h (in percent of the 24h concentration).

However, these kinds of simulation have some limitations and can only be lifted by confirming the efficacy and per-

sistence of the product directly on target species. The first limitation is the conditions of the pharmacokinetics studies, especially the administered dose, limited information is available on the self-induction of AR elimination. If such modulation exists, the pharmacokinetics of AR might be different according to the administered dose. The second main limitation is the behavior of wild rodent regarding to the poisoned bait. All new baits have to be tested in vivo in at least a baits choice feeding efficacy test, to check that the bait palatability is sufficient to ensure an adequate intake of active products in one or more times.

In conclusion, this study shows that four difethialone stereoisomers are able to inhibit the wild *Vkorc1* rat enzyme with the same efficiency. Moreover, the pharmacokinetics of these stereoisomers are widely different which allows the creation of new baits that might assure the efficiency of difethialone and modulate a persistence adapted to the treated environment. Finally, this article presents a model to select better difethialone formulation. The developed model brings a new powerful method to design more efficient ARs and could be applied to other ARs stereoisomers formulation studies.

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5.3.2.3 Article :

Comparative pharmacokinetics of difethialone stereoisomers in male and female rats and mice : development of an intra- and inter-species model to predict the suitable formulation mix

Comparative pharmacokinetics of difethialone stereoisomers in male and female rats and mice: development of an intra- and inter-species model to predict the suitable formulation mix

Antoine Rached^{1,2}, Virginie Lattard*¹, Ambre Fournoux¹, Hervé Caruel², Isabelle Fourel¹, Etienne Benoit¹, Sébastien Lefebvre¹

Abstract

The ecotoxicity of anticoagulants used for rodent pests' management is a major concern, particularly with second generation anticoagulants, which are more persistent in the body of rodents and therefore more likely to cause secondary exposure in their predators. One of the solutions envisaged to mitigate this risk is to use stereoisomers of these anticoagulants, each of which has particular pharmacokinetics. However, the few studies published to date have considered only one species and one sex. Here, we study the pharmacokinetics of the 4 stereoisomers of 3.4 mg/kg of difethialone in rats (*Rattus norvegicus*) and 3 mg/kg in mice (*Mus musculus*) in both sexes and propose a model to choose the optimal stereoisomer efficacy/ecotoxicity mixture for the management of all these animals. Our results show that while the most persistent stereoisomer (E3-cis) is common to both species and sexes, the pharmacokinetics of the other stereoisomers show marked differences between sexes and species. Thus, the area under curve (AUC) of E4-trans in male rats is four times lower than in females or mice, making it a priori unusable in male rats. Conversely, our modeling seems to show that the E1-trans stereoisomer seems to offer the best compromise AUC persistence. In conclusion, we highlight that studies on anticoagulants must necessarily integrate research on the effect of gender and species both on efficacy and with regard to the ecotoxicity of these molecules.

Keywords

Modeling — Difethialone — Rodents—Ecotoxicology—Anticoagulant rodenticide

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Introduction

Anticoagulant rodenticides are highly effective for rodent control in agriculture and urban environment because of their delayed action that prevents rodents from developing food aversion. The first generation of anticoagulant rodenticides (FGARs) was developed and used from the 1950s. Unfortunately, their efficacy was rapidly limited by resistance phenomena associated with mutations in the *Vkorc1* gene, present in some rodent populations.

To overcome this resistance, a “second generation” of anticoagulant rodenticides (SGARs) -including difenacoum, bromadiolone, flocoumafen, brodifacoum and difethialone- was developed. This generation is more toxic (lower concentration to induce mortality) and presents greater duration of action (longer persistence in target organ)¹⁻⁶. This group of molecules was also referred to as superwarfarins and long-acting anticoagulant rodenticides. Despite these properties, resistance to some SGARs has been reported in different regions due to their widespread use^{7,8}. In addition, intoxication

of non-target animals is the most serious drawback of SGARs. Indeed, direct access of domestic or wild animals to the poisoned bait or repeated consumption of poisoned rodents by some predators and scavengers lead to fatal poisonings^{9–11}. Although ingestion of intoxicated rodents does not lead systematically to the death of the animal, many predators and scavengers have been shown to be chronically exposed to SGARs due to the long tissue-persistence of these molecules and the consequences of this exposure on populations are not fully understood^{12,13}.

To overcome this drawback, several studies have suggested the development of ecological anticoagulant rodenticides based on the stereochemical principle. Thus, since the SGARs are all composed of 4 stereoisomers, it has been shown that these stereoisomers associated by couple of trans- or cis-isomers presented different biological properties within the same SGAR. Indeed, they presented a different hepatic half-life with systematically a couple of stereoisomers eliminated more rapidly than the other, while their inhibitory efficacy remains comparable^{14,15}. This difference is clear for bromadiolone, difenacoum and brodifacoum: the systematic use of the least persistent diastereoisomeric pair would reduce the ecotoxicity associated with their use.

Surprisingly, this difference is less clear for difethialone, which is the most recent SGAR. This limited difference prompted Lefebvre et al. to investigate the pharmacokinetic properties of the two enantiomers of each diastereomer of difethialone. In contrast to the diastereomers, the four stereoisomers individually have different liver half-lives in male rats and reformulation of the active ingredient in favor of certain stereoisomers would improve its ecotoxicological profile. A mathematical modeling approach based on liver half-lives has been proposed to optimize the formulation of rodenticide mixtures¹⁶.

Could this bait improvement be generalized to female rats, or even mice whose populations are also managed primarily with anticoagulant rodenticides? This seems crucial as both the Norway rat (*Rattus norvegicus*) and the house mouse (*Mus musculus*) are commensal rodents. They both live near humans, even though their habitat is often separated because of the aggressiveness of one towards the other. The half-lives of current anticoagulants have been described as very different between species¹⁷. Optimizing the composition of an active ingredient must therefore take this into account. In addition, as sex differences in response to anticoagulant rodenticides have been demonstrated in rats^{18–21}, this factor must also be considered. Would the composition of a remixed difethialone bait be the same if all of these criteria were considered, or should a compromise be sought?

In this work, we compared first the pharmacokinetics of the four individual stereoisomers of difethialone in male and female rats and mice, and thus we tried to identify the best theoretical mix composition of difethialone stereoisomers to satisfy the need for efficiency of rodenticides while controlling the ecotoxic risk as best as possible.

1. Material and methods

1.1 Chemicals

Difethialone (3-[3-[4-(4-bromophenyl)phenyl]-1,2,3,4-tetrahydronaphthalen-1-yl]-2-hydroxythiochromen-4-one) with a purity above 98 % composed of the four stereoisomers was provided by Liphatech (Pont-du-Casse, France). It was a mixture containing 45 % of the cis-isomers in racemic proportion (1S, 3R and 1R, 3S) and 55 % of the trans-isomers in racemic proportion (1S, 3S and 1R, 3R). Isoflurane and vitamin K1 were purchased from Alcyon, (Miribel, France).

1.2 Animals

Forty male and female, seven-week-old, CD 1-mice and 21 eight-week-old female OFA-Sprague Dawley rats were obtained from a commercial breeder Charles Rivers, (l'Arbresle, France). Animals were housed three per standard cage (Euro-standard, Type IV, Tecniplast, Limonest, France) for rats and five per cage for mice in the institutional animal facility in a controlled environment (ambient temperature 20 ± 1 °C and 12-h light/dark cycle) with access to standard food (Scientific Animal Food and Engineering, reference A04) and water ad libitum. Animals were acclimatized for at least one week prior to use and were maintained in accordance with the European and French legislation guidelines on animal studies.

1.3 Pharmacokinetic study

Experimental research on the rodents was carried out according to an experimental protocol following international guidelines and approved by the ethics committee of the Veterinary School of Lyon (authorization n°201704190941578).

Female OFA-Sprague Dawley rats received through per os administration 3.4 mg/kg of difethialone dissolved in 10 % DMSO and 90 % vegetable oil. Rats were maintained in life by daily subcutaneous administration of vitamin K1 (10 mg.kg⁻¹). 4, 9, 24, 48, 120, 168, and 216 hours after difethialone administration, three rats were anesthetized with isoflurane, and blood was taken by cardiac puncture into citrated tubes. After blood sampling, rats were euthanized with CO₂ and the liver of each rat was immediately collected and stored at -80 °C until analysis.

Data concerning the pharmacokinetic study of difethialone in male rats are taken from our previously published article Lefebvre et al. 2020¹⁶.

Male and female CD1-mice received through per os administration 3 mg/kg of difethialone dissolved in 10 % DMSO and 90 % vegetable oil. Mice were maintained in life by daily subcutaneous administration of vitamin K1 (10 mg.kg⁻¹). 24, 72, 168 and 336 hours after difethialone administration, five mice of each sex were anesthetized with isoflurane and blood was taken by cardiac puncture into citrated tubes. After blood sampling, mice were euthanized with CO₂ and the liver of each mouse was sampled and stored at -80 °C until analysis.

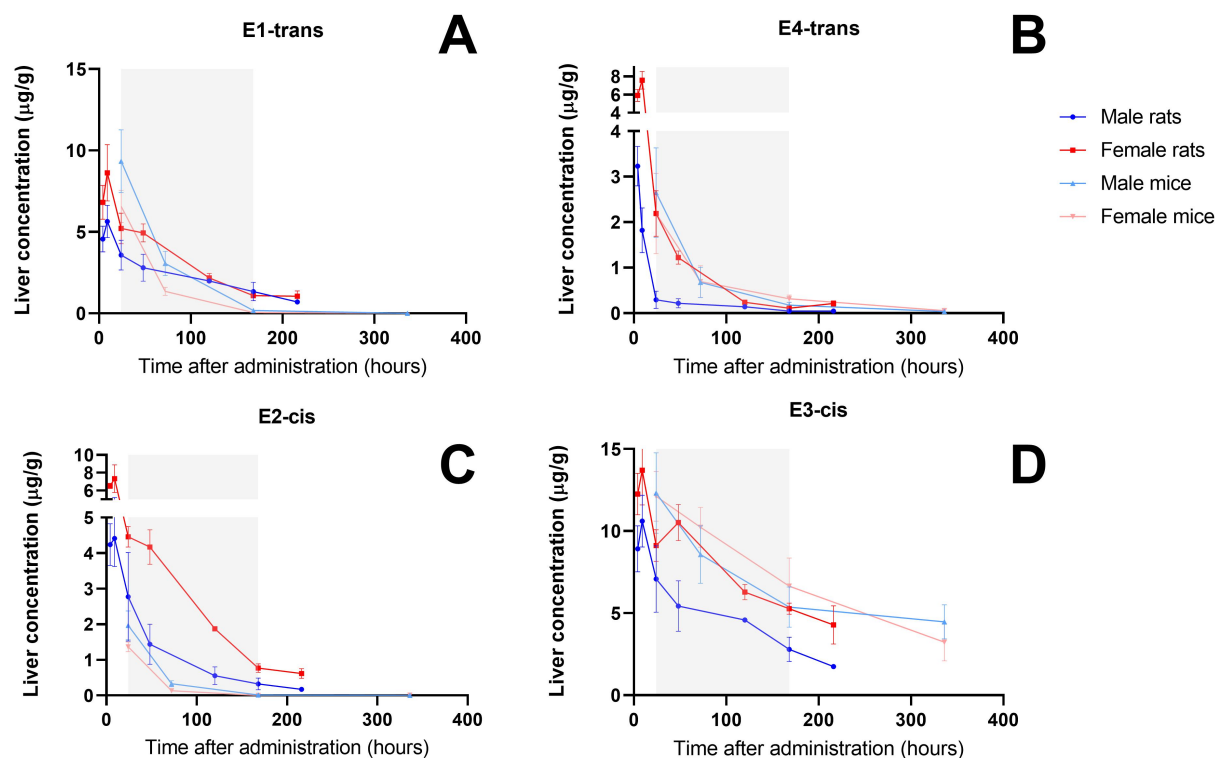


Figure 1. Concentration of each stereoisomer of difethialone over the time after a per os administration of 3.4mg/kg in rats or 3mg/kg in mice of difethialone. Values are presented as mean and standard deviation. The grey area is the one that is analyzed for interspecies comparison and modeling in the rest of the article. Data for male rats are from Lefebvre 2020¹⁶

1.4 Dosages

Difethialone stereoisomers were dosed as described previously in Fourel et al.²².

1.5 Data Analysis

Data handling and statistical analyses were performed with R 4.0.5²³ and the following package PK (1.3.5)²⁴. The non-compartmental analysis was performed by applying the Bailer's method for sparse sampling as described in Nedelman²². Results were expressed as mean values \pm Standard Error or fitted value and Confidence Interval. Wilcoxon test or if relevant Dunn's multiple comparison test was used with $\alpha < 0.05$ in order to statistically compare the results among groups.

2. Results

2.1 General pharmacokinetics of each stereoisomer and effect of sex on pharmacokinetic parameters

Hepatic concentrations of each stereoisomer of difethialone (E1-trans, E4-trans, E2-cis and E3-cis, called according to their order of elution on the chromatographic chiral column and according to their belonging to the diastereoisomeric

couple) versus time are shown in figure 1 A, B, C and D, respectively. Remarkably, the stereoisomer with the highest concentration and the most persistent by far was E3-cis, regardless of species or sex. Considering that the sample times and doses were different among species, the results were first analyzed according to the sex within a species. Tables 1 and 2 present the non-compartmental analysis of the pharmacokinetics of each stereoisomer for rats and mice, respectively. In rats, the AUC_{4-216h} were significantly higher in females than in males for all four stereoisomers as the $AUC_{4h-infinite}$ except for the E1-trans stereoisomer where no significant difference was found. C_{max} was statistically different between males and females for only the E4-trans stereoisomer. T_{max} was also different for the latter stereoisomer with a T_{max} of 4h and 9h in males and females, respectively. The mean residence time (MRT) was significantly lower in females for both trans-stereoisomers and higher for E2-cis stereoisomer.

In mice, significant differences in AUCs were highlighted only for the E1-trans and E2-cis stereoisomers with higher AUCs in males compared to females. These higher AUCs were associated with significantly higher C_{max} in males for both stereoisomers but no statistical differences for MRT.

	Cmax (µg/g)		Tmax observed (h)		AUC _{0-216h} (µg·h/g)		AUC _{0-∞} (µg·h/g)		MRT _{0-216h} (h)	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
E1-trans	5.64±0.57	8.64±1.00	9	9	458±27.1	650±25.1*	660±103	767±25.9	77.9±3.94	68.5±2.06*
E2-cis	4.41±0.46	7.33±0.91	9	9	226±24.17	536±17.2*	252±30.1	606±19.4*	53.2±4.50	64.9±1.39*
E3-cis	10.6±0.91	13.7±1.22	9	9	952±50.5	1538±30.3*	1434±166	2821±177*	82.3±3.23	87.8±2.50
E4-trans	3.23±0.25	7.56±0.56*	4	9	54.5±4.52	219±9.21*	59.5±4.9	226±9.09*	45.6±2.71	39.2±1.39*

Table 1. Pharmacokinetic parameters of the non-compartmental analysis of each difethialone stereoisomer in the liver of rats. Data are presented with standard error. Data for male rats are from Lefebvre et al. 2020¹⁶. *Statistical significant between male and female < 0,05

	Cmax (µg/g)		Tmax observed (h)		AUC _{0-216h} (µg·h/g)		AUC _{0-∞} (µg·h/g)		MRT _{0-216h} (h)	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
E1-trans	9.3±0.86	6.6±0.44*	24	24	469±32.5	264±13.4*	469±32.5	264±13.4*	54.4±2.58	47.4±1.63
E2-cis	1.9±0.18	1.4±0.06*	24	24	73.2±5.16	43.2±20.4*	73.3±5.2	43.6±20.3*	44.0±1.93	39.6±1.65
E3-cis	12.3±1.1	12.1±0.68	24	24	1996±103	2176±117	3429±384	2911±258	148±5.19	137±4.94
E4-trans	2.7±0.44	2.2±0.39	24	24	157±15.9	149±15.5	160±15.9	154±15.8	83.5±5.71	90.3±6.51

Table 2. Pharmacokinetic parameters of the non-compartmental analysis of each difethialone stereoisomer in the liver of mice. Data are presented with standard error. *Statistical significant between male and female < 0,05

	Male Rat			Female Rat			Male Mice			Female Mice		
	MRT _{0-216h} (h)	Half-life (h)	R ²	MRT _{0-216h} (h)	Half-life (h)	R ²	MRT _{0-216h} (h)	Half-life (h)	R ²	MRT _{0-216h} (h)	Half-life (h)	R ²
E1-trans	82.7 ^a	107.6 ^a	0.0064	72.3 ^a	66.09 ^a	0.01049	50.1 ^b	27.53 ^b	0.02518	43.8 ^b	21.13 ^b	0.03280
	77.8-82.0	72.73 - 220.9	0.003137 - 0.009531	70.0-74.6	54.14 - 83.94	0.008257 - 0.01280	47.1-53.0	23.65 - 34.56	0.02006 - 0.02931	41.6-45.8	18.99 - 24.17	0.02867 - 0.03651
E2-cis	65.6 ^a	44.5 ^a	0.01558	71.4 ^a	61.93 ^a	0.01119	41.4 ^b	19.15 ^b	0.03620	35.4 ^b	14.57 ^b	0.04756
	58.0-73.6	31.73 - 77.21	0.008978 - 0.02185	70.0-73.0	52.13 - 77.95	0.008893 - 0.01330	38.9-44.6	16.78 - 23.04	0.03009 - 0.04132	33.0-37.5	13.59 - 17.21	0.04027 - 0.05101
E3-cis	85.5	121.7	0.005696	83.5	152.7	0.004538	81.0	119.6	0.005796	84.4	165.7	0.004182
	81.1-90.6	78.14 - 259.2	0.002675 - 0.008871	81.5-88.4	110.0 - 236.8	0.002928 - 0.006304	76.8-85.0	88.81 - 186.7	0.003713 - 0.007805	80.3-88.4	123.6 - 279.3	0.002481 - 0.005610
E4-trans	76.1	63.7	0.01088	55.1	31.44	0.02205	31.92 ^c	0.02171	0.8159	45.69	0.01517	0.7233
	70.5-83.4	38.15 - 144.8	0.004788 - 0.01817	53.0-57.6	27.11 - 37.30	0.01858 - 0.02557	47.5-59.3	25.89 - 47.39	0.01463 - 0.02677	57.6-69.3	34.53 - 71.87	0.009645 - 0.02007

Table 3. MRT and half-life of each difethialone stereoisomer according to species and sex between 24 and 168 h after a per os administration of difethialone.

2.2 Non-compartmental analysis of the four individual stereoisomers of difethialone according to species

Since the 24-hour to 168-hour period of the pharmacokinetics (i.e., corresponding to 24, 72, 168-hours sample times for mice and 24, 48, 120, and 168-hours sample times for rats) was common among the four groups, the analysis of the results was continued by considering only this relevant part in order to compare the efficacy and the ecotoxicity of difethialone between species and gender. Because the amount of difethialone administered per weight was higher in rats than in mice, only the non-inferiority of the concentrations or AUC results obtained in rats versus mice (i.e., intra- or inter-gender comparison) was statistically analyzed. Results are presented in figure 2.

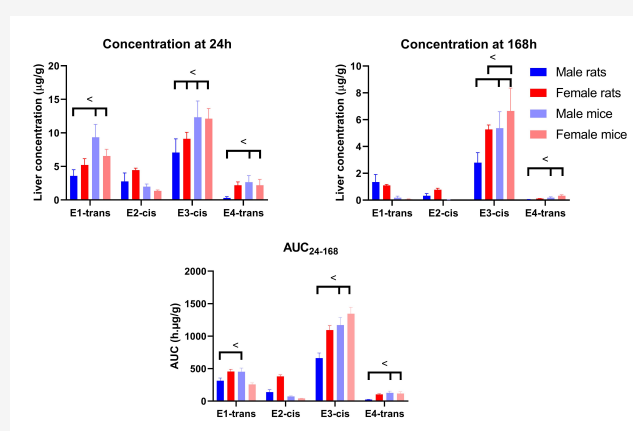


Figure 2. Parameters of the non-parametric pharmacokinetic between 24 and 168h for each stereoisomer according to the sex and the species. For each stereoisomer, for all pairs (rats-mice) of histograms designated < symbol, the values of the mice are significantly higher than those of the rat

Considering the E1-trans stereoisomer, both male and female mice (9.3 and 6.6 $\mu\text{g/g}$, respectively) had higher liver concentration than male rats (3.6 $\mu\text{g/g}$) 24 hours after the difethialone administration. The significance of difference was not tested at 168h as the E1-trans concentration was not tested. Indeed, at this time the rats had hepatic concentrations of the order of the microgram (0.7 $\mu\text{g/g}$ for males and 1.1 $\mu\text{g/g}$ for females) when the mice had a hepatic concentration of the order of a hundred of nanograms (170 ng/g for males and 60 ng/g for females). Moreover, only the $\text{AUC}_{24-168h}$ of male mice was higher than that in male rats (453 $\mu\text{g}\cdot\text{h/g}$ versus 314 $\mu\text{g}\cdot\text{h/g}$ respectively).

For the E3-cis stereoisomer, the liver concentrations 24 hours after the administration were significantly greater in mice (both sexes, 12.3 $\mu\text{g/g}$ in males and 12.1 $\mu\text{g/g}$ in females) than in rats (both sexes, 7.1 $\mu\text{g/g}$ in males and 9.1 $\mu\text{g/g}$ in females). However, at 168 hours if male and female mice liver concentrations (5.4 $\mu\text{g/g}$ and 6.6 $\mu\text{g/g}$ respectively) were significantly higher than that of male rats (1.7 $\mu\text{g/g}$), only female mice concentration was greater than that of female rats

(4.3 $\mu\text{g/g}$). $\text{AUC}_{24-168h}$ of male and female mice (1170 $\mu\text{g}\cdot\text{h/g}$ and 1346 $\mu\text{g}\cdot\text{h/g}$ respectively) were twice as high as that of male rats (662 $\mu\text{g}\cdot\text{h/g}$), however no significant difference was highlighted with female rats (1093 $\mu\text{g}\cdot\text{h/g}$).

Considering E2-cis stereoisomer, concentration at 24 hours and 168 hours and $\text{AUC}_{24-168h}$ were lower in mice than in rats so they have not been tested for a significant difference. However, it is noteworthy that the concentration at 168h was in the order of a few hundred nanograms for both male and female rats (0.17 $\mu\text{g/g}$ and 0.61 $\mu\text{g/g}$ respectively), versus a few nanograms for both male and female mice (0.015 $\mu\text{g/g}$ and 0.003 $\mu\text{g/g}$ respectively).

Finally, considering E4-trans stereoisomer, the liver concentrations at 24 hours of both male and female mice (2.7 $\mu\text{g/g}$ and 2.2 $\mu\text{g/g}$ respectively) were significantly higher than that of male rats (0.29 $\mu\text{g/g}$). The same kind of differences were observed for the concentration at 168 hours with values of 0.18 $\mu\text{g/g}$ for male mice and 0.32 $\mu\text{g/g}$ for female mice that were both significantly higher than the value of 0.046 $\mu\text{g/g}$ obtained for male rats. The $\text{AUC}_{24-168h}$ with values of 127 $\mu\text{g}\cdot\text{h/g}$ for male mice and 117 $\mu\text{g}\cdot\text{h/g}$ for female mice were also both significantly higher than the 23 $\mu\text{g}\cdot\text{h/g}$ for male rats.

In addition to the MRTs, the half-lives of each stereoisomer was determined between 24h and 168h for each species-sex pair (Table 3 and figure 3). Statistical differences were observed between rats and mice considering the MRTs and half-lives of E1-trans and E2-cis. For these two stereoisomers, MRTs and half-lives were shorter in mice than in rats for both sexes (two times more regarding the half-lives), while no difference was pinpointed for others (table 3). Even if the half-life of E4-trans stereoisomer (63.7h) in male rats seemed to be higher than that for female rats or mice, the difference was not statistically significant.

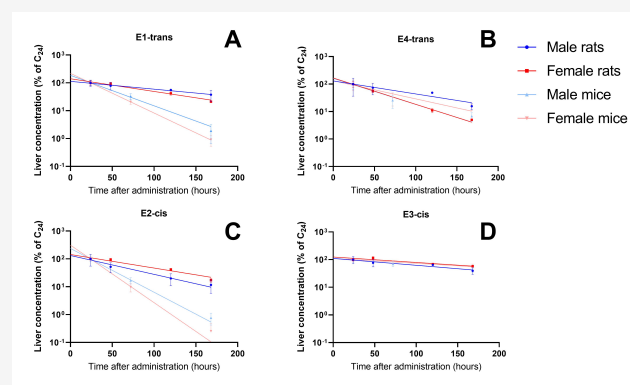


Figure 3. Linearization of the concentration of each stereoisomer as a function of time by using a logarithmic scale base ten. Values are presented in percent of the concentration at 24h post-administration as mean and standard deviation

3. Discussion

In our previous article, that included only the data of male rats, we have proposed a model to predict the efficacy and the ecotoxicity of different mixes of difethialone to optimize the mix of difethialone by increasing its AUC and thus their efficacy while decreasing their persistence and thus their ecotoxicity. The results of this previous study showed that a mixture of E1 and E2 could achieve a good compromise between these two opposing criteria¹⁶. Considering the results of the current study, is this mix still a good opportunity if we extend the use of such a bait to female rats or mice of both sexes? Should we evolve the model to adapt it to the constraints of sex and species? Is there an optimal mix for managing rat populations on the one hand, and mouse populations on the other hand, or even the infestation of both populations?

This study was conducted to answer these questions and to extend the first proposed model to the different rodent populations that can be managed with anticoagulant rodenticides (ARs). Nevertheless, two major limitations of this study must be mentioned, the dose of difethialone administered orally to rats and mice at T0 slightly differs (3,4 mg/kg for rats and 3 mg/kg for mice) and that the sampling times are different for each species. These two aspects of the experiment have been considered in the analysis of pharmacokinetics and particularly in comparisons between the two species. One of the purposes of our modeling is to limit the use of laboratory animals in rodenticide research by predicting the best mixture before testing. In accordance with this objective and the principle of the 3R that rules the experiments on animals, we chose not to retest but to use unpublished results to build our model. However, in order to reduce the importance of these biases, we performed the analysis in two steps. A first analysis was performed by comparing the sexes within each species (same dose and same sampling time). Then a second one by comparing the species between them in period where the sampling times overlap for both species with a common start and finish.

In male rats, a mixture of E1 and E2 allows by modulating the ratio between both stereoisomers to reach a wide range of AUC, from simple to double, with a simple to triple increase in persistence at 168h. Thus, this simple pair of stereoisomers would allow creating a variety of baits that adapt to the various conditions by an optimization of the effectiveness/ecotoxicity compromise. If such baits were used in female rats they would, a priori, retain their efficacy, since the $AUC_{24-168h}$ of each of the two stereoisomers are higher than those of the males. However, in female rats, the difference between E1-trans and E2-cis, although significant ($p=0.035$), is greatly reduced, the $AUC_{24-168h}$ of E1-trans being only 20 % higher than that of E2-cis. Moreover, the persistence of the E2-cis stereoisomer is only 30 % lower than that of the E1-trans ($p=0.040$). Thus, in females, if a mixture of difethialone composed only of E1-trans and E2-cis (i.e., a stereoisomer that is a priori more effective but more persistent and another that is less effective and less persistent) was recommended

as in male rats, part of the modularity and flexibility of use that such a mixture allows in male rats would be lost. Indeed, the difference in properties between the two stereoisomers in female rats is much smaller than in male rats. In mice, the persistence of both stereoisomers is lower in both sexes than in rats, especially for E2-cis. However, for the latter this low persistence is accompanied by a strongly decreased $AUC_{24-168h}$. Thus, the effectiveness and value of adding the E2-cis stereoisomer in a bait for mice is questionable. Again, the benefit of a mixture composed of E1-trans and E2-cis, as presented in our previous article, cannot be extended from the male rats.

The mechanisms underlying these sex and species differences have not been explored here. However, the literature provides different hypotheses to explain those differences. Considering the effect of sex on pharmacokinetics, in agreement with previous studies on the pharmacokinetics of difethialone, each of the stereoisomers has a higher AUC in female rats compared to male rats. The difference between males and females is particularly large in rats, with a 1.4- (E3-cis) to 4-fold (E4-trans) higher AUC_{4-216h} in females. Nevertheless, the origin of this difference is not clearly identified here. Indeed, the MRT_{4-216h} is only 21 % higher in females than in males for the E2-cis stereoisomer and the MRT_{4-216h} of the other stereoisomer is lower in females or not significantly different. The origin might be intestinal absorption or hepatic capture efficiency of the female, although the statistical power of our data does not allow us to conclude. Indeed, although the maximum concentration at peak is 1.3, 1.5, 1.7, and 2.3 times higher in females for E3-cis, E1-trans, E2-cis, and E4-trans respectively, there is only a statistically significant difference for E4-trans. However, the pharmacokinetics of the latter stereoisomer seems to be strongly sex-dependent in rats. Indeed, the hepatic elimination rate of this stereoisomer appears to be much higher in males, resulting in an earlier C_{max} and a low liver concentration at 24h. Although the stealthiness of the passage of this stereoisomer in the liver seems to argue against its use as a rodenticide in male rats, it raises many questions about its hepatic metabolism. This difference could suggest that difethialone E4-trans metabolic enzymes are part of the sex-specific cytochromes P450 in rats (CYP2C11, CYP2C13, CYP3A2, and CYP2C12)²⁵ or that the tissue distribution is different between sexes. Considering the mice, significant differences are only pinpointed for $AUC_{24-336h}$ of the E1-trans and E2-cis stereoisomers, with a lower value in females. The AUC results are consistent with the maximal concentrations, which are significantly lower in females for both stereoisomers. The lack of significant difference for the corresponding MRT may suggest, as for rats but conversely for the genders, that a higher liver bioavailability of these two stereoisomers in male mice might explain the observed difference on AUC. Further studies focusing on the absorption of the different stereoisomers and their metabolism are needed to confirm, or not, this hypothesis.

These observations, in addition to ruling out the stereoisom-

$$C_{d,f_{1...4}}(t) = \frac{d}{D} \sum_{i=1}^4 \frac{f_i}{F_i} * C_{D,i,24} * e^{-\lambda_i(t-24)}$$

1. Equation used to model the liver concentration evolution in time of different mixes of difethialone stereoisomers. *i* is the index of each stereoisomer; *f* the fraction of each stereoisomer in the modeled mix; *F* the fraction of each stereoisomer in the experimental mix (*F*₁ = 0.275, *F*₂ = 0.225, *F*₃ = 0.225, *F*₄ = 0.275); *d* is the dose of difethialone to model; *D* is the dose of difethialone used in our experimental plan (in mice *D* = 3.0 mg/kg, in rats *D* = 3.4 mg/kg); *C*_(*D*,*i*,24) is the average concentration obtained experimentally for the respective stereoisomer at 24h and with the *D* dose of difethialone; *λ*_{*i*} is the decrease constant of the respective stereoisomer obtained experimentally with data between 24 and 168 hours; *t* is the time variable that can take values between 24 and 168 hours.).

meric pair selected with male rats for use in female rats or mice, also eliminate the use of the model in a multi-gender, multi-species approach. Indeed, one of the assumptions used in its construction is that absorption is not a determining factor in the observed differences in AUC and residues. However, our analysis shows that the differences in pharmacokinetics observed between gender and species cannot be explained by elimination alone. Thus, it is necessary to reconstruct a multi-gender and multi-species model considering an absorption criterion while keeping the spirit of the previous model to plot a graph with an efficiency indicator (the AUC) expressing as a function of an ecotoxicity indicator (the amount of molecule remaining in the liver five days after taking the bait, i.e., when the animal dies). To take into account the differences in hepatic capture between species and sexes and as there are not enough early samples (between 0 and 12 hours after per os administration) to determine an absorption coefficient, we have added the consideration of the observed *C*₂₄ concentration of each stereoisomer and included the notion of the administered dose in the model. For the administered dose and its relationship with *C*₂₄, we chose a linear relationship, which seems relevant for an oral administration of difethialone in the order of milligrams. Indeed, in one of our previous studies in rats, a dose of 10 mg/kg was administered orally and the liver concentrations of difethialone at 24h were about 3 times higher than those observed in the present study²⁶. Then, equation 1 is used to model the pharmacokinetic of difethialone stereoisomeric mixture. For each stereoisomer *i*, *C*_{*D*,*i*,24} is defined with *C*₂₄ concentration from figure 2 and *λ*_{*i*} with elimination constants of table 3.

The implementation of the model results in the creation of the graphs presented in figure 4. First, if a mixture with an AUC similar to the commercial product (represented with a star in figure 4) but with a maximum residue reduction at 5 days was created, the formulation would be identical for

rats. However, for mice, it would be preferable to use an E1-trans/E3-cis mixture instead. For example, a mixture of 65 % E1-trans and 35 % E3-cis would decrease the hepatic residues of difethialone in male and female mice by 20 % without decreasing the AUC compared to the current product. However, if such a product was used in rats, the concentration at 120h in male rats would be increased by 15 %. But, should the AUC of difethialone baits be maintained at all costs, or should a more balanced approach towards low ecotoxicity be preferred when, in some urban territories, difethialone is the main anticoagulant found in birds of prey²⁷? Thus, to continue using difethialone, which is an effective method of controlling resistant rodent populations, it is essential to reduce its ecotoxicity. This ecotoxicity is a priori largely due to the E3-cis stereoisomer, which is the most persistent stereoisomer among sexes and species. Therefore, as much as technically possible, baits should be formulated without E3-cis. Based on this assumption, E1-trans seems to be the best compromise for mice and rats with an AUC corresponding to 70 % or 53 % of that of the current product for rats and mice respectively at a dose of 3 mg/kg. Nevertheless, this decrease in AUC is accompanied by a reduction in residues at 120h of 48 % for female rats, 82 % for male mice, and 90 % for female mice. Only the residues at 120h of male rats decreased in the same proportion as the AUC, i.e., by about 20 %. Since the reported LD50 of the current product are 0.5 mg/kg in rats and 1.3 mg/kg in mice (Pelfrène 2010), corresponding, according to our model, to liver AUC values, of 226 h.µg/g in male rats, 332 h.µg/g in female rats, 875 h.µg/g in male mice and 956 h.µg/g in female mice, a male rat (≈300 g) or a female rat (≈250 g) would have to eat 10 g of bait; a male mouse (≈30 g), 3.6 g of bait and a female mouse (≈25 g), 2.9 g of bait to achieve such concentrations with a 25 ppm E1-trans bait. These amounts are about twice as much as with the current bait, but the residual hepatic dose at 5 days would be equivalent for the male rat, decreased by 20 % for the female rat, divided by 3 for the male mouse and by 5 for the female mouse. Of course, it will be necessary to verify experimentally the effectiveness of this product and technically assess its production challenges in order to provide a cost-effective commercial AR.

Finally, the E2-cis stereoisomer may also be of interest. Indeed, compared to the current product, this stereoisomer allows in rats, for a reduction of about half of the AUC, to decrease the residues at 120h of about 75 % in males and 60 % in females compared to the same dose of the current product. However, the decrease in AUC is even more drastic in mice, over 90 %. This might seem to be a disadvantage, but it could also be a sign of the selectivity that this stereoisomer would allow. Indeed, if this mouse specificity is shared with other small mammals that are often victims of primary intoxication to ARs^{11,28}, this would provide the way to the creation of ARs effective on rats but with limited primary and secondary ecotoxicity on non-target fauna. Further investigations on non-target species should be implemented to explore this

Comparative pharmacokinetics of difethialone stereoisomers in male and female rats and mice: development of an intra- and inter-species model to predict the suitable formulation mix — 8/10

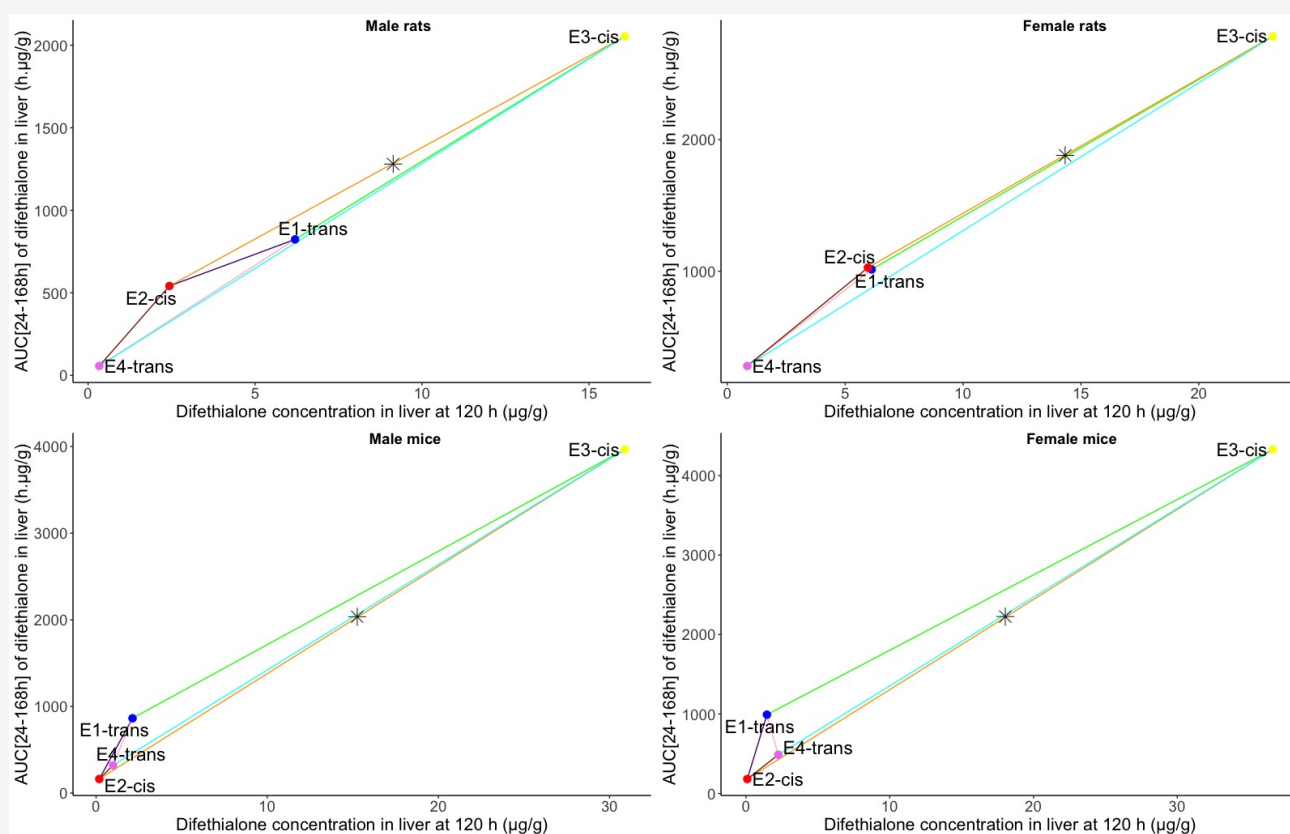


Figure 4. AUC_{24–168} and concentration of difethialone at 168h, obtained by calculation after a theoretical administration of 3 mg/kg of a couple of difethialone stereoisomers. Points represent the values for one stereoisomer alone. Lines represent values obtained with mixed of connected stereoisomer. Star point represents the results obtained with actual commercial difethialone

hypothesis.

In conclusion, and despite the biases of our study, we highlight that studies on anticoagulants must necessarily integrate research on the effect of gender and species both on efficacy (which is already done in regulatory tests), but also with regard to the ecotoxicity of these molecules. To date, many studies have simply extended the pharmacokinetic results for all rodents from rat or mouse data, often obtained only in males. However, as if significant differences are observed in our study performed on two laboratory strains belonging to the same phylogenetic super-family, one must question the need to avoid extrapolation from one species to the other, at least until the underlying mechanisms of SGAR stereoisomers metabolism and distribution, which are still in question, have been elucidated. Furthermore, as it is not possible to perform pharmacokinetic studies on all non-target species that may be exposed to anticoagulants, it is essential to characterize which stereoisomers are found in wildlife and which are not in order to assess the ecotoxic potential of each stereoisomer, as it is performed today with diastereoisomers^{29–31} and ultimately improve pest management by limiting effects on non-target fauna.

4. Author contributions

Conceptualization: VL, EB, SL; Methodology: AR, VL, AF, IF, SL; Data curation: AR, SL; Formal analysis and investigation: AR, VL, SL; Writing - original draft preparation: AR, VL, SL; Writing - review and editing: AR, VL, AF, HC, IF, EB, SL; Funding acquisition: VL, HC; Resources: VL; Supervision: VL, SL.

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5.4 Amélioration de l'expérimentation animale

5.4.1 Mise en place des points limites

5.4.1.1 poster :

Early end point

5.4.2 Amélioration du suivi des expérimentations

5.4.2.1 Article :

Monitoring of antivitamin K-dependent anticoagulation in rodents
– Towards an evolution of the methodology to detect resistance in rodents

Monitoring of antivitamin K-dependent anticoagulation in rodents – Towards an evolution of the methodology to detect resistance in rodents

Sébastien Lefebvre¹, Claire Hascoët¹, Marlène Damin-Pernik¹, Benoit Rannou¹, Etienne Benoit¹, Virginie Lattard¹

Abstract

Vitamin K antagonists are used as rodenticides for pest control management. In rodents, prothrombin time is used to monitor their effect despite its limits and the emergence of many coagulation methods. The aim of this study is to explore different coagulation monitoring methods in order to propose the best method and the best parameter to monitor vitamin K antagonists effect in rodents.

The coagulation function was thus monitored with global coagulation assays and specialty assays after difethialone administration in rats.

Despite many parameters obtained by thromboelastometry, only clotting time and clot formation time obtained by EXTEM were modified. Their evolution was fast with doubling time respectively of 4.0 h and 3.7 h but their increases were delayed with a lag time higher than 8 h. Conversely, prothrombin time evolution presented a lag time of only 2 h, but a higher doubling time of 7.2 h. The measurements of factor VII and X activities were the most sensitive assays to monitor vitamin K antagonists effect with almost no lag time and the fastest evolution. Nevertheless, factor X was shown to be the only key factor driving prothrombin time.

Monitoring factor X activity enables to follow most effectively the anticoagulation status in rats after rodenticides administration.

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Introduction

For over half century, vitamin K antagonists (VKA) have been used in human medicine in prevention and treatment of thromboembolic disorders and as rodenticides for pest control management. VKA acts as inhibitors of the vitamin K cycle. This cycle is a key component of the blood coagulation. Indeed, four clotting factors of the clotting cascade are vitamin K dependent – i.e. factor II, VII, IX and X. Their activity is conditioned by the gamma-carboxylation of their glutamate residues [1]. The gamma-carboxylation is performed in endoplasmic reticulum of hepatic cells by the enzyme gamma-glutamyl carboxylase with vitamin K as cofactor. For each gamma-carboxylation, vitamin K hydroquinone has to be converted in vitamin K epoxide. Then, vitamin K is regenerated by an enzyme, the vitamin K epoxide reductase complex 1 (VKORC1). VKA are specific inhibitors of this enzyme [2]. This inhibition blocks the regeneration cycle, diminishing the vitamin K hydroquinone available for the gamma carboxylation of vitamin K-dependent glutamate residues. Hence, the vitamin K-dependent clotting factors are not functional, impairing the coagulation function.

VKA are used for pests control management because of their delayed action. This delayed action is crucial to success

of the population management by avoiding the connection between these two events. Nevertheless, since the sixties, many cases of VKA resistances have been described [3], [4], [5]. Since this discovery, at least 25 different mutations of the *Vkorc1* gene have been described in Norway rats (*Rattus norvegicus*), some of these mutations lead to different levels of resistance against anticoagulant compounds [6]. Moreover, other resistance mechanisms have been described or suspected, some involving an overexpression of cytochromes P450 [7], [8] and others with origins still unknown.

With the emergence of resistance mechanisms to vitamin K antagonists in rodents, the diversity of VKA used for pest control management and the future development of new VKA, the need for proper coagulation testing has emerged. Initially, to evaluate resistance level, VKA efficiency tests were based on a survival challenge after a VKA administration. These challenges were quite long (many days) and have many shortcomings concerning the animal welfare and the repeatability. Later, new methods based on blood clotting response tests – i.e., the prothrombin time (PT), have been developed [9], [10], [11]. This new method reduces the experimentation time to 24 h, but is associated with major issues – i.e., the reproducibility and the repeatability of the test. Indeed, the PT measurement is widely dependent of the thromboplastin used. Thus, to compare the results the same reagent has to be used. In 2007, Prescott and al. have proposed to use the International Normalized Ratio (INR) in tests in order to increase the reproducibility [12]. However this concern has not been completely solved by the use of International Normalized Ratio as reported by Prescott in [13]. Moreover, in human medicine, evolving clinical experience has made practitioners doubting the value of the PT test in the management of VKA anticoagulation [14], [15], [16]. Therefore, in human medicine, many coagulation tests have been explored to monitor VKA anticoagulation. Recent guidelines suggest the use of thromboelastometry (TEM) in the management of VKA anticoagulation [17]. With the diversity of blood clotting tests developed in human medicine, it might be today relevant to explore the interest of these tests to follow an anticoagulation by VKA in rodents to propose a better monitoring solution.

In this study, we monitored VKA anticoagulation by four clotting tests including PT and Activated Partial Thromboplastin time tests (aPTT) and TEM test with two different kinds of reagents. We completed this exploration by the measurement of the activity of the four vitamin K dependent clotting factors. Indeed, only gamma-carboxylated vitamin K dependent clotting factors are released in the blood of rats which allow their easy dosing [18]. Most of these blood clotting parameters have never been assessed or compared before in the perspective of the VKA-dependent coagulation studies. The aim of this study is to compare the kinetic evolution of different blood clotting parameters during the first 24 h after the administration of a lethal dose of VKA and to study the possible correlation between the evolutions of these parameters. Our result might be useful in order to refine the

standardized efficiency test of VKA.

1. Material and methods

1.1 Animals

OFA-Sprague-Dawley rats of 8 weeks (250–300 g) were obtained from a commercial breeder (Charle Rivers, l'Arbresles, France) and acclimated for a minimal period of 5 days. The rats were housed four per cage under a constant photoperiod and ambient temperature. Experimental research was performed following international guidelines on animal welfare with approval from the ethics committee of the Veterinary School of Lyon. Animals were checked every day before difethialone administration and at least every 8 h after, with a special attention to potential bleeding adverse event. In order to study the decay kinetics of the vitamin K-dependent blood clotting factors after administration of anticoagulants, rats (35 males) have been fed with vitamin K3-deficient food (Reference U8220 Version 231 from Scientific Animal Food and Engineering, Augy, France), at least 48 h before the beginning and during the experience. To define the basal levels of blood parameters, a first group of 8 rats were killed 48 h after the beginning of the vitamin K3-deficient feeding. Blood was collected in citrate tubes (3.2%; 1:9 v/v) by intracardiac taking and liver was collected and frozen at - 80 °C. The other animals received by force-feeding a per os administration of 10 mg/kg of difethialone (kindly provided by Liphatech) 48 h after the beginning of the vitamin K3-deficient feeding, then a group of 3 rats was killed 1, 2, 4, 8, 10, 13, 16, 20 and 24 h after the difethialone administration. Blood was collected in citrate tubes (3.2%; 1:9 v/v) by intracardiac taking and liver was collected and frozen at - 80 °C.

In order to explore the relation between the parameters, additional data were obtained with 33 male rats. They receive the same protocol at the beginning, they have been fed with vitamin K3-deficient food then they have received by force-feeding a per os administration of 10 mg/kg of difethialone in the same condition as the other rats. 48 h after the difethialone administration rats had received an oral dose of vitamin K (10 mg.kg⁻¹). Then a group of 3 rats was killed at 135, 150, 165, 180, 210, 240, 270, 330, 360, 720 and 1440 min after the vitamin K administration. Blood was collected in citrate tubes (3.2%; 1:9 v/v) by intracardiac taking and liver was collected and frozen at - 80 °C.

1.2 Clotting times

Activated partial thromboplastin time (aPTT) and prothrombin time (PT) were measured using a STA Compact coagulation analyzer (Diagnostica Stago, Asnières, France) and Diagnostica Stago reagents. For aPTT determination, 100 μL of plasma was incubated at 37 °C for 3 min with 100 μL of cephalin and activator (CK Prest, Stago), then 100 μL of 0,025 M CaCl₂ was added and the clotting time was recorded. For PT determination, 100 μL of plasma was added to 200 μL of tissue factor and calcium (Neoplastine, Stago)

prewarmed to 37 °C and the clotting time was recorded. The device automatically stopped if no clot appeared in 600 s.

1.3 Thromboelastometry

The measures of thromboelastometry are realized according to the protocol described by [19]. Analyses were performed using the thromboelastometer Rotem Delta (TEM international GmbH, Munich, Germany) and reagents provided by the manufacturer of the instrument. Those analyses were performed on whole citrated blood 30 min after blood collection according to recommendations. Briefly, 300 µL of prewarmed (37 °C) citrated whole blood samples was added to clotting reagents in the cup. The analyses were then run for 60 min. Two types of clotting reagents were used to perform two profiles: intrinsic TEM (inTEMs reagent) to evaluate the intrinsic pathway (with activation by ellagic acid), and extrinsic TEM (exTEMs reagent) to evaluate the extrinsic pathway (with tissue factor activation). For these tests, the following parameters were recorded: clotting time (CT), the time until an amplitude of 2 mm; clot formation time (CFT), the time between 2 mm amplitude and 20 mm amplitude; alpha-angle (α), angle between the baseline and a tangent to the clotting curve through the 2 mm point; maximum clot firmness (MCF), the maximum amplitude reached during the test. Furthermore, 1 parameter based on exTEM and InTEM was calculated: the thrombodynamic potential index (TPI) which shows thrombodynamic potential of the temogram, calculated from maximal clot elasticity (MCE; $MCE = (100 \times MCF) / (100 - MCF)$) using the following equation: $TPI = MCE / CFT$.

1.4 Determination of clotting factor concentrations

Vitamin K-dependent clotting factors activities (II, VII, IX, X) were measured on citrated plasma using a chromogenic method on Konelab 30i (Thermoscientific) with Hyphen Biomed reagents.

More precisely, factor II was activated by incubation of citrate plasma with Echis carinatus venom (0.25 mg/mL) in Tris-buffer (0,1 mM Tris HCl, pH 8.3 at 25 °C, 0.2% BSA) at 37 °C for 10 min. Thus, 50 µg of thrombin chromogenic substrate was added and absorbance was monitored at 405 nm for 1 min.

Factor VII activity was assayed by incubation of citrate plasma with calcium and tissue factor in excess to allow the formation of tenase complexes and addition of a saturating concentration of human factor X activated in factor Xa proportionally to the quantity of tenase complexes formed. Thus, activity of factor Xa was monitored at 405 nm for 2 min after the addition of a specific chromogenic substrate generating para-nitroaniline in the presence of factor Xa.

Factor IX activity was assayed by an initial incubation of thrombin, calcium, phospholipids and Factor XIa with citrate plasma. The addition of a second reagent allowed to add factor VIII:C and factor X in the mixture. Thus, 50 µg of thrombin chromogenic substrate was added and absorbance was monitored at 405 nm for 1 min.

Factor X was activated by the addition of Russel's viper venom. Thus, 50 µg of thrombin chromogenic substrate was added and absorbance was monitored at 405 nm for 2 min.

1.5 Statistical analysis and mathematical model

Statistical analysis was performed with R (Version 3.1.2, R Foundation for Statistical Computing, Vienna, Austria). Results were expressed as mean values \pm SD or fitted value and confidence interval. For the evolution of each parameter against the time after the administration of difethialone, data were linearized with a logarithmic function. Then the slope of the regression curves was assessed with a Fisher test. The null hypothesis is that the slope is null.

For the evolution of each clotting factor against the prothrombin time, clotting factor and prothrombin time were linearized with a logarithmic function. Regression curves were compared on the slope and origin parameters with a Fisher test on the variance. The null hypothesis is that curves have the same slope or the same origin.

2. Results

2.1 Assessment of VKA-dependent hypocoagulability by coagulation assays

VKA-dependent hypocoagulability was assessed during the first 24 h following the administration of 10 mg/kg of difethialone by traditional coagulation tests – i.e. PT and Activated Partial Thromboplastin Time (aPTT) and by more global coagulation assay that is TEM using exTEM or inTEM reagents (Fig. 1). To facilitate the analysis of each technique, results were thus represented comparatively to the corresponding results measured before difethialone administration then linearized with the natural logarithm function (Fig. 2, Fig. 3). The lag time before a significant modification of the parameter and the slope of parameter evolution after this lag time are resumed in Table 1.

The four coagulation assays enabled to obtain a clotting time parameter (Fig. 2). Only the clotting time obtained by TEM using the inTEM reagent did not significantly differ from basal level during the first 24 h ($P = 0.693$). The others clotting time present a lag time ranged between 2 h for PT method and 10 h for TEM method using the exTEM, then significantly increase. The fastest increase was observed for the evolution of the clotting time obtained by TEM using the exTEM reagent with a slope of 0.179 h^{-1} ($r^2 = 0.925$). The evolution of the clotting time obtained by PT and aPTT methods was slower with a slope of respectively 0.096 h^{-1} ($r^2 = 0.972$) and 0.030 h^{-1} ($r^2 = 0.825$).

In addition to the clotting time, four other parameters were assessed by TEM with exTEM or inTEM reagents. The evolution of CFT, α , MCF and TPI is represented in Fig. 3. No significant evolution was observed for any parameters obtained by TEM using the inTEM reagent during the 24 h following difethialone administration. Concerning parameters obtained by TEM using the exTEM reagent only CFT

Table 1. Values of parameters from linear regressions performed on linearized data of each clotting parameter evolution after per os administration of difethialone. Values are presented with their 95% confidence interval.

Clotting parameters	Lag time (h)	During the lag time		After the lag time			
		Slope (h ⁻¹)	p-Value	Slope (h ⁻¹)	p-Value	R square	Doubling time (h)
CT exTEM	10	- 0.003 (- 0.035 to 0.028)	0.819	0.173 (0.083 to 0.263)	0.0088	0.925	4.0 (2.6 to 8.4)
α exTEM	>20	- 0.002 (- 0.005 to 0.0002)	0.069	N/A	N/A	N/A	
MCF exTEM	>24	- 0.002 (- 0.004 to 0.0004)	0.098	N/A	N/A	N/A	
CFT exTEM	8	- 0.038 (- 0.079 to 0.003)	0.059	0.187 (0.065 to 0.308)	0.013	0.819	3.7 (2.3 to 10.7)
TPI exTEM	>24	0.008 (- 0.001 to 0.018)	0.086	N/A	N/A	N/A	
CT inTEM	>24	0.003 (- 0.013 to 0.018)	0.693	N/A	N/A	N/A	
α inTEM	>24	0.0004 (- 0.00006 to 0.0009)	0.089	N/A	N/A	N/A	
MCF inTEM	>24	0.001 (- 0.0003 to 0.002)	0.176	N/A	N/A	N/A	
CFT inTEM	>24	- 0.005 (- 0.013 to 0.001)	0.103	N/A	N/A	N/A	
TPI inTEM	>20	- 0.017 (- 0.042 to 0.008)	0.158	N/A	N/A	N/A	
Prothrombin time	2	0.044 (- 0.022 to 0.111)	0.161	0.096 (0.089 to 0.103)	<0.0001	0.972	7.2 (6.7 to 7.8)
aPTT	4	0.015 (- 0.059 to 0.090)	0.661	0.030 (0.023 to 0.037)	<0.0001	0.825	22.9 (18.7 to 30.1)

increased significantly with a slope of 0.187 ($r^2 = 0.819$) after a lag time of 8 h.

2.2 Assessment of VKA-dependent hypocoagulability by direct measurement of vitamin K-dependent clotting factors activity

Activities of vitamin K-dependent clotting factors II, VII, IX and X were measured during the first 24 h after difethialone administration (Fig. 1). To facilitate the analysis, for each clotting factor activity, results are expressed comparatively to the corresponding clotting factor activity before difethialone administration then linearized with the natural logarithm function (Fig. 4). A lag time was pinpointed only for factor IX and X, of respectively 2 h and 1 h. Conversely, 1 h after the difethialone administration, prothrombin and factor VII activities were already significantly decreased (respectively $P = 0.048$ and $P = 0.037$). Factor VII activity had the most important decrease with a slope of $- 0.26 \text{ h}^{-1}$ ($r^2 = 0.978$), following by the factor X activity with a slope of $- 0.14 \text{ h}^{-1}$ ($r^2 = 0.989$). Prothrombin and factor IX activity decreases were similar with a slope of respectively $- 0.07 \text{ h}^{-1}$ ($r^2 = 0.937$) and $- 0.06 \text{ h}^{-1}$ ($r^2 = 0.843$). Concerning the factor VII, its activity at 24 h was under the limit of quantification. The lag time before a significant modification of the clotting factor activity and the slope of clotting factor activity evolution after this lag time are resumed in Table 2.

2.3 Assessment of the correlation between coagulability parameters and clotting factors

In order to evaluate the correlation between coagulability parameters and clotting factors evolution, we added data ob-

tained from male rats which received a per os administration of $10 \text{ mg}\cdot\text{kg}^{-1}$ of difethialone, then 48 h after an administration of $10 \text{ mg}\cdot\text{kg}^{-1}$ of vitamin K1. Owing to the important lag time observed with TEM parameters and the evolution weakness of the aPTT the correlation was successfully performed only with PT. Clotting factors and PT were expressed comparatively to the corresponding basal level before any administration, then transformed by natural logarithm function. Each vitamin K-dependent clotting factors evolution are represented in function of the PT in Fig. 5. For each factor, slope and origin obtained after the linear regression of data during the decrease of clotting were compared with those obtained after the vitamin K1 administration. Only the evolution of factor IX against PT was significantly different regarding slopes between decrease and regeneration phases ($P = 0.009$). Concerning prothrombin and factor VII, slopes between both phases were not significantly different (respectively $P = 0.150$ and $P = 0.952$) but their origins were different (respectively $P < 0.0001$ and $P = 0.007$). Finally, the linear regression parameters for the factor X evolution did not differ significantly between both phases for slopes ($P = 0.884$) or origins ($P = 0.793$).

3. Discussion

In human medicine the prothrombin time is the main parameter of VKA therapy monitoring, in the form of the International Normalized Ratio form [20], [21]. The aim of this monitoring is to avoid VKA adverse event and to follow possible change in patient physiology (hepatic induction). The purpose of the monitoring of parameters in rodent research is

Table 2. Values of parameters from linear regressions performed on linearized data of each clotting factor evolution after per os administration of difethialone. Values are presented with their 95% confidence interval

Clotting factor	Lag time (h)	During the lag time		After the lag time			
		Slope (h^{-1})	p-Value	Slope (h^{-1})	p-Value	R square	Half-life (h)
Prothrombin	0	NA	NA	- 0.072 (- 0.078 to - 0.065)	<0.0001	0.937	9.7 (8.9 to 10.7)
Factor VII	0	NA	NA	- 0.258 (- 0.275 to - 0.241)	<0.0001	0.972	2.7 (2.5 to 2.9)
Factor IX	2	- 0.078 (- 0.307 to 0.150)	0.441	- 0.062 (- 0.074 to - 0.050)	<0.0001	0.843	11.0 (9.6 to 13.1)
Factor X	1	- 0.049 (- 0.274 to 0.177)	0.630	- 0.144 (- 0.150 to - 0.137)	<0.0001	0.989	4.9 (4.7 to 5.1)

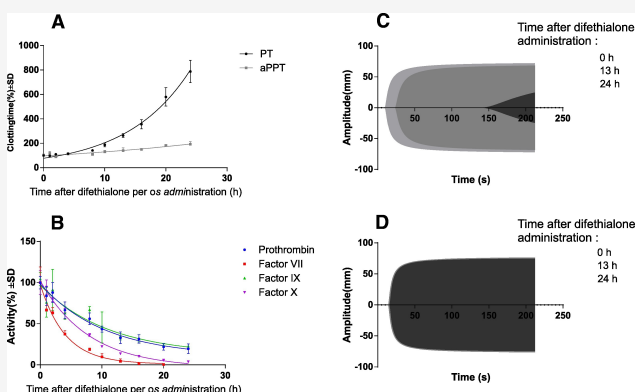


Figure 1. Results given by different coagulation monitoring methods after per os administration of 10 mg.kg⁻¹ of difethialone. (A) Clotting time tests. Values are presented for each time as the relative mean of clotting time and their standard deviation; for each kind of test, the basal mean of clotting times is set at 100%. (B) Vitamin K dependent clotting factors' activities. Values are presented for each time as the relative mean of activities and their standard deviation; for each factor, the basal activities' mean is set at 100%. (C) Silhouettes of the thromboelastometry graph with exTEM reagents. (D) Silhouettes of the thromboelastometry graph with inTEM reagents.

to test VKA molecules or rodent resistance strains [9], [10]. They were standardized by the Rodenticide Resistance Action Committee in order to obtain a discriminating dose for each VKA based on the work of Prescott et al. [12], [22]. Discriminating doses of anticoagulants, called ED50 (i.e., effective dose 50), were determined in rodents to characterize the susceptibility of rats to anticoagulants. ED50 corresponds to the dose leading to 50% of animals tested with a 5-fold increase of the International Normalized Ratio 24 h after the administration of anticoagulants. International Normalized Ratio is calculated from PT. Consequently, in rodent VKA research, PT is also the key parameter too. There are some concerns about the use of ED50 method, on the one hand, the reproducibility as reported in [13], on the other hand, the number of animals used which may be important for this kind of test [23]. Consequently, it might be relevant to study other parameters in order to diminish the duration of the resistance test, with parameters which evolve quickly than prothrombin

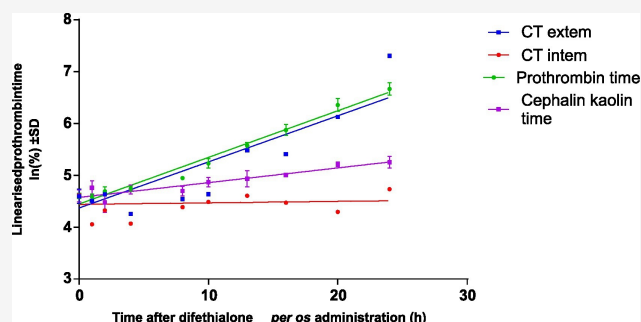


Figure 2. Evolution of different clotting times after per os administration of 10 mg.kg⁻¹ of difethialone. For each parameter, values are expressed in percentage relative to the basal values' mean (values obtained at 0 h), then linearized by a natural logarithmic function. Each point represents the mean of linearized values and its standard deviation.

after AVK administration, and the number of animals used, with parameters which have less inter-individual variability, and to improve the reproducibility of the method. Indeed, many techniques have been described assessing the blood coagulation in rats. Some of these techniques explore the blood capacity to coagulate. The simplest techniques as PT or aPTT give only a clotting time whereas the TEM using exTEM reagent – i.e. containing tissue factor - or inTEM reagent – i.e., containing ellagic acid or calcium - assess the global formation of the clot by providing many data. TEM is expected to become the future cornerstone in patient bleeding risk assessment [24]. It has been yet used in some transfusion protocol and in cardiac and liver surgery [25], [26] and recent studies try to increase its application scope [27]. In rats, TEM is increasingly used to measure coagulability in research [28], [29]. Thus, it might help to refine ED50 test in rats by replacing INR. Finally, the last kind of methods explores the activity of the vitamin K dependent clotting factors themselves. All these tests were applied in this study in order to determinate the best method and the best parameter(s) to follow a VKA administration in rats and the possible correlation between clotting times and vitamin K dependent clotting factors.

To cause experimental anticoagulation in laboratory rats, we chose to administer difethialone orally. The oral administration corresponds to the normal route of administration of anticoagulant rodenticides in rodents. Difethialone is one

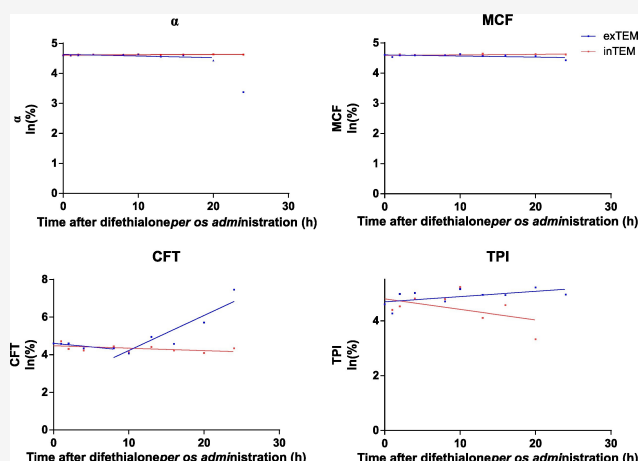


Figure 3. Evolution of different TEM parameters after per os administration of $10 \text{ mg}\cdot\text{kg}^{-1}$ of difethialone. For each parameter, values are expressed in percentage relative to the basal values' mean (values obtained at 0 h), then linearized by a natural logarithmic function. Each point represents the mean of linearized values and its standard deviation.

of the most powerful anticoagulant molecules currently available. In addition, to be sure to have completely inhibited the vitamin K regeneration cycle, a dose 25 times higher than the ED50 was administered. The ED50 of difethialone for the male rats is of $0.4 \text{ mg}\cdot\text{kg}^{-1}$, according to the technical monograph of 2003 [22]. This excessive dose was used to be sure to completely inhibit VKORC1 and thus the recycling of vitamin K. The use of such a dosage avoid to evaluate in the same time partial activation of new synthesized clotting factors and elimination of activated clotting factors. In this way, we were able to determine the maximal evolution speed of clotting parameters following a VKA administration leading to a complete inhibition of the vitamin K cycle and thus to assess which parameter has the best potential in the monitoring of vitamin K dependent coagulation. After administering the anticoagulant, coagulation was assessed by the various methods mentioned above. A good parameter to study of VKA efficiency has to be reliable and to vary quickly with the shortest lag time. Fig. 6 presents the half-lives and doubling times resulting of the evolution of different parameters during the first 24 h after administration of difethialone. The current reference assay, the PT, presented in our study a lag time of two hours, then a doubling time of 9.7 h with a good accuracy (0.972). As expected, aPTT evolved slower than PT. Conversely, two parameters determined by TEM performed with exTEM reagent, CT and CFT, seemed to change faster, after the lag time, than other clotting formation assessment parameters, and peculiarly than PT. Nevertheless, both parameters obtained by TEM using the ExTEM reagent showed an important lag time before increasing; 10 h for CT and 8 h for the CFT. Moreover, doubling time of these parameters presented very wide confidence ranges. These large ranges were due, on the one hand, to a lack of reliability of this method

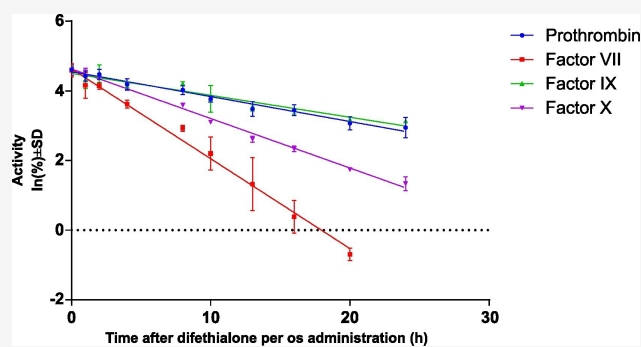


Figure 4. Evolution of different clotting factors activity after per os administration of $10 \text{ mg}\cdot\text{kg}^{-1}$ of difethialone. For each clotting factor, values are expressed in percentage relative to the basal values' mean (values obtained at 0 h), then linearized by a natural logarithmic function. Each point represents the mean of linearized values and its standard deviation.

in these conditions, and on the other hand, to the number of rats at each time used for the TEM method. Indeed, we respected the recommendations to perform thromboelastometry in rodents [19]. These recommendations impose time constraints in samples processing. Samples have to be ran within 2 h following the sampling. Freezing the sample before the analysis is not possible, conversely to PT measurement where the sample can be stored at -70°C until one month without significant differences [30]. In our case we had to adapt our protocol schedule and to restrict the measure to one specimen for each time for TEM methods. Another concern is about the quantity of blood needed which is of $300 \mu\text{L}$ against $100 \mu\text{L}$ of plasma for prothrombin time, making it difficult for its use in little rodent as mice, mainly if animal has to stay alive. Moreover, standardized method and reference values have not been defined for whole animals. Finally, the other parameters measured by TEM using exTEM reagent and all other parameters measured with inTEM reagent did not show significant evolution during the 24 h following difethialone administration. Consequently, concerning methods which assess the blood capacity to coagulate and considering their lag time, their importance of evolution, their reliability and their simplicity of implementation in research schedule, PT is the best method to assess the effectiveness of VKA during the first 24 h. This conclusion is in line with a previous study in human treated with warfarin [31].

Contrary to the parameters determined by the global coagulation tests, almost no lag time was observed for the evolution of clotting factors activities. Lag times for factor IX and X were very limited, respectively of 2 and 1 h and factors II and VII activities evolved immediately after VKA administration. Moreover, two of them, factors VII and X, evolved faster than the gold standard prothrombin time. Consequently, factors VII and X can be interesting to study the kinetic and the efficiency of VKA in rodents, especially since they seem to be the subject of few inter-individual variations. The great ad-

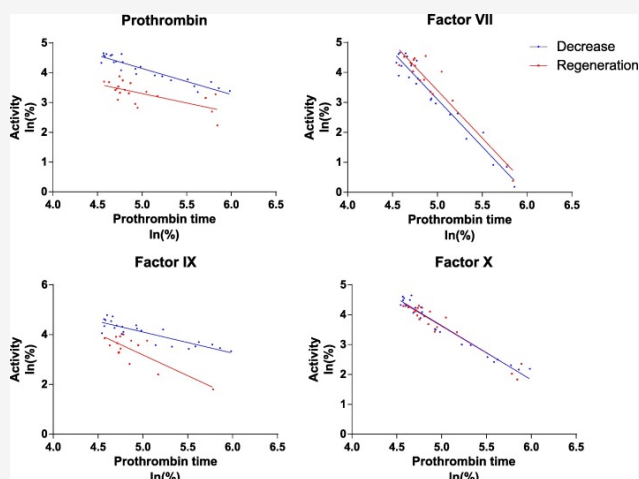


Figure 5. Evolution of clotting factors activity against prothrombin time during the decrease of coagulation capacity or its regeneration. For each couple “factor activity-prothrombin time”, values are expressed in percentage relative to the basal values’ mean of their respective parameters, then linearized by a natural logarithmic function.

vantage of factor VII is its short half-life which may be useful to study the effect of VKA few hours after its administration. But 24 h after the VKA administration, no more activity is detected for factor VII. Therefore 24 h after VKA administration, factor X measurement should be preferred to monitor VKA efficiency. In addition to these benefits, clotting factors activities seem to be stable over storage time [32]. Moreover, the quantification of vitamin K dependent clotting factor activities is fairly simple in rats. Indeed, only gamma-carboxylated vitamin K dependent clotting factors are released in the blood of rats. However in other species of rodents such as mice, both factors, gamma- and non-gamma-carboxylated, are released. It could be an issue for prothrombin quantification. Indeed, chromogenic assay used to quantify the prothrombin doses gamma- and non-gamma-carboxylated forms of prothrombin. Consequently, the concentration of active prothrombin cannot be precisely determined in some species with this method. In order to quantify only active prothrombin, it would be necessary to purify prothrombin or to use a quantification by a prothrombin time based assay (the test plasma is completed by deficient II plasma) as used in human medicine [33]. Nevertheless this method has not been assessed for rodent use.

In order to use the measurement of vitamin K dependent clotting factors activity to monitor the VKA action instead of prothrombin time, it is essential to study the correlation between these parameters. In human medicine many studies have been performed to assess the importance of each clotting factor on prothrombin time by using dilution of clotting factors [34]. Moreover, many in vitro studies have been performed to characterize each reaction from the human coagulation cascade, which allows to model mathematically the

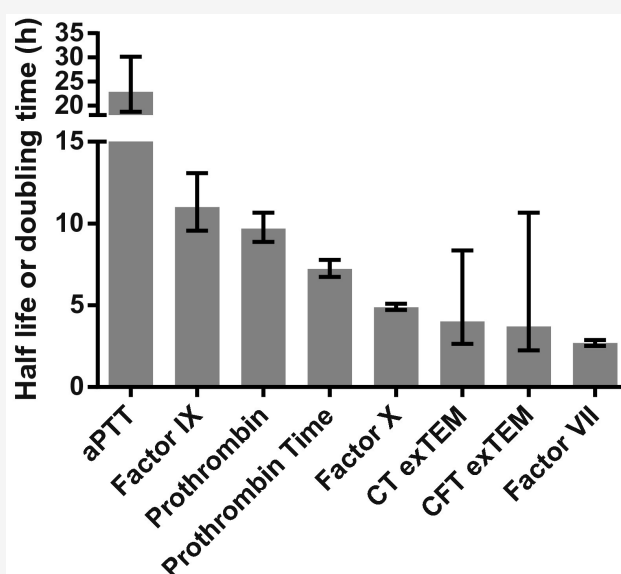


Figure 6. Values half-life or doubling time of each parameter evolution after per os administration of difethialone. Values are presented with their 95% confidence interval. Parameters with no significant evolution are not represented.

cascade and to assess the importance of each factor [35], [36]. However, concerning the rat, there is too little information on the cascade to create such model. But the study of the evolution of clotting factors relatively to prothrombin time might help to identify which clotting factor determine prothrombin time during VKA intoxication because it is the restraining element. The difficulty of this analysis is that all vitamin K dependent decrease and so it is not possible to individualize the action of one. We hypothesized that if one vitamin K dependent clotting factor leads to the variation in the decrease of the clotting capacity, it might also lead to it in the restoration of this capacity. Thus for this clotting factor the curve obtained when clotting capacity decreased and that one obtained during the regeneration of clotting capacity might be the same. In our experiment, only the factor X fulfilled this requirement. This result is consistent with the hypothesis of Kerins and MacNicol [37]. that the factor X has greater effect on the prothrombin time of rats than others, whereas, in human, the dilution assays and the mathematical model results give prothrombin as the critical factor for the prothrombin time [36], [38]. This relation between prothrombin time and factor X might be useful in order to refine ED50 test. Indeed, the activity of the factor X enables to monitor more efficiently a VKA-dependent anticoagulation in rodents than PT, while being totally correlated with the evolution of PT. Further investigations have to be conducted to confirm this opportunity by using lower dose of VKA. Nevertheless, because factor X activity is totally correlated with the evolution of PT, the use of factor X activity measurement could certainly diminish the duration of ED50 tests even if doses of VKA used are lower and thus improve the monitoring of VKA action in rodent

research.

4. Conclusion

During the 24 h following administration of an antivitamin K anticoagulant, prothrombin time assay seems to be a better method than thromboelastometry to monitor coagulation in rodents. Nevertheless, measuring the activities of vitamin K-dependent clotting factors would enable to detect an anticoagulation status earlier. Table 3 summarizes the applicability of each technique to monitor VKA-anticoagulation in rodents and their benefits. Factor X is the critical factor which determine the prothrombin time in rats under an antivitamin K anticoagulant and enables to follow most effectively the anticoagulation status. Further studies should be performed to confirm the relevance of this factor to follow the anticoagulation in the different strains of resistant rats.

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Table 3. Summary of the upside and downside of each technique to monitor a VKA-dependent anticoagulation in rodents. Three characteristics, precocity, reliability and amplitude of evolution are respectively assessed according to lag time, R-square and absolute slope. A, applicable, Q, questionable, N, not applicable.

Assay	Parameter	Applicability	Precocity	Reliability	Amplitude of evolution	
Global coagulation assays	PT-Quick	Clotting time	A	+	++	+
	APTT	Clotting time	Q	+	-	-
		Clotting time	A	-	+	++
	TEG/TEM-TF (ExTEM)	α	N			
		MCF	N			
		CFT	Q	-	-	++
		TPI	N			
		Clotting time	N			
	TEG/TEM-CA (InTEM)	α	N			
		MCF	N			
		CFT	N			
		TPI	N			
Factor II		A	+++	+	+	
Specialty assays	Clotting factor activity	Factor VII	A	+++	++	+++
		Factor IX	A	+	-	+
		Factor X	A	++	+++	++

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5.4.2.2 Article :

Thrombin generation test : A reliable tool to evaluate the pharmacodynamics of vitamin K antagonist rodenticides in rats

Thrombin generation test: A reliable tool to evaluate the pharmacodynamics of vitamin K antagonist rodenticides in rats

Jourdi Georges¹, Lefebvre Sebastien², Le Bonniec Bernard¹, Curis Emmanuel³, Gaussem Pascale⁴, Lattard Virginie², Siguret Virginie⁵

Abstract

Vitamin K antagonist rodenticide pharmacodynamics (PD) is studied in rodents with traditional laboratory tests. We wondered if thrombin generation test (TGT) could add value.

Difethialone (10mg/kg) was administered per os to 97 OFA-Sprague Dawley rats. PD was studied over a 72h-period using the Calibrated Automated Thrombogram on platelet poor plasma before and after intoxication (3 female and 3 male rats for each 13 time points) and TGT parameters were compared with the prothrombin time (PT) and vitamin K dependent factor activities previously reported.

Following intoxication, preliminary tests evidenced rapid and full inhibition of thrombin generation triggered with 5 or 20pM human recombinant tissue factor. To study the evolution of TGT parameters following difethialone intake, we adapted the test by complementing intoxicated rat samples with pooled normal rat plasma (3/1, v/v). Adapted TGT confirmed the known higher procoagulant basal level in females compared to males through higher endogenous thrombin potential (ETP) and peak height (PH) ($p < 0.0001$ and $p = 0.0003$, respectively). An exponential model fitted well the PH and ETP decay after intoxication. In contrast to PT, the decreases were observed immediately following VKA intake and had comparable time to halving values: 10.5h (95% CI [8.2; 13.6]) for ETP and 10.4h (95% CI [7.8; 14.1]) for PH. The decrease of FVII and FX preceded that of PH, ETP and FII while FIX decreased later on, contributing to the severe hypo-coagulability.

We demonstrated that TGT performed in samples of intoxicated rats complemented with normal plasma is a reliable tool for evaluation of VKA rodenticide PD in rats.

Keywords

Thrombin generation test — Difethialone — Rodents

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Introduction

Vitamin K antagonists (VKA) have been used as rodenticides for more than sixty years¹. They exert their anticoagulant effect by targeting the vitamin K 2,3-epoxide reductase complex subunit 1, therefore blocking the recycling of vitamin K 2,3-epoxide in vitamin K hydroquinone². Decreased availability of vitamin K hydroquinone, a co-substrate of the γ -carboxylase, results in hypo- γ -carboxylated vitamin K dependent clotting factors thus in dramatically decreased clotting activity of factors II, VII, IX and X (FII, FVII, FIX, FX)^{3,4}.

VKA rodenticides are characterized by a wide inter-individual variability of response between rats due to gender, environmental and genetic factors^{1,5,6}. The origin of the gender differences in the response to VKA rodenticides has been reported in different rat strains: in Sprague-Dawley rats, a higher basal activity of FVII and FX has been found in females compared to males, in agreement with results of older studies in Wistar rats^{6,7}. Besides, resistance to VKA rodenticides due to genetic factors has been reported in both genders from many countries in Europe but also in United States, Canada, Japan and Australia resulting in a serious concern for pest control^{8–10}. Consequently, warfarin and diphacinone which had been introduced in the early 1950s, were replaced in the 70s by more potent molecules such as difenacoum or bromadiolone, and 10 years later by brodifacoum or flocoumafen. Nevertheless, genetic resistance to these compounds developed in parallel^{1,5,11,12}. Difethialone was the latest VKA rodenticide developed for pest control and is currently considered as one of the most powerful for which no resistance has been described so far¹³.

Published data on the rodent PD profile of VKA rodenticides are scarce and report results obtained using coagulation tests of human clinical practice performed with rodent plasma samples^{5,7,14,15}. Semi-global coagulation assays, namely prothrombin time (PT) and activated partial thromboplastin time are reliable tools for the exploration of coagulation system in rodents, especially to assess the anticoagulant resistance¹⁶. However, they mainly explore the initiation phase of coagulation, which requires a minimal amount of thrombin generated (i.e. 10–30nM) necessary for clot formation¹⁷. More recently, additional global tests, among which the thromboelastometry and the thrombin generation test (TGT), have been proposed to evaluate thrombin generation beyond clot formation^{18–20}, thus to follow rodents anticoagulation by VKA and propose a better rodenticides management. Rotational thromboelastometry (Rotem) did not provide a very good insight of coagulability in this setting since only two parameters obtained with Extem were modified in intoxicated rat Platelet Poor Plasma (PPP) with lag time higher than 8h and very wide confidence ranges of their doubling time along with the relatively large sample volume needed. Consequently, PT remains the best method to monitor VKA rodenticides induced hypocoagulation²⁰. In TGT, the addition into plasma of a fluorescent substrate cleaved by thrombin allows measuring a fluorescence signal over time, which is not disturbed by the turbidity

of a forming clot, in contrast to optical density for a chromogenic substrate²¹. In humans, TGT accurately evaluates coagulation disorders associated with bleeding or thrombosis. For example, TGT provides another insight on anticoagulation improving the monitoring of factor replacement therapy in hemophilic patients beyond clotting times^{22–25}. However, this method has never been developed in rats intoxicated with VKA rodenticide in order to evaluate their impact on the coagulation system and therefore the rodenticide efficacy.

In a recent study, our group determined the pharmacokinetic/pharmacodynamic profile of difethialone in male and female Sprague-Dawley rats over a 72-hour period following difethialone intoxication using routine coagulation tests²⁰. The aim of the present study was (i) to adapt the standard Calibrated Automated Thrombography (CAT) protocol to ensure the reliability of TGT to evaluate anticoagulant rodenticide PD in rat plasma samples and (ii) to determine the kinetic profiles of TGT parameters from individual intoxicated male or female rats and built models of TGT parameters kinetics after VKA intoxication. Finally, we compared TGT parameter decays to those of PT and vitamin K dependent clotting factor activities.

1. Materials and methods

1.1 Animals and blood sampling

All research procedures were performed according to an experimental protocol following international guidelines (86/609/EEC) and approved by the ethic committee of VetAgro Sup. A total of 97 eight-week old male and female OFA-Sprague-Dawley rats (Charles River, l'Arbresles, France) were used in the present study. All rats were acclimatized for at least five days and housed in the animal facility, four rats per cage as already reported in details²⁰. Just before blood sampling, rats were anesthetized with isoflurane and euthanized with CO₂ immediately after sampling.

1.1.1 Blood sampling and preparation of pooled normal rat plasma (PNrP)

Since female have higher basal procoagulant level compared to male rats²⁰, we chose to use only male rats to prepare a pool of normal rat plasma. Therefore a group of 10 male rats were fed with standard diet Scientific Animal Food and Engineering, referenced A04 (Augey, France). Blood was collected by cardiac puncture in 3.5mL tubes containing sodium citrate (3.2% 0.105M; 1:9v/v; Greiner, Alcyon, Nancy, France) and gently inverted five times to ensure adequate anticoagulation. Tubes were then double centrifuged at 2000g 15min at 20°C and PPP samples were pooled and served as Pooled Normal rat Plasma (PNrP). PNrP was aliquoted, frozen and stored at -80°C until use. PT and aPTT results measured on PNrP were in the reference intervals²⁰.

1.1.2 Blood sampling and preparation of PPP from intoxicated rats

In order to adapt the CAT protocol for intoxicated rat PPP samples, 9 male rats were fed with vitamin K3-deficient food

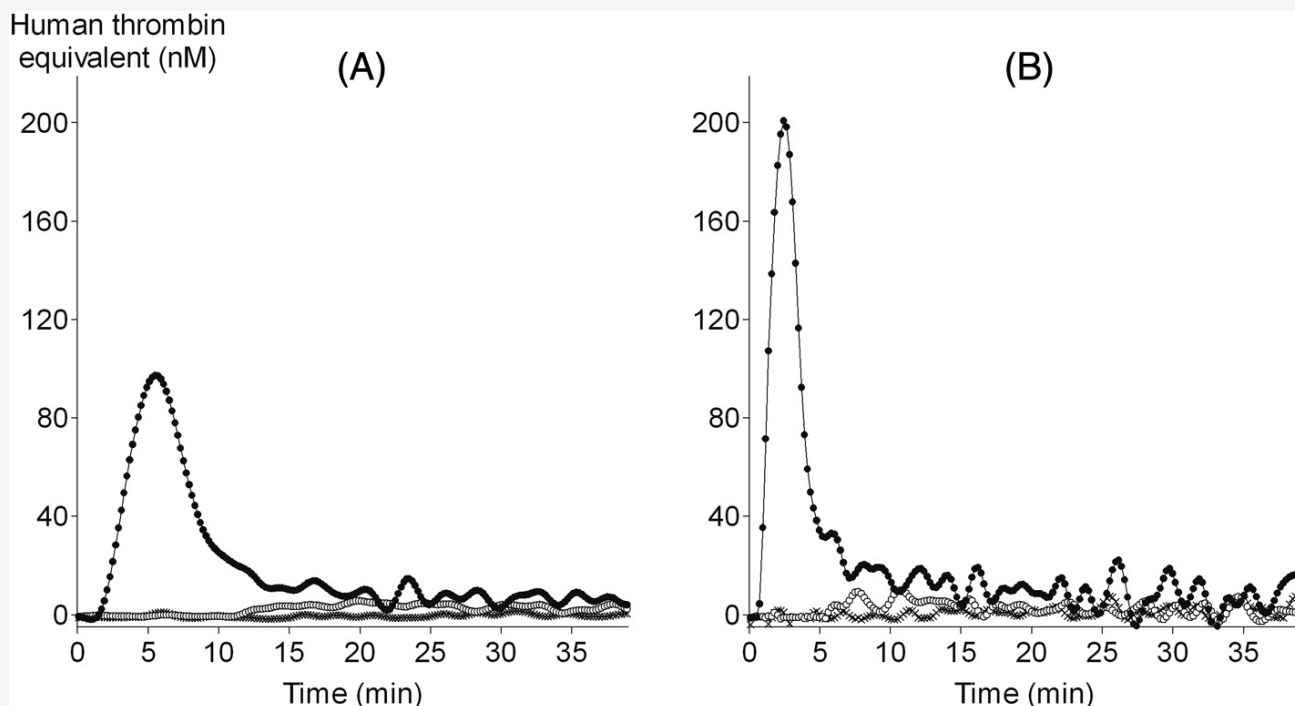


Figure 1. TGT with rat PPP. TGT were performed in pooled PPP samples from 3 male rats using the CAT standard protocol triggered with either PPP-reagent (1A) or High PPP-reagent (1B). In both panels, closed circles represent TGT performed at T0 (prior to intoxication), open circles TGT at T24 after difethialone intoxication, and crosses at T72. PH at T0 doubled whereas TTP halved when TGT was triggered with 20 instead of 5pM tissue factor. Thrombin levels are expressed as equivalent human thrombin. With both triggers, thrombin generation was completely inhibited at T24 and T72 post difethialone intoxication.

(Scientific Animal Food and Engineering), at least 48h before the beginning and throughout the experience as described²⁰. Animals received by force-feeding a per-os administration of 10mg/kg difethialone (kindly provided by Liphatech, Pont-du-Casse, France), corresponding to 20-fold the lethal dose LD50. Pooled PPP samples from 3 male rats obtained at T0, T24 and T72 after difethialone intoxication were prepared and stored as described above.

In a second phase and in order to study coagulation system after intoxication with difethialone, 39 male and 39 female rats were force-fed as above. Groups of 3 males and 3 females were anesthetized prior to blood sampling as described above at different times (T) (in hours) after difethialone administration: T0, T1, T2, T4, T8, T10, T13, T16, T20, T24, T32, T48 and T72. All PPP samples were thawed in a 37°C water bath just before use.

1.2 Thrombin generation test in rat PPP

1.2.1 CAT assay

TGTs were performed on a CAT system according to the method described by Hemker et al.²¹. TGTs were measured using a microplate fluorometer (Fluoroskan Ascent™, ThermoLabsystems, Helsinki, Finland). Rodent PPP tends to have short lag time and rapid thrombin generation resulting that if all of the 96 wells of a microplate are used simultaneously, lack of the early points for the second half of the microplate

precludes accurate ETP evaluation²⁶. Using only the first half of the 96-well plate allows starting on time all fluorescence measurements following initiation. Consequently, half of a round bottom 96-well plate (Greiner, Frickenhausen, Germany) was used each time. PPP samples were run in duplicate using either 20μL PPP-reagent (5pM tissue factor and 4μM phospholipid vesicles; Stago, Asnières sur Seine, France) or 20μL High PPP-reagent (20pM tissue factor and 4μM phospholipid vesicles; Stago), and 20μL Thrombin Calibrator (Stago) in parallel; average of the duplicates was used for analysis and mean CV values varied between 1.5% for temporal TGT parameters and 3% for the others. TGT plate containing the mixture of 80μL PPP and 20μL triggering reagent or Thrombin Calibrator was incubated 5min in the machine to warm to 37°C. TGT was initiated by autodispensing 20μL of FluCa solution (Stago) containing calcium ions and the fluorogenic substrate, Z-Gly-Gly-Arg-7-amino-4-methylcoumarin, to each well. Generated thrombin cleaves the fluorogenic substrate and fluorescence increase was monitored at 37°C for 120min. Thrombinoscope software (Thrombinoscope® BV, version 5.0.0.742, Maastricht, Netherlands) was used to calculate the amount of thrombin generated over time expressed as human thrombin equivalent taking into account for each sample the calibrator activity. We recorded and interpreted the following parameters: lag time (LT; in min: time from test triggering to signal detection), time to peak (TTP;

in min: time necessary for thrombin concentration to reach its maximal value), peak height (PH; in nM: maximal human thrombin equivalent generated amount) and endogenous thrombin potential (ETP; in nM.min: area under the thrombin concentration curve).

1.2.2 Protocol

TGTs were first performed without complementation in pooled PPP samples from 3 male rats before (T0), and after administration of difethialone (at T24 and T72), using either PPP- or High PPP-reagent. TGT were subsequently performed in PPP samples complemented with PNrP at different ratios (PPP/PNrP; v/v) to determine the optimal complementation providing reliable results in TGT.

In a second phase of experiments, TGT triggered with the PPP-reagent were performed in female and male PPP samples obtained at various time-points post intoxication. There, samples were complemented with PNrP at the previously determined ratio of 3/1 PPP/PNrP (v/v) to evaluate the kinetics of TGT parameters following VKA intoxication.

1.3 Statistical analyses

TGT parameters from the Thrombinoscope software were further analyzed using the R software (version 3.3.1; R Foundation for Statistical Computing, Vienna, Austria). Intra-assay coefficient of variation (CV) were computed from the parameters of TGT performed in PNrP and triggered with PPP-reagent; their 95% confidence intervals (95% CI) were obtained as the 2.5 and 97.5 percentiles of their bootstrap distribution (9999 random draws).

TGT parameters, assigned as y , were modeled as a function of time (t) after rodenticide administration, using an exponential decay model, $y=A e^{-k \cdot t}+B+\varepsilon$, where $t=0$ at the administration time, A is the basal value, B is the residual value of the considered parameter, k is the rate constant related to the half-life ($t_{\frac{1}{2}}$) by the relation $t_{\frac{1}{2}}=\frac{\log(2)}{k}$, and ε is an error term, assumed to be Gaussian (“normal”), centered and of constant variance. The model was fitted using non-linear least-squares (nls function in R). Gender effect was tested using sex and A , sex and k , or sex and B interaction terms, and the resulting models were compared using nested models, with an asymptotic likelihood ratio test. Results were expressed as mean with their 95% CI obtained by profiling. Tests were considered significant if $p < 0.05$. Initial values for the non-linear regression were obtained, for B , by averaging the last values, then, for A and k , by a linear regression of $\log(y-B)$ on time. Adequation of the model to the data was checked based on the fit residuals: no significant heteroskedasticity, approximate normal distribution, no clear residual curvature.

2. Results

2.1 Adaptation of TGT standard protocol to study difethialone PD in rats

TGT were first triggered with PPP-reagent (5pM tissue factor) in pooled PPP samples from 3 male rats obtained at T0,

T24 and T72. Before difethialone administration (T0), TGT resulted on the average in a thrombin generation culminating at 100nM PH for a 5min TTP. Complete inhibition of thrombin generation was observed at both T24 and T72 post difethialone administration over the 2h of fluorescence measurement (Figure 1A).

When TGT were triggered with High PPP-reagent (20pM tissue factor) the PH at T0 doubled whereas TTP halved compared to the values obtained using the PPP-reagent. Nevertheless High PPP-reagent also failed to trigger thrombin generation in the T24 and T72 samples following difethialone administration (Figure 1B).

As complete inhibition of thrombin generation was observed at T24 with pooled PPP from intoxicated rats triggered with either 5 or 20pM tissue factor reagent, pooled PPP at T24 was complemented with PNrP at different PPP/PNrP ratios (v/v) (9/1, 4/1, 7/3, 1/1 and 1/3) (Table 1). TGT performed with PNrP and triggered with 5pM tissue factor resulted in a CV of 4.6% (95% CI [3.7; 6.2]) for TTP, 4.4% (95% CI [3.1; 6.3]) for PH, and 7.8% (95% CI [6.2; 11.2]) for ETP. Since fluorescence was not measured continuously in the Thrombinoscope, it resulted that all LT values obtained with PNrP were short and equal to 1.33min. As the PNrP fraction increased, PH and ETP of TGT triggered with the PPP reagent progressively increased up to the values obtained with PNrP alone. As little as one volume of PNrP for 9 volumes of plasma from intoxicated rats already considerably shortened LT and TTP (Figure 2). Similar results were obtained through PNrP complementation of PPP collected at T72 (data not shown). The 3/1 PPP/PNrP ratio was found optimal to obtain reliable TGT parameters, especially PH and ETP. Such complementation therefore allowed studying the 72h kinetic of TGT parameters following per os administration of the rodenticide.

2.2 Changes of thrombin generation over time after difethialone per os intoxication

TGTs were then performed in individual PPP samples from male and female rats obtained at the 13 pre-specified time points over a 72h-period following difethialone intoxication. Each sample was complemented (PPP/PNrP 3/1). During the 72h-period and in both genders, PH and ETP decreased up to a similar plateau value (Figure 3 A and B) whereas, LT and TTP were not significantly modified (Figure 3C and D).

Different PD models were built taking into account the complementation with PNrP. Regarding ETP and PH, the exponential decay models fitted well the experimental data in males as well as in females and over than 80% of the total variance are explained by the models (Table 2). Before intoxication (T0), the basal PH values estimated by the model (parameter A) were significantly higher in females (94nM 95%CI [82; 107]) than in males (70nM [57; 83], $p=0.0003$; Figure 3 A). The same observation held for estimated basal ETP (352 [315; 390] vs 253nMmin [213; 295], $p < 0.0001$; Figure 3 B). After intoxication, rat gender did not signifi-

		LT (min)	ETP (nMmin)
PNrP		2.8	301
PPP/PNrP	1/3 (v/v)	3.1	234
PPP/PNrP	1/1 (v/v)	3.1	169
PPP/PNrP	7/3 (v/v)	3.3	143
PPP/PNrP	4/1 (v/v)	3.6	97
PPP/PNrP	9/1 (v/v)	4	57
PPP non complemented		Below the LOQ	Below the LOQ

Table 1. Pooled PPP samples of 3 male rats obtained 24h after difethialone intoxication were tested by TGT with or without complementation with pooled normal rat plasma (PNrP) at different PPP/PNrP ratios (v/v) (9/1, 4/1, 7/3, 1/1 and 1/3). TGT were triggered with PPP reagent (5pM tissue factor). LT corresponds to the time necessary to reach 10nM human thrombin equivalent and ETP the area under thrombin concentration curve following coagulation activation. As PNrP fraction increased, LT values progressively decreased while ETP increased up to reach that of PNrP alone.

cantly influence the k parameter in both PH and ETP models ($p=0.1808$ and $p=0.1922$, respectively). No significant differences were observed between males and females on the residual values of PH and ETP (parameter B; $p=0.88$ and $p=0.94$, respectively).

Consequently, times to halving PH and ETP were comparable in males and females ($p=0.1808$ and $p=0.1922$, respectively), leading to values of 10.4h (95%CI [7.8; 14.1]) for PH and 10.5h (95%CI [8.2; 13.6]) for ETP (Table 3).

Half-lives of the vitamin K dependent factors following difethialone intoxication had been previously estimated in aliquots collected during this same study⁷ and reported: 2.9h for FVII, 5.5h for FX, 11h for FII and 12.6h for FIX. Based on the time to halving values, ETP and PH were ranked: in both genders, FVII decreased first, followed by FX, then ETP, PH, and FII then finally by FIX.

3. Discussion

In the present study, we adapted and validated the CAT as a reliable TGT method to evaluate the PD of VKA rodenticides in intoxicated rats. TGT had been proposed previously as a complementary approach to study coagulation system in rodents^{14,19,27-30}, but, to our knowledge, this method had never been used on rodents plasma after VKA intoxication.

TGT has the advantages to globally study the coagulation system including initiation, amplification and propagation phases, resulting in large amounts of generated thrombin overtime controlled by physiological inhibitors albeit TGT performed without the addition of thrombomodulin did not

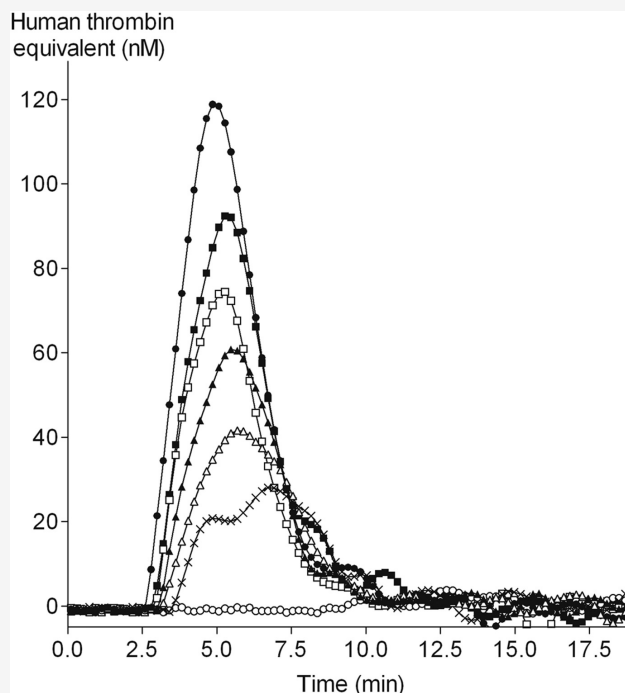


Figure 2. TGT with complemented rat PPP. Pooled PPP samples from 3 male rats at T24 after difethialone intoxication were complemented with PNrP at different ratios: PNrP only (closed circles), PPP/PNrP 1/3 (v/v) (closed squares), 1/1 (open squares), 7/3 (closed triangles), 4/1 (open triangles), 9/1 (crosses) and no complementation (open circles). Thrombin levels are expressed as equivalent human thrombin. Highest complementation (ratio 1/3) restored PH and ETP just below the values observed with PNrP alone. Lowest complementation (ratio 9/1) already considerably decreased LT and TTP likely by restoring the initiation phase of coagulation.

allow exploring the protein C system. Hyper- as well as hypo-coagulability states including responsiveness to anticoagulants might be explored reliably through TGT parameters³¹. Data derived from thrombinography can be used to determine temporal parameters (LT and TTP) as well as thrombin generated (PH and ETP), useful parameters for assessing coagulation state³².

In a paper recently published by our group regarding the PD of difethialone (7), traditional semi-global coagulation tests such as PT were used besides coagulation factor activity measurement. However, PT has some limitations. In PT test, coagulation is triggered with calcified thromboplastin reagent containing large amounts of tissue factor resulting in clot formation as soon as 10–30nM of thrombin are generated, thus limiting the PT exploration to the initiation phase of coagulation³¹. Moreover, the large excess of tissue factor contained in thromboplastin reagent hardly encountered real-life patho-physiological situations. Appropriate laboratory tests sensitive to early, transient as well as long lasting hypo-

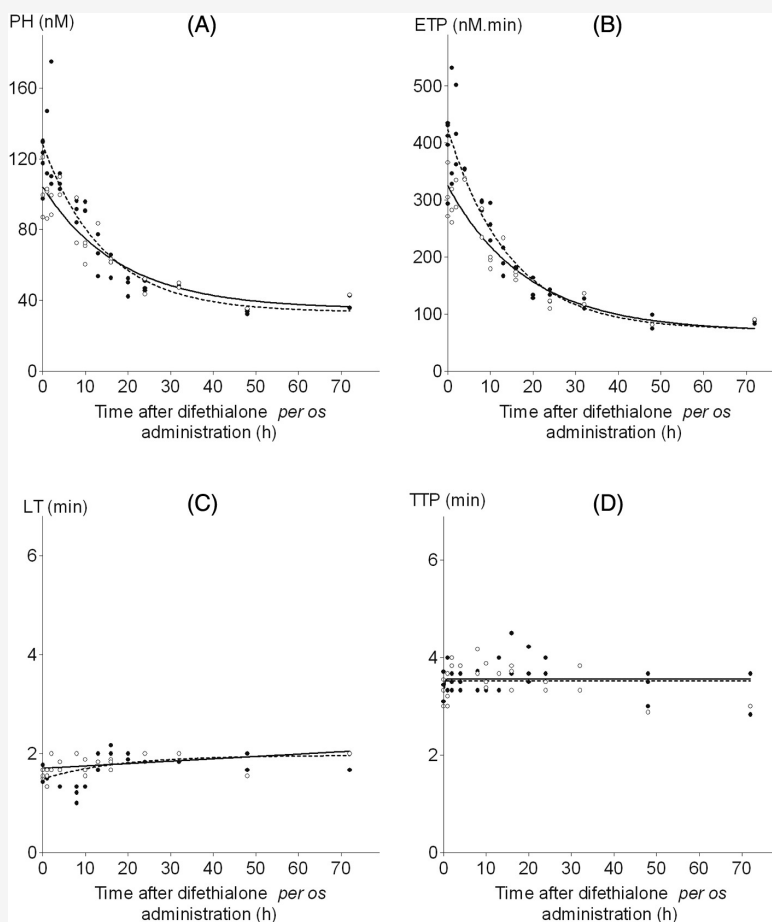


Figure 3. Changes of thrombin generation parameters over time after difethialone intoxication: PH (3A), ETP (3B), LT (3C) and TTP (3D). Graph represents the resulting parameters of TGT performed with PPP reagent as a function of the time (in hours) following intoxication (T0, T1, T2, T4, T8, T10, T13, T16, T20, T24, T32, T48 and T72). The PPP/PNrP ratio was 3/1 (v/v). Male and female individuals are represented by open and closed circles, respectively. PH (panel 3A) and ETP (panel 3B) were significantly higher at T0 in females than in males ($p=0.0015$ and $p<0.0001$, respectively). Both decreased progressively after difethialone intoxication. Time to halving ETP and PH values did not differ significantly between males and females ($p=0.1922$ and $p=0.1808$, respectively). Same residual values of PH and ETP were observed, owing to PNrP used for PPP complementation in both groups. LT (panel 3C) and TTP (panel 3D) did not change over time reflecting the sufficient amount of clotting factors added by PPP complementation with PNrP to normally initiate coagulation.

or hyper-coagulation would be ideal tools in VKA rodenticide development strategy. Yet PT responds only partially to this challenge since in rats it pictures hypo-coagulation with a delay (2h in male and 8 in female rats) rapidly decreasing to undetectable level thereafter.

For all these reasons, we chose to perform TGT with rat PPP after VKA rodenticide intoxication, namely, difethialone in the present study. This rodenticide was chosen because it is known as one of the most potent inhibitor of vitamin K 2,3-epoxide reductase complex subunit 1 and that to date no resistance to difethialone has been reported in rodents. The dose of 10mg/kg used in the protocol corresponds to nearly 20-fold the lethal dose 50³³ ensuring that all vitamin K dependent factors are not functional. It is worth to note that unlike in humans and mice, in rats, proteins induced by vitamin K

antagonists are predominantly retained in the liver and thus are not released into the bloodstream³⁴. Consequently they do not affect TGT in VKA intoxicated rats.

Since thrombin generation was completely inhibited at T24 and T72 after difethialone intoxication, even when triggered with high tissue factor concentration (20pM), we successfully adapted TGT protocol by complementation of the samples with PNrP. Functional γ -carboxylated factors brought by PNrP restored in part thrombin generation and thus allowed studying the kinetics of two of the TGT parameters, namely PH and ETP.

At baseline, TGT performed in complemented PPP samples confirmed a significant higher hypercoagulability state in female compared to male rats, as reflected by significantly higher ETP and PH. This gender difference is in agreement

Rats	female (n=39)			male (n=39)			Residual error	Explained variance
	Parameters	A	k	B	A	k		
PH	94.5 [81–108]	0.069 [0.050–0.095]	33.4 [21.1–43.8]	68.9 [49.7–98.3]	0.055 [0.024–0.106]	35.2 [2.9–52.34]	13.1	83.1%
ETP	352 [311–393]	0.069 [0.052–0.091]	72.4 [33.5–105.8]	255 [194–344]	0.054 [0.027–0.094]	69.5 [26.7–125.5]	40.9	87.5%

Table 2. Pooled PPP samples of 3 male rats obtained 24h after difethialone intoxication were tested by TGT with or without complementation with pooled normal rat plasma (PNrP) at different PPP/PNrP ratios (v/v) (9/1, 4/1, 7/3, 1/1 and 1/3). TGT were triggered with PPP reagent (5pM tissue factor). LT corresponds to the time necessary to reach 10nM human thrombin equivalent and ETP the area under thrombin concentration curve following coagulation activation. As PNrP fraction increased, LT values progressively decreased while ETP increased up to reach that of PNrP alone.

Parameters	Times to halving (hours) [95% CI]				p value
	Both genders (n=78)	Females (n=39)	Males (n=39)		
ETP	10.5h [8.2; 13.6]	10.1 [7.8; 13.1]	12.7 [8.7; 18.9]		0.1922
PH	10.4h [7.8; 14.1]	9.9 [7.4; 13.5]	13.1 [8.4; 20.6]		0.1808

Table 3. Time to halving ETP and PH after difethialone intoxication of male and female rats. Times to halving ETP and PH were calculated using k value of each exponential decay model. Mean with the 95% CI were reported for female and male rats and showed no significant difference between genders (p=0.1922 for ETP and p=0.1808 for PH). Common value was consequently estimated at 10.5h for ETP and 10.4h for PH after VKA intoxication. p value : Comparison between females and males.

with previous results obtained by our group using PT, activated partial thromboplastin time and clotting factor activities (FVII, FX) measurements. Early after intoxication, thrombin generation rapidly decreased over time resulting in an exponential decay of PH and ETP, in both genders. Experimental data fitted well with the decay models as shown by >80% of the total variance explained by the models. On the opposite, we have already reported that following intoxication PT significantly increases only after remaining unchanged for 2h in males and 8h in females [7]. Our study therefore outlines the low sensitivity of TP compared to TGT for an early detection of hypo-coagulability in rats.

Interestingly, ranking ETP and PH parameters along with vitamin K dependent factor decays based on the time to halving values suggested that ETP and PH mostly reflected FII level. Since FVII and FX added by PNrP complementation already normalized LT and TTP, they could not further decrease ETP and PH. TGT is a valuable tool allowing PD evaluation of VKA rodenticides in one test instead of a panel of four single clotting factor activity assays. Noteworthy, PH and ETP reached a plateau around 48h after intoxication, corresponding to the effect of the functional factor amounts brought by the added PNrP at a fixed ratio. Since VKA indirectly exert their anticoagulant effect by targeting vitamin K 2,3-epoxide reductase complex subunit 1, they do not inhibit functional factors added by the complementation, thus not hampering the kinetics study.

Our study has some limitations. Firstly, intoxicated rat samples were complemented with PNrP obtained from only male rats. Female rats are known to be hypercoagulable,

therefore this complementation in PNrP from male rats might reduce the difference in basal coagulation state between the two genders. Yet ETP and PH values at T0 remained significantly higher in female compared to male rats, thus these results suggest that our TGT protocol is well adapted to study the coagulability state in rats. Secondly, temporal parameters of the TGT, i.e. LT and TTP, remained stable over time probably due to PNrP complementation providing sufficient amounts of functional factors to restore clotting initiation, thus making their interpretation inadequate, unlike ETP and PH. Thirdly, in the present paper, we studied difethialone as rodenticide; however, we can reasonably consider that the adapted TGT can be applied to the PD study of other VKA rodenticides since they all inhibit vitamin K 2,3-epoxide reductase complex subunit 1 and that difethialone is one of the most potent inhibitor recently developed for rat control.

In conclusion, we propose TGT as a reliable additional tool to evaluate rodenticide PD and efficacy in rodents control strategy. TGT allows global evaluation of hypercoagulability state at baseline as well as early evaluation of VKA rodenticide anticoagulant effect in rats contrary to traditional tests.

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Chapitre 6

Analyse rétrospective et perspective

6.1 Analyse de l'activité de recherche

En raison d'un tempérament curieux et d'une tendance à m'impliquer là où mes compétences ou ma force de travail peuvent être utiles, ma participation aux travaux de l'unité a été diversifiée, au risque de manquer de lisibilité dans le parcours de ma carrière. Toutefois, cette implication étendue m'a permis, au fil des années, de me spécialiser dans certains secteurs et de proposer aujourd'hui mes propres thématiques de recherche. Dans ce qui suit, je présente les points clés qui ont jalonné ma progression scientifique et influencé ma manière d'encadrer la recherche, à travers sept publications et un poster.

6.1.1 Construction scientifique

6.1.1.1 Trouver sa place

Bien que le devoir de répondre aux attentes de la société ait été un guide majeur dans ma décision de rejoindre cette unité de recherche et dans le choix de mes thématiques, cette orientation n'est pas en soi une compétence déterminante pour se faire une place au sein d'une unité de recherche. En effet, cette compétence s'acquiert progressivement par des approches originales qui apportent une valeur ajoutée dans l'abord des problématiques de recherche.

En rétrospective, l'une de mes premières initiatives majeures en recherche a été prise dans le cadre de la préparation et de la rédaction du chapitre présenté en section 5.2.1.1. Cette publication, la première en dehors de ma thèse, devait initialement être une revue bibliographique peu originale, traitant de manière générale les empoisonnements par les anticoagulants anti-vitamine K (AVK) chez l'humain et les animaux non cibles, qu'ils soient domestiques ou sauvages. Après discussion, j'ai proposé et effectué une analyse sur neuf années de données du Centre National d'Informations Toxicologiques Vétérinaires de Lyon (CNITV). Ces données, constituées de 150 000 appels dont 10% concernaient des

AVKs, ont permis d'apporter une part d'originalité à cette publication et de mettre en lumière les répercussions positives de la réglementation sur les boîtes d'appâtage en termes de réduction du nombre de cas d'exposition chez les animaux domestiques.

Cette initiative a contribué à me positionner au sein de l'unité de recherche comme l'un des référents en matière d'analyse de données, notamment lorsque celles-ci requièrent, souvent en raison de leur taille ou de leur complexité, l'utilisation du langage de programmation 'R'.

6.1.1.2 Développer une approche originale

Cette approche analytique a été mise en évidence dans un second article, présenté en section 5.3.2.2. Cet article décrit la pharmacocinétique des quatre stéréoisomères de la diféthialone chez le rat mâle. Bien que novateur, ce n'était pas de mon initiative. La problématique qui s'est posée lors de la rédaction concernait l'infinité de possibilités offertes par ces quatre stéréoisomères. En effet, chacun d'entre eux présentait une demi-vie hépatique différente, allant de 6 à 82 heures, tout en ayant une constante d'inhibition identique. Ceci ouvrait la possibilité de réduire la rémanence de l'AVK en sélectionnant un stéréoisomère à demi-vie courte, mais au détriment de l'efficacité, mesurée par l'aire sous la courbe (AUC). Pour trouver le meilleur compromis, un graphique a été généré, mettant en relation chaque rémanence avec l'AUC résultant du mélange optimal des stéréoisomères (voir figure 6.1).

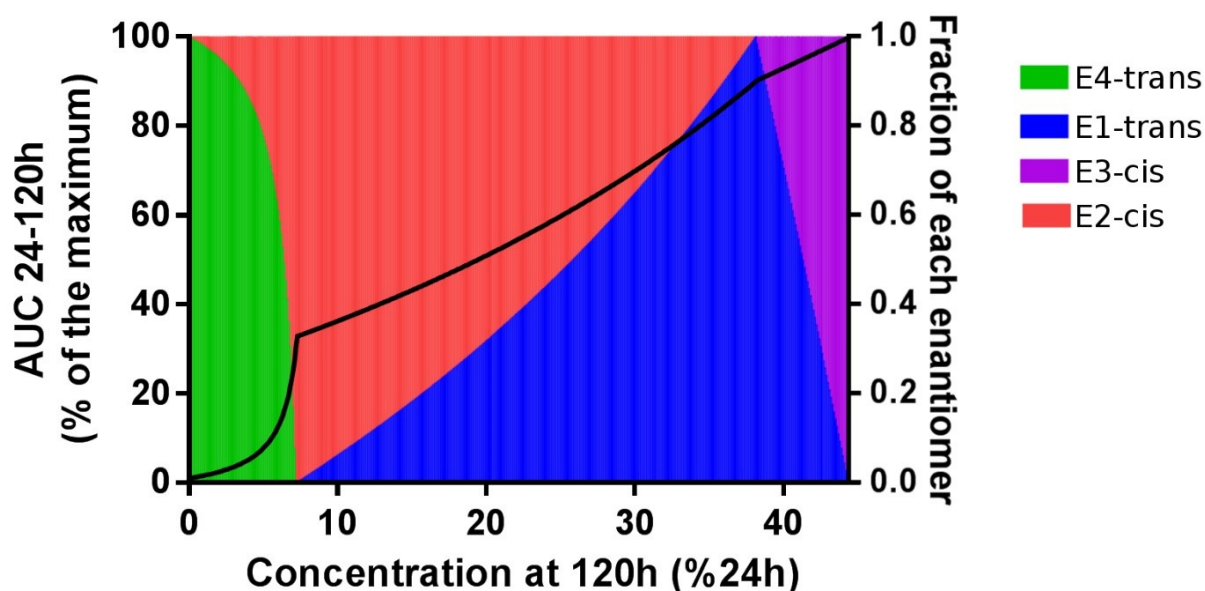


FIGURE 6.1 – Évolution de l'AUC de la diféthialone entre 24h et 120h (courbe et axe de gauche), simulée avec le meilleur mélange d'appât des quatre stéréoisomères de la diféthialone (zone colorée et axe de droite), pour une concentration résiduelle fixe de la diféthialone à 120h (en pourcentage de la concentration à 24h).

Bien que cette approche présente de nombreuses limites, notamment l'absence de prise

en compte des contraintes de synthèse, des interactions entre les stéréoisomères et de la variabilité individuelle, elle occupe une place particulière dans mes travaux. Elle ouvre la voie à une nouvelle méthode complémentaire, augmentant la valeur des recherches *in vivo*. L'objectif est d'inférer les résultats possibles *in vivo*, non pour les remplacer entièrement, mais pour ne réaliser que les tests les plus pertinents, sauf si les prédictions s'avèrent inexactes. De plus, l'ajout de ce niveau d'abstraction permettrait de tester de nouvelles hypothèses, toujours en confrontation avec la réalité.

6.1.2 Étendre vers une thématique propre

À partir des ébauches d'approche des rodenticides par la modélisation réalisées précédemment, il serait intéressant de développer des modèles plus complexes pour simuler leur action sur les rongeurs. Ceci permettrait d'estimer les chances de réussite d'un test d'homologation ou les effets d'un AVK sur une espèce non-cible. Cette méthode pourrait contribuer à surmonter les obstacles majeurs limitant l'étude des rodenticides : **i**/ la diversité des molécules ayant des propriétés pharmacocinétiques différentes, même si elles partagent un mode d'action similaire; **ii**/ la diversité des espèces à étudier, y compris les espèces protégées; **iii**/ l'acceptabilité sociétale de l'expérimentation animale. Ces contraintes nous empêchent d'avoir une approche globale de la problématique des AVKs avec des moyens raisonnables, qu'ils soient financiers ou éthiques.

Cette approche numérique est déjà utilisée depuis de nombreuses années en médecine humaine, où elle permet d'évaluer les risques associés à l'administration de traitements à base d'AVK et d'en estimer le dosage^{1,2}. Ces modèles prennent en compte de nombreux paramètres, tels que le génotype du patient, son sexe, et son âge. Dans notre contexte, la problématique est plus vaste car elle intègre de nombreuses espèces et un plus grand nombre de molécules.

Le nouvel axe que je souhaite insuffler au sein de l'équipe est de valoriser l'ensemble des données provenant de différents champs (études de terrain, de laboratoire, etc.) par la modélisation, dans le but d'enrichir la compréhension des AVKs. Il est nécessaire de prioriser les axes pour lesquels le plus de données sont disponibles, afin de faciliter la construction et la validation d'un modèle. Par la suite, ce modèle pourra être étendu dans les domaines où de l'inférence de paramètres est attendue.

Ainsi, le choix a été fait de se concentrer sur les tests d'homologation en laboratoire. Ces tests offrent un cadre standardisé : les rongeurs sont hébergés individuellement et se voient proposer pendant 5 jours un appât contenant le rodenticide (période d'exposition), avant d'être suivis pendant 16 jours supplémentaires. Ce travail s'inscrit dans la continuité de mes études précédentes, notamment deux d'entre elles qui portent sur l'évolution de biomarqueurs de la pharmacodynamique des AVKs en conditions expérimentales^{3,4}, ainsi que celles qui décrivent la pharmacocinétique des AVK^{5,6}.

À partir des paramètres pharmacocinétiques déterminés expérimentalement, nous construisons des modèles pharmacocinétiques et pharmacodynamiques (PKPD), comme celui présenté ci-dessous en équation 6.1. Ce modèle est divisé en deux phases : la pharmacocinétique hépatique de l'AVK et la cinétique d'un facteur de coagulation qui permettra de déterminer la mortalité. Pour la cinétique de l'AVK, dont la concentration en fonction du temps est représentée par $C(t)$, nous avons opté pour un simple modèle mono-compartimental. La phase d'absorption est négligée et remplacée par C_{\max} théorique, proportionnel à la dose absorbée (proportion obtenue par les expérimentations), et la cinétique d'élimination est déterminée par le coefficient k_{el} .

Pour le facteur de coagulation, nous avons choisi de nous concentrer sur l'activité du facteur 2 plasmatique en raison de sa corrélation avec le temps de prothrombine³. La régénération est définie en fonction de l'activité du facteur 2 à l'équilibre $F2_{\max}$, et du coefficient d'élimination du facteur 2 k_{elF2} . Cette régénération est modulée par une fonction sigmoïde dépendant de la concentration hépatique en AVK. C_{i50} est la concentration hépatique d'AVK qui divise par deux la régénération du facteur 2. La mortalité est définie par une baisse de 99% de l'activité du facteur 2, pour correspondre à la cinétique de mortalité pour une unique dose d'AVK.

$$\begin{aligned} \frac{dC(t)}{dt} &= -k_{el} \times C(t) \\ \frac{dF2(t)}{dt} &= \frac{k_{elF2} \times F2_{\max}}{1 + \left(\frac{C(t)}{C_{i50}}\right)^\beta} - k_{elF2} \times F2(t) \end{aligned} \quad (6.1)$$

L'objectif de ce modèle simple est de déterminer si, avec les informations disponibles dans la littérature et les données de consommation d'appât, il est possible d'ajuster un modèle qui estime les chances de réussite d'un essai et les concentrations terminales au jour de la mort en utilisant des simulations de Monte-Carlo. Les résultats des simulations seront comparés aux données réelles. D'autres modèles plus complexes qui prennent en compte l'absorption ou des probabilités de mortalité sont également envisagés. Les études des modèles sont en cours.

Au-delà de l'enjeu de prédire l'efficacité d'un rodenticide, l'approche par modélisation et simulation vise à mieux comprendre la pharmacocinétique dans les populations de rongeurs afin d'évaluer les stratégies de réduction de l'écotoxicité des AVKs. Elle pourrait également permettre d'inférer des données sur des espèces difficilement étudiables, comme les rapaces. Cependant, pour généraliser l'utilisation de cet outil au-delà des tests standards, il est nécessaire d'acquérir de nouvelles données concernant d'autres espèces ou des mutations résistantes. Deux demi-bourses de thèse ont été obtenues pour ce projet, afin de créer un modèle PKPD basique sur les rongeurs et de l'étendre aux rapaces.

L'obtention de financements dans ce domaine n'est pas toujours aisée. Bien que l'aspect éthique soit évident, les retombées scientifiques et économiques sont plus limitées. Des appels à projets français sur la thématique des 3 Rs existent, portés par le GIS FC3R^a. Une demande de financement sera renouvelée cette année, en prenant en compte les remarques précédentes pour améliorer l'architecture du projet. D'autres pistes de financements sont également envisagées, notamment auprès des autorités, car les approches de modélisation et simulation pourraient aider à détecter les fraudes dans les dossiers.

6.1.3 Encadrement

Les projets de recherche m'ont permis d'encadrer des étudiants à différents niveaux (liste des encadrements en annexe section 9), dont trois doctorants : Meg Anne Moriceau (20%, 2018-2021), Antoine Rached (50%, 2019-2022), et Flore Viillard (70%, débutée en mai 2023).

6.1.3.1 Initier l'encadrement

On ne naît pas encadrant, on le devient souvent en apprenant de ses erreurs. Ma première expérience en tant qu'un des encadrants principaux était pour l'article de revue de la littérature Rached et al. 2020 (section 5.2.2.1⁷). Cet article aborde la problématique qui nous affecte dans les études d'écotoxicité : différencier l'exposition de l'intoxication aux anticoagulants chez les animaux sauvages. L'enjeu est l'attribution de la causalité de la mort. Actuellement, pour identifier une intoxication aux AVKs, l'approche la plus courante est la mesure des résidus de rodenticides anticoagulants dans le foie ou le plasma d'animaux morts ou intoxiqués présentant des symptômes cliniques. Cependant, la littérature manque actuellement d'une valeur seuil de concentration hépatique ou plasmatique pour différencier l'exposition de l'intoxication. Bien que certains auteurs aient fait des propositions de valeurs seuil⁸, celles-ci sont assez empiriques et ne prennent pas suffisamment en compte la variation des propriétés pharmacologiques inter- et intra-espèces. Il est nécessaire d'identifier des biomarqueurs qui permettraient de pointer l'action des AVKs dans les différentes espèces. Dans cette revue, un aperçu des paramètres toxicodynamiques et toxicocinétiques des rodenticides anticoagulants dans différentes espèces animales est présenté. Nous examinons différents types de biomarqueurs utilisés pour caractériser et différencier l'exposition et les effets toxiques des rodenticides anticoagulants, en montrant les forces et les faiblesses de chacun. Enfin, nous décrivons de nouveaux biomarqueurs possibles et mettons en évidence leurs capacités.

Ce travail colossal d'analyse de la littérature (224 articles étudiés) a été effectué principalement par mes deux doctorants, Mme Moriceau et M. Rached. Malgré des propositions de voir l'article durant sa rédaction, ils avaient décidé d'avancer en autonomie, si bien

a. Groupement d'intérêt scientifique, centre français de 3R

que l'article n'a été transmis que deux semaines avant la date de soumission (publication invitée pour un numéro spécial). Il est alors apparu que l'article nécessitait encore une grande quantité de travail pour pouvoir être soumis, notamment en raison de défauts rédactionnels et de structure. Avec l'aide du Dr. Lattard, un plan a été redéfini, et nous avons organisé un plan de travail et de relecture intensif pour pouvoir soumettre une publication de qualité dans les temps. Cela a été possible grâce à la grande qualité des notes qu'avaient prises les doctorants sur les articles.

Ce travail m'a appris à mieux doser mon encadrement, notamment dans les premières phases de préparation des articles. À présent, le travail des étudiants chercheurs sous ma responsabilité est plus jalonné, avec des validations à chacune des étapes pour éviter qu'ils ne partent dans de mauvaises directions.

6.1.3.2 Transmettre

Précédemment, l'importance de l'article Lefebvre et al. 2020 dans l'avancée de ma réflexion a été soulignée. La publication Rached et al. 2022, qui est la continuité du premier, constitue également un jalon important dans ma carrière. Dans cet article, l'étude de la pharmacocinétique des différents stéréo-isomères de la diféthialone est étendue aux femelles et aux souris. Bien que nous nous attendions à des différences entre les sexes et les espèces, notamment en raison de la littérature existante, nous supposions que le classement relatif des demi-vies et des AUC serait conservé. Cependant, l'expérience nous a montré le contraire, ajoutant ainsi une complexité au modèle précédemment établi. Des hypothèses simplificatrices, telles qu'une absorption indifférenciée entre les stéréoisomères, ont dû être remplacées. Le modèle reste simple et est loin de celui développé en 6.1.2, mais il a permis la création de diagrammes facilitant une analyse graphique des possibilités offertes par les différents mélanges dans les différentes espèces (figure 6.2). Cela permet de tenir compte soit du souhait d'un appât commun pour les rats et les souris, soit à l'inverse d'un appât plus sélectif pour les rats et moins toxique pour d'autres petits mammifères (point qui sera à confirmer par d'autres études). Cet article souligne les limites de la transposition de la pharmacocinétique d'une espèce à l'autre, et même d'un sexe à l'autre.

En termes d'encadrement, en confiant la rédaction de cet article à M. Rached, il a été nécessaire de lui transmettre et de faciliter son appropriation des réflexions issues du premier article. Cela a permis de construire ensemble une approche plus complète et plus nuancée que la précédente. De même, cet article a été l'occasion de le former aux bases de l'analyse pharmacocinétique. Cette transmission des approches, des réflexions et des méthodes constitue, à mon sens, le succès de l'encadrement de ce travail.

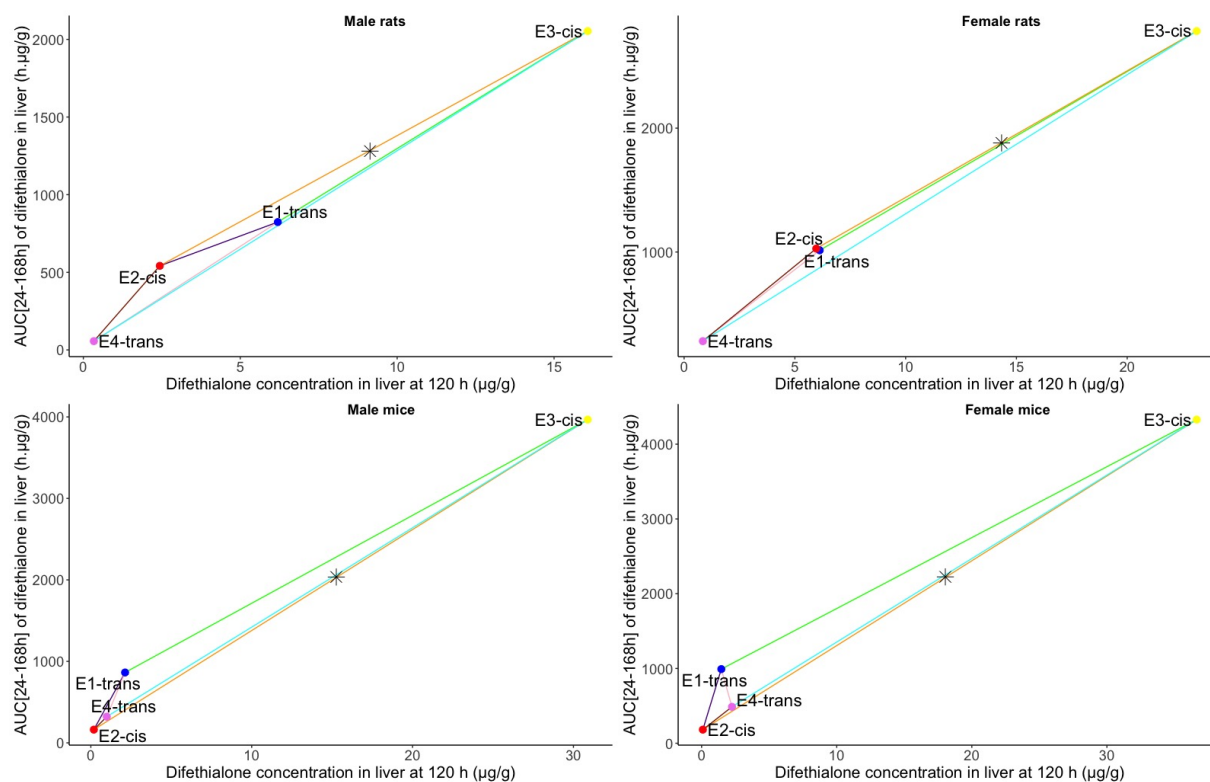


FIGURE 6.2 – $AUC_{24-168h}$ et concentration de diféthialone à 168h, obtenues par calcul après une administration théorique de 3 mg/kg d’une paire de stéréoisomères de diféthialone. Les points représentent les valeurs pour un seul stéréoisomère. Les lignes représentent les valeurs obtenues avec un mélange de stéréoisomères liés. Le point étoilé représente les résultats obtenus avec le diféthialone commercial actuel.

6.1.3.3 Encadrer en autonomie

Dernièrement, j’ai encadré un article en autonomie sur le sujet de la nutrition (Viallard et al. 2023, section 3.3.5). Cet article reprend l’étude réalisée par Flore Viallard lors de son stage de fin d’études dans le cadre de sa formation d’ingénieur. L’objectif était de quantifier les modifications comportementales induites par la mise en place d’un régime dit “sans fruit” pour cinq espèces différentes de primates. En effet, les fruits cultivés, qui ont été retirés dans cette étude, contiennent des sucres à fort pouvoir sucrant, responsables de comportements addictifs chez les primates. L’étude démontre que le remplacement de ces fruits cultivés par des légumes permet d’améliorer le comportement et le bien-être des animaux, notamment pendant la prise alimentaire.

Mon inclusion dans cette étude fut assez tardive, le plan expérimental ayant déjà été programmé. Mon rôle en tant qu’encadrant principal s’est principalement concentré sur l’analyse des données, la bibliographie et la rédaction de l’article. Ce premier article en nutrition a été l’occasion de mettre en pratique l’expérience acquise en nutrition et en encadrement ces dernières années. Cependant, la principale difficulté résidait dans le fait de ne pas avoir été impliqué dès la mise en place de l’étude et dans sa réalisation. Ceci

est principalement dû au fait qu'à l'époque, la production d'une publication n'était pas prévue. Ainsi, des biais évitables ont persisté. De même, lors de la rédaction, des incompréhensions sur la manière d'effectuer les mesures ont ralenti son avancée, sans entacher sa sincérité. À présent, et notamment en considérant l'encadrement de la thèse CIFRE qui se fait en partie à distance (voir section 3.1.2), je prévois d'assurer un suivi plus rigoureux, tant en distanciel qu'en présentiel. Ceci afin d'avoir une meilleure prise de conscience des conditions opérationnelles, de lever les biais le plus tôt possible, et surtout pour assurer mon accessibilité au doctorant.

6.2 Analyse générale et perspective

Le choix initial de dissocier la thématique d'enseignement de celle de la recherche a entraîné un surcroît de travail, nécessitant le développement de compétences dans deux domaines distincts. D'autant plus que les lacunes dans la matière de la nutrition des carnivores ont exigé le développement d'un enseignement et d'une expertise non présents au sein de l'école. Certes, il aurait été plus aisé de me concentrer uniquement sur la thématique nutritionnelle en réalisant une résidence dans ce domaine, une formation auprès d'un spécialiste du collège européen. Cette voie aurait permis de mieux préparer le développement de la partie nutrition. Cependant, la préparation de cette spécialité aurait pris entre 3 et 6 ans, dont la moitié du temps se serait déroulée dans une autre université. Ainsi, le développement de la nutrition clinique sur l'établissement aurait été retardé d'autant, de même que mon investissement dans la recherche. Cette stratégie présentait des risques, notamment celui de l'isolement professionnel vis-à-vis des collègues européens et français, et de ne pas fournir un enseignement à la pointe des avancées. Par conséquent, il était nécessaire d'adopter une attitude volontariste pour exister et participer activement à la communauté nutritionnelle. On pourrait arguer, à raison, que cet objectif aurait été plus facilement atteint en maintenant une activité de recherche en nutrition, comme prévu dans la fiche de poste initiale. Pour rappel, celle-ci attribuait mon poste à une équipe de recherche en nutrition humaine. Néanmoins, cette unité était éloignée tant physiquement qu'administrativement (absence de lien d'UMR et de collègues de VetAgro Sup dans l'équipe), ce qui compliquait le lancement d'une activité de recherche. Cela m'a incité à retourner dans l'unité de recherche où j'avais réalisé ma thèse.

Ici encore, cette décision comportait le risque de me maintenir dans une zone de confort. Toutefois, deux éléments ont présidé à cette décision : les responsabilités professionnelles et l'opportunité de co-encadrer des thèses. En ce qui concerne le premier point, lors de ma prise de poste en tant que maître de conférences, j'étais également vétérinaire sanitaire, responsable de la pharmacie et chargé du bien-être animal des animaleries de l'USC 1233, sans possibilité de transférer ces astreintes à moyen terme (des rôles que je continue d'assu-

mer). Quant au second point, l'USC 1233 présentait un flux de doctorants assez important pour la taille de l'unité, et la possibilité de co-encadrer des thèses dans les premières années de poste me permettrait d'acquérir des compétences dans ce domaine.

Comme montré précédemment, j'ai pu, au sein de cette unité de recherche, co-encadrer deux doctorants. Ce co-encadrement m'a permis de prendre progressivement des responsabilités au sein de l'encadrement doctoral et ainsi d'acquérir une expérience dans de bonnes conditions pour les doctorants. Cet investissement dans la recherche s'illustre également par des publications dans des positions majeures ; sur 20 publications, 15 sont signées soit parmi les deux premiers auteurs (11), soit parmi les deux derniers auteurs (4). Une transition vers des positions d'encadrement s'est opérée ces dernières années (figure 6.3) .

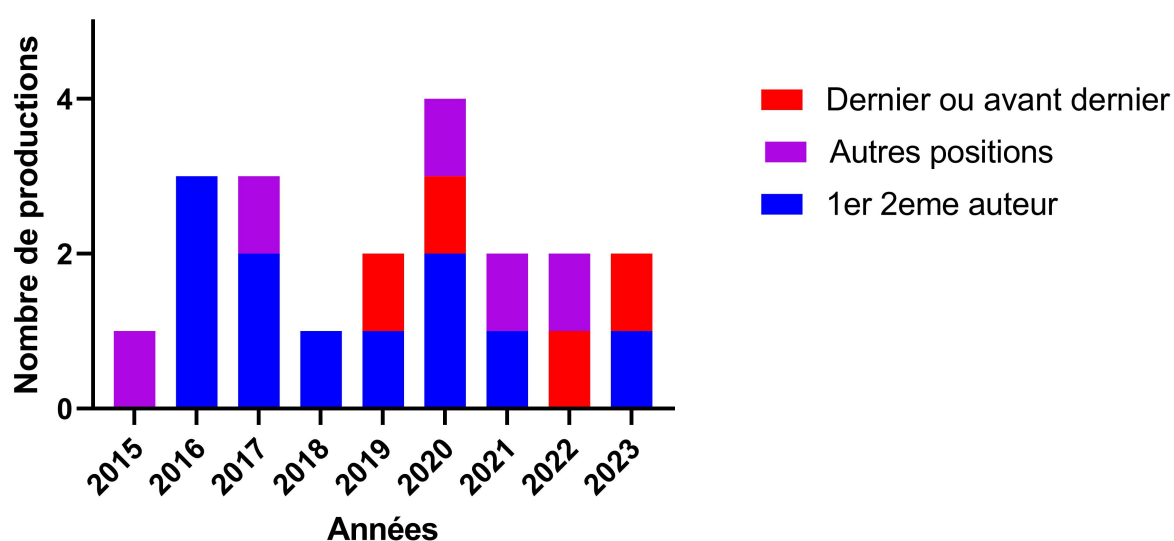


FIGURE 6.3 – Position dans les articles et chapitres de livre à comité de lecture en fonction du temps.

Ces productions m'ont permis d'engager une approche originale en adéquation avec les attentes sociétales. De par mes responsabilités au niveau des animaleries, j'ai pu mesurer les difficultés associées à la mise en place de l'approche 3R au sein de la thématique de l'unité, notamment dans le cadre de la soixantaine de tests réglementaires réalisés. J'avais d'ailleurs critiqué ces tests lors d'une conférence de l'EVPMC^b en 2019, en soulignant leurs limites⁹. En conjuguant ces problématiques avec un intérêt pour l'analyse de données, j'ai initié une thématique propre de modélisation/simulation de l'action des AVKs, en complément de l'approche animale, afin de réduire la sévérité des tests et d'en augmenter la qualité. Cette approche balbutiante doit à présent être consolidée. En effet, au-delà des simples tests réglementaires, la problématique de la recherche sur les rodenticides est bien plus vaste. Les limitations expérimentales sur les espèces non cibles nous incitent au

b. European Vertebrate Pest Management Conference

développement d'analyses innovantes, exploitant au mieux les données *in vitro* dans des modèles permettant une meilleure compréhension de l'écotoxicité des AVKs et offrant la possibilité de tester *in silico* de nouvelles approches pour limiter cette écotoxicité.

Bien qu'éloigné de la nutrition, les compétences d'encadrement acquises en recherche m'ont permis d'initier une activité exploratoire modeste dans ce domaine. Ceci s'est notamment manifesté par l'encadrement de 25 thèses d'exercice vétérinaires et d'un stage de fin de cursus d'ingénieur. En raison de l'absence de laboratoire, ces activités ont été plus modestes et se sont concentrées sur des études sociologiques, la création d'outils, et des études en parc zoologique. Elles ont néanmoins conduit à la réalisation de plusieurs posters¹⁰⁻¹², la création d'outils tels que des logiciels et des livres, et la rédaction d'un article de recherche¹³. Ces efforts ont abouti au lancement d'une thèse universitaire en nutrition des animaux sauvages, débutée en mai 2023. Il reste du chemin à parcourir, notamment pour augmenter le rayonnement international de cette thématique. Malgré des moyens limités, les compétences acquises dans un domaine où j'ai été guidé m'ont permis de développer une activité en adéquation avec mon domaine d'enseignement. La thèse récemment débutée permet de fusionner ces deux domaines, car elle intègre l'étude des campagnols terrestres pour mieux cerner leurs particularités nutritionnelles et préférences alimentaires.

Avec l'expérience acquise en nutrition et compte tenu du manque de recherche sur l'alimentation des animaux de compagnie et des animaux en zoo en France, la question de la création d'une équipe dédiée pourrait se poser. Toutefois, en l'absence d'un soutien financier solide de la part des pouvoirs publics, ce projet risquerait de dépendre uniquement de fonds privés, notamment de l'industrie du *petfood*, ce qui compromettrait l'indépendance que j'ai souhaitée et qui est attendue par la profession et les propriétaires. Cette démarche n'est donc pas la plus aisée pour garantir un avenir à cette initiative. Il est nécessaire de former un vivier de docteurs possédant les prérequis statutaires et les compétences pour postuler aux postes au sein des écoles vétérinaires. Cette formation ne peut se faire sans une équipe d'encadrement solide et un accès à un capital humain et technologique pour mener à bien les projets de thèse, ce que permet mon positionnement actuel au sein de l'USC 1233. J'ai ainsi pu accompagner mon ancien doctorant, M. Rached, dans un poste de nutrition animale à VetAgro Sup. Nous travaillons actuellement à renforcer ses compétences en nutrition et à développer les enseignements et expertises liés à cette chaire, notamment l'alimentation des équidés et la toxicologie végétale.

En conclusion, l'accueil de mon ancien doctorant en tant que collègue académique et l'initiation d'une première thèse universitaire en nutrition animale marquent une transition vers des responsabilités d'encadrement de plus en plus substantielles. Après avoir navigué de manière atypique entre les domaines de la nutrition et de la toxicologie, il devient impératif de consolider mon rôle dans les fonctions d'encadrement. L'objectif est double : d'une part, sécuriser et amplifier la démarche qui a permis à VetAgro Sup de regagner

une position significative dans le domaine de la nutrition des animaux de compagnie, et d'autre part, contribuer activement à l'innovation dans les méthodologies de gestion des populations de rongeurs. Cette orientation s'inscrit dans un engagement à fournir un service public d'enseignement et de recherche qui répond aux attentes sociétales.

6.3 Bibliographie

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- [2] Seongho KIM et al. "Simplified Warfarin Dose-response Pharmacodynamic Models". In : *Biomedical engineering : applications, basis, and communications* 27.1 (fév. 2015), page 1550001. ISSN : 1016-2372. DOI : 10.4015/S1016237215500015. (Visité le 04/09/2023) (cf. page 353).
- [3] Sébastien LEFEBVRE et al. "Monitoring of Antivitamin K-dependent Anticoagulation in Rodents – Towards an Evolution of the Methodology to Detect Resistance in Rodents". In : *Pesticide Biochemistry and Physiology* (2017). ISSN : 0048-3575. DOI : 10.1016/j.pestbp.2017.02.003. (Visité le 28/02/2017) (cf. pages 353, 354).
- [4] Georges JOURDI et al. "Thrombin Generation Test : A Reliable Tool to Evaluate the Pharmacodynamics of Vitamin K Antagonist Rodenticides in Rats". In : *Pesticide Biochemistry and Physiology* 146 (fév. 2018), pages 19-24. DOI : 10.1016/j.pestbp.2018.02.004 (cf. page 353).
- [5] Sebastien LEFEBVRE et al. "Origin of the Gender Differences of the Natural Resistance to Antivitamin K Anticoagulants in Rats". In : *Toxicology* (fév. 2016). DOI : 10.1016/j.tox.2016.02.002. (Visité le 18/10/2019) (cf. page 353).
- [6] Marlène DAMIN-PERNIK et al. "Management of Rodent Populations by Anticoagulant Rodenticides : Toward Third-Generation Anticoagulant Rodenticides". In : *Drug Metabolism and Disposition* 45.2 (fév. 2017), pages 160-165. ISSN : 0090-9556, 1521-009X. DOI : 10.1124/dmd.116.073791. (Visité le 29/10/2018) (cf. page 353).
- [7] Antoine RACHED et al. "Biomarkers Potency to Monitor Non-target Fauna Poisoning by Anticoagulant Rodenticides". In : *Frontiers in Veterinary Science* 7 (2020). ISSN : 2297-1769. DOI : 10.3389/fvets.2020.616276. (Visité le 11/01/2021) (cf. page 355).
- [8] I. (Ian) NEWTON et al. *Empirical Evidence of Side-Effects of Rodenticides on Some Predatory Birds and Mammals*. 1999 (cf. page 355).
- [9] Sebastien LEFEBVRE. "Rodenticide Bait Trials : How to Conciliate Scientific Relevance and 3R Principles. Comparison between European and USA Regulatory Requirements". In : *12th European Vertebrate Pest Management Congress*. Clermont-Ferrand, France, sept. 2019. (Visité le 15/12/2020) (cf. page 359).
- [10] E THIERCY et al. *Labelled Analytical Constituents of Commercial Dog Foods According to the Main Point of Sale. Are There Any Clues as to the Origin of Phosphorus ?* Sept. 2020. (Visité le 15/12/2020) (cf. page 360).

- [11] Sebastien LEFEBVRE, Denis GRANCHER et L ALVES DE OLIVEIRA. “You Said ”gastro-Intestinal” Food? Approach of Petfood Diversity during a Student-Conducted Consultation.” In : Turino, sept. 2019. (Visité le 27/02/2020) (cf. page 360).
- [12] Laurent ALVÈS DE OLIVEIRA, Denis GRANCHER et Sebastien LEFEBVRE. “Reflection and Proposition of a Tool for Teaching Small Animal Nutrition in Veterinary Courses”. In : *23. Congress of the European Society of Veterinary & Comparative Nutrition*. Turin, Italy, sept. 2019, np (cf. page 360).
- [13] Flore VIALARD et al. “Multi-Criteria Study on a Change to a Fruit-Free Diet Change in Cebidae and Cercopithecidae”. In : *Journal of Zoo and Aquarium Research* (2023) (cf. page 360).

Troisième partie

Annexes

Chapitre 7

Curriculum Vitae

Sébastien LEFEBVRE

PhD, Docteur vétérinaire



Age 33 ans
Établissement VetAgro Sup – Campus vétérinaire de Lyon
Discipline Nutrition alimentation animale
Grade Maître de conférences depuis 7 ans

Expérience professionnelle

Depuis septembre 2022:

Expert ANSES au Comité d'expert spécialisé Alimentation Animale

Depuis octobre 2015:

Maître de conférences en nutrition et alimentation animale.

Enseignements dans le cursus initial vétérinaire et les formations continues

Création d'un enseignement en nutrition clinique des carnivores domestiques.

Création d'un logiciel de calcul de ration pour carnivore domestique.

Recherche au sein de l'USC 1233 (18 articles, 2 chapitres de livres, 11 communications avec actes, 1 livre et 24 thèses vétérinaires encadrées)

Thèses d'université encadrées : 2 soutenues et 2 en cours

Juin 2015 :

Enseignant temporaire.

VetAgro Sup, Campus Vétérinaire de Lyon. (Responsable : Dr Laurent Alves de Oliveira).

Enseignement dans le cursus initial vétérinaire. (visites d'élevage et leurs évaluations).

Aout 2015 :

Stagiaire de recherches.

Institut fédéral de protection des plantes (Julius Kühn Institut), département de recherches sur les vertébrés. Münster (Allemagne).

Janvier 2015 à Juillet 2015 :

Stagiaire aux affaires réglementaires.

Société Merial (Lyon-France). Chargé des mises à jour et des extensions des autorisations de mise sur le marché (AMM) des vaccins porcins et bovins au niveau de la division Europe de la société.

Octobre 2014 à octobre 2015 :

Autoentrepreneur. Création de sites internet.

Mars 2012 à octobre 2015 :

Téléconseil téléphonique.

Centre antipoison vétérinaire de Lyon (C.N.I.T.V).

Autres expériences :

Depuis novembre 2012 :

VetBrain : Création d'une association pour le développement de solutions innovantes à destination des vétérinaires. Réalisation de nombreux projets dont un carnet de santé pour animaux de compagnie qui a reçu le prix de l'innovation remis par Véolia au Concours Campus Création (édition de 2013).

Diplômes et formations complémentaires

Diplômes et titres universitaires :

- 2016 Doctorat de biochimie**, Sujet : Etude de l'impact du genre sur la coagulation vitamine K dépendante chez le rat. Jury : Rapporteurs : Martine KAMMERER (ONIRIS) et Claude PETIT (ENVT). Examineurs : Léa PAYEN (Université Claude Bernard), Virginie SIGURET (Paris Descartes), Caroline MOREAU (Université de Rennes), Etienne BENOIT (VetAgro Sup) et Virginie LATTARD (VetAgro Sup).
- 2015 Doctorat en Médecine vétérinaire**
- 2015 Master en Sciences du médicament** (spécialité pharmacie vétérinaire).
- 2015 Certificat d'aptitude à l'expérimentation animale** – niveau I concepteur
- 2014 Diplôme d'études fondamentales vétérinaires**

Formations complémentaires :

- 2017 Formation à la pédagogie du ministère de l'agriculture**
- 2014 Building Mobile Experiences** (21W.789x). Organisé par MITx sur la plateforme edX. (cours en ligne, auditeur libre).
- 2014 Introduction to computer science** (CS50x). 2014. Organisé par Harvardx sur la plateforme edX . (cours en ligne, auditeur libre).
- 2013 Gestion de Projets**. 2013. Organisé par l'école Centrale de Lille. (cours en ligne, auditeur libre).
- 2013 Pass'entrepreneur**. 2013. Organisé par la fondation pour l'Université de Lyon. Certificat de réussite (formation du concours d'entrepreneuriat Campus Création).

Langues

- Français** : langue maternelle
- Anglais** : Courant (obtention du TOEIC (845/990) en avril 2015)

Informatique

- Maîtrise des **logiciels de bureautique traditionnels** : Microsoft Word® (traitement de texte), Microsoft Excel® (tableur), Microsoft PowerPoint® (diaporama), Zotero (gestion informatisée des références bibliographiques).
- Maîtrise du **langage de bureautique et d'édition avancé** : LaTeX.
- Maîtrise de l'utilisation de **logiciels de statistiques** : R.
- Maîtrise des **langages de programmation**, dont Java (Réalisation de nombreux logiciels et applications mobiles)

Publications : <https://cv.archives-ouvertes.fr/sebastien-lefebvre>

Chapitre 8

Liste des productions

8.1 Article dans des revues à comité de lecture

T : article de thèse, E : encadrement, (Impact Factor). Souligné : les doctorants encadrés.

1. T (3.04) Louvet, M.-S. ; Gault, G. ; **Lefebvre, S.** ; Popowycz, F. ; Boulven, M. ; Besse, S. ; Benoit, E. ; Lattard, V. ; Grancher, D. Comparative inhibitory effect of prenylated coumarins, ferulenol and ferprenin, contained in the ‘poisonous chemotype’ of *Ferula communis* on mammal liver microsomal VKORC1 activity. *Phytochemistry* 2015, 118, 124–130, doi :10.1016/j.phytochem.2015.08.012.
2. T (4.09) **Lefebvre, S.** ; Rannou, B. ; Besse, S. ; Benoit, E. ; Lattard, V. Origin of the gender differences of the natural resistance to antivitamin K anticoagulants in rats. *Toxicology* 2016, 344–346, 34–41, doi :10.1016/j.tox.2016.02.002.
3. T (1.05) Trouchon, T. ; **Lefebvre, S.** A Review of Enrofloxacin for Veterinary Use. *OJVM* 2016, 06, 40–58, doi :10.4236/ojvm.2016.62006.
4. (3.23) 4. Damin-Pernik, M. ; Espana, B. ; Lefebvre, S. ; Fourel, I. ; Caruel, H. ; Benoit, E. ; Lattard, V. Management of Rodent Populations by Anticoagulant Rodenticides : Toward Third-Generation Anticoagulant Rodenticides. *Drug Metab Dispos* 2017, 45, 160–165, doi :10.1124/dmd.116.073791.
5. (2.75) **Lefebvre, S.** ; Hascoët, C. ; Damin-Pernik, M. ; Rannou, B. ; Benoit, E. ; Lattard, V. Monitoring of antivitamin K-dependent anticoagulation in rodents – Towards an evolution of the methodology to detect resistance in rodents. *Pesticide Biochemistry and Physiology* 2017, 138, 29–36, doi :10.1016/j.pestbp.2017.02.003.
6. (2.75) Georges, J. ; **Sebastien, L.*** ; Bernard, L.B. ; Emmanuel, C. ; Pascale, G. ; Virginie, L. ; Virginie, S. Thrombin generation test : A reliable tool to evaluate the pharmacodynamics of vitamin K antagonist rodenticides in rats. *Pesticide Biochemistry and Physiology* 2018, 146, 19–24, doi :10.1016/j.pestbp.2018.02.004.

7. (4.54) Debaux, J.V.; Hammed, A.; Barbier, B.; Chetot, T.; Benoit, E.; **Lefebvre, S.**; Lattard, V. Establishment of the Variation of Vitamin K Status According to Vkorc1 Point Mutations Using Rat Models. *Nutrients* 2019, 11, 2076, doi :10.3390/nu11092076.
8. (2.2) Gault, G.; **Lefebvre, S.**; Benoit, E.; Lattard, V.; Grancher, D. Variability of ferulenol and ferprenin concentration in French giant fennel (*Ferula* sp.) leaves. *Toxicon* 2019, 165, 47–55, doi :10.1016/j.toxicon.2019.04.010.
9. (3.57) Chetot, T.; Mouette-Bonnet, M.; Taufana, S.; Fourel, I.; **Lefebvre, S.**; Benoit, E.; Lattard, V. Differences in teratogenicity of some vitamin K antagonist substances used as human therapeutic or rodenticide are due to major differences in their fate after an oral administration. *Toxicology Letters* 2020, 333, 71–79, doi :10.1016/j.toxlet.2020.07.034.
10. (5.06) **Lefebvre, S.**; Fourel, I.; Chatron, N.; Caruel, H.; Benoit, E.; Lattard, V. Comparative biological properties of the four stereoisomers of difethialone, a second-generation anticoagulant rodenticide, in rats : development of a model allowing to choose the appropriate stereoisomeric ratio. *Arch Toxicol* 2020, 94, 795–801, doi :10.1007/s00204-020-02662-0.
11. E (4.79) Moriceau, M.-A.; **Lefebvre, S.**; Fourel, I.; Benoit, E.; Rattner, B.A.; Lattard, V. Accidental chlorophacinone exposure of lactating ewes : Clinical follow-up and human health dietary implications. *Food and Chemical Toxicology* 2020, 143, 111518, doi :10.1016/j.fct.2020.111518.
12. E (2.24) Rached, A.; Moriceau, M.-A.; Serfaty, X.; **Lefebvre, S.**; Lattard, V. Biomarkers Potency to Monitor Non-Target Fauna Poisoning by Anticoagulant Rodenticides. *Front. Vet. Sci.* 2020, 7, doi :10.3389/fvets.2020.616276.
13. E (10.75) Moriceau M.-A.; Sébastien Lefebvre, Isabelle Fourel, Etienne Benoit, Florence Buronfosse, et al.. Exposure of predatory and scavenging birds to anticoagulant rodenticides in France : Exploration of data from French surveillance programs. *Science of the Total Environment*, Elsevier, 2021, 810, pp.151291. DOI10.1016/j.scitotenv.2021.151291.
14. (8.43)Abi Khalil, R.; Barbier, B.; Fafournoux, A.; Mahamat, A.B.; Marquez, A.; Poissenot, K.; Keller, M.; Desvars-Larrive, A.; Fernandez-De-Simon, J.; Coeurdassier, M.; Benoit E.; **Lefebvre S.**; Pinot A.; Lattard V. Seasonal Diet-Based Resistance to Anticoagulant Rodenticides in the Fossorial Water Vole (*Arvicola Amphibius*). *Environmental Research* 2021, 200, 111422, doi :10.1016/j.envres.2021.111422.
15. E (5.06) Rached, A.; Lattard, V.; Fafournoux, A.; Caruel, H.; Fourel, I.; Benoit, E.; **Lefebvre, S.** Comparative Pharmacokinetics of Difethialone Stereoisomers in

Male and Female Rats and Mice : Development of an Intra- and Inter-Species Model to Predict the Suitable Formulation Mix. *Arch Toxicol* 2022, 96, 535–544, doi :10.1007/s00204-021-03210-0.

16. (1.6) Mahjoub, T. ; Krafft, E. ; Garnier, L. ; Mignard, A. ; Hugnet, C. ; **Lefebvre, S.** ; Fourel, I. ; Benoit, E. ; Lattard, V. Asymptomatic Anticoagulant Rodenticide Exposure in Dogs and Cats—A French and Belgian Rural and Urban Areas Study. *Front. Toxicol.* 2022, 4, 907892, doi :10.3389/ftox.2022.907892.
17. E (5.78) Rached, A. ; Mahjoub, T. ; Fafournoux, A. ; Barbier, B. ; Fourel, I. ; Caruel, H. ; **Lefebvre, S.** ; Lattard, V. Interest of the Faecal and Plasma Matrix for Monitoring the Exposure of Wildlife or Domestic Animals to Anticoagulant Rodenticides. *Environmental Toxicology and Pharmacology* 2023, 104033, doi :10.1016/j.etap.2022.104033.
18. E Viallard, F. ; **Lefebvre, S.** ; Petry, A. ; Vonfeld, I. ; Quintard, B. Multi-Criteria Study on a Change to a Fruit-Free Diet Change in Cebidae and Cercopithecidae. *Journal of Zoo and Aquarium Research* 2023.

* : article en co-premier auteur.

8.2 Publication de chapitre de livre, avec comité de lecture :

1. **Lefebvre, S.** ; Benoit, E. ; Lattard, V. Comparative Biology of the Resistance to Vitamin K Antagonists : An Overview of the Resistance Mechanisms. In *Anticoagulation Therapy* ; Basaran, O., Biteker, M., Eds. ; InTech, 2016 ISBN 978-953-51-2666-9.
2. **Lefebvre, S.** ; Fourel, I. ; Queffélec, S. ; Vodovar, D. ; Mégarbane, B. ; Benoit, E. ; Siguret, V. ; Lattard, V. Poisoning by Anticoagulant Rodenticides in Humans and Animals : Causes and Consequences. In *Poisoning - From Specific Toxic Agents to Novel Rapid and Simplified Techniques for Analysis* ; Malangu, N., Ed. ; InTech, 2017 ISBN 978-953-51-3681-1.

8.3 Communication avec Acte dans des congrès internationaux

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2. Fettata, S. ; Gault, G. ; **Lefebvre, S.** ; Benoit, E. ; Lattard, V. ; Grancher, D. Asphodel Exposure of Sheep in Algeria. In Proceedings of the Proceedings for the 10th International Symposium on Poisonous Plants (ISOPP) ; St Georges, United States, September 2018.
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8.4 Logiciels

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3. LEFEBVRE S. (2015-2020). VetNutri. Logiciel de calcul et d'analyse de rations, Windows, Mac et Linux. Deux mises à jours majeures par an. (remplacé par VetNutri 2, qui est une refonte totale)
4. LEFEBVRE S. (2016). Logiciel de gestion d'animalerie
5. LEFEBVRE S. (2021-2023). VetNutri 2. Logiciel de calcul et d'analyse de rations, Windows, Mac et Linux. Deux mises à jours majeures par an.

Chapitre 9

Liste des encadrements

9.1 Encadrements de stage

Nom	Niveau	Année	Titre
Kevin Valette	Pharmacie	2017	Etude de la régénération de la coagulation
Iris Deixonne	IUT	2018	La vitamine K chez le rat
Hélène Gatto	Pharmacie	2018	Etude de la régénération de la coagulation chez le rat femelle hypocoagulé après administration de vitamine K
Flore Viillard	Ingenieur	2021	Mise en place d'une ration sans fruit et étude des impacts sur la digestion et le comportement pour les primates au parc zoologique et botanique de Mulhouse

TABLE 9.1 – Stages d' IUT, de pharmacie ou d'ingénieur, encadrés ou en cours d'encadrement. Les années entre parenthèse indique une thèse en cours (année prévue de fin).

9.2 Encadrements de thèse d'université

Nom	Taux	Année	Titre
Meg-Anne Moriceau	20%	2021	Intérêt et exploration des marqueurs cliniques et biologiques de la toxicité primaire et secondaire des rodenticides anticoagulants
Antoine Rached	50%	2022	Exploration des pistes de gestion du risque écotoxicologique associé aux anticoagulants antivitaminaires K utilisés comme rodenticides
Flore Viillard	70%	(2026)	Etude de la régénération de la coagulation chez le rat femelle hypocoagulé après administration de vitamine K

TABLE 9.2 – Thèse d'université encadrés ou en cours d'encadrement. Les années entre parenthèse indique une thèse en cours (année prévue de fin).

9.3 Thèses vétérinaires

Nom	Année	Titre
Agathe Champetier	2018	Nutrition Et Cancer Chez Les Carnivores Domestiques : Création D'Un Guide Sur La Prise En Charge Nutritionnelle
Anne-Cécile Cael	2018	Réalisation D'Un Guide De Prise En Charge Du Surpoids Et De L'Obésité Chez Le Chien Et Le Chat À Destination Du Vétérinaire Et Du Propriétaire
Camille Trameni	2019	Evolutions Récentes De La Prise En Charge Du Chat Diabétique
Alyzée Galy	2019	Rédaction D'Un Guide À L'Attention Du Propriétaire Pour La Réalisation Pratique D'Une Ration MénagÈre Pour Un Chien
Charlotte Garot	2019	Nouvelles Tendances Nutritionnelles Chez Le Chat : Élaboration D'Un Guide Sur Les Différents Types De Rations
Alma Taleux	2019	Influence De La Ration Alimentaire À L'Entrainement Et En Course Sur Le Score Corporel, Les Performances Sportives Et La Prévalence D'Affections Chez Des Chiens De Traîneau : Exemple Lors De La Grande Odyssée
Marine Riffart	2020	Comparaison Des SystÈmes De Rationnement Français (INRA) Et Américain (NRC) Chez Le Cheval Et Le Poney
Ernelle Thiery	2020	Comparaison Des Aliments Industriels Physiologiques ComplÈts Pour Chiens Et Chats En Fonction Des Informations Fournies Par Leur Étiquetage, De Leur Prix Et De Leur Positionnement Mercatique
Corentin Treich	2020	Positionnement De La Structure Vétérinaire Libérale Vis-À-Vis De La Vente D'Aliments Pour Animaux De Compagnie
Tristan Michel	2021	Etude De L'Impact Du Type De Ration Sur Les Performances De Reproduction En Élevage Canin. Exemple Du Berger Australien
Gamou Gueye	2021	Démarche D'Utilisation Des Compléments Alimentaires Chez Le Cheval : Rédaction D'Un Guide Pratique
Léa Chevalier	2021	Perception De La Qualité Et Confiance Des Vétérinaires Praticiens Dans Les Aliments Industriels Pour Chien Et Chat : Une Étude Qualitative
Emilie Hauwiller	2021	Lecture Critique D'ouvrages De Vulgarisation Sur L'alimentation Commerciale Non Thérapeutique Des Chiens Et Des Chats De Compagnie
Simon Goddet	2022	Elaboration De Documents Supports Accompagnant La Prise En Charge Et Le Suivi Des Animaux De Compagnie En Nutrition
Solenne Dutreuil	2022	Mise En Place D'Un Système Permettant La Prescription D'Aliment Basée Sur Les Preuves En Cas De Diabète Sucré, D'Insuffisance Rénale Ou D'Arthrose Chez Le Chien
Quentin Mainfroy	2022	L'alimentation Sans Céréale Et Ses Conséquences Chez Le Chien Et Le Chat
Caroline Chambon	2023	Étude De L'Espérance De Vie Des Chiens Et Des Chats En France Entre 2003 Et 2020, Et Influence Des Conditions Socio-Économiques Des Propriétaires
Lucile Mange	2023	Étude De L'Évolution De La Médicalisation Des Chien Et Des Chats En France Entre 1997 Et 2020
Clelia Marra	2023	Aliments industriels physiologiques pour chiens et chats : analyse du crédit accordé aux informations fournies par le fabricant et a l'équilibre global de l'aliment, grâce a l'élaboration d'une grille d'évaluation de la créance
Marie Bouvier	(2024)	État Des Lieux Et Évolution De L'Alimentation Des Chiens Et Des Chats De 1997 À 2020
Laura Gioanni*	(2025)	État Des Lieux De La Vulgarisation Des Connaissances En Nutrition À Disposition Des Propriétaires De Carnivores Domestiques, Des Demandes Et Besoins De Ces Propriétaires Face À L'offre Grandissante Des Marques.
Maiwen Moreau	(2025)	Compréhension Des Facteurs De Risques Professionnels Et Personnels Motivant La Décision D'interrompre La Pratique Clinique Vétérinaire Chez Les Diplômés De Moins De 5 Ans. Étude Qualitative Au Sein Des Territoires Ruraux De La Région Auvergne Rhône-Alpes.
Vulcain Grosdenier	(2025)	Création d'un système d'analyse de ration à destination des primates du nouveau monde
Thomas Fernandez	(2025)	Création d'un système d'analyse de ration à destination des primates de l'ancien monde

TABLE 9.3 – Thèses vétérinaires encadrés ou en cours d'encadrement. Les noms d'étudiant en gras indiquent les thèses avec production originale et non uniquement bibliographiques. Les années entre parenthèse indique une thèse en cours (année prévue de fin). * Étudiante de l'ENVA