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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.

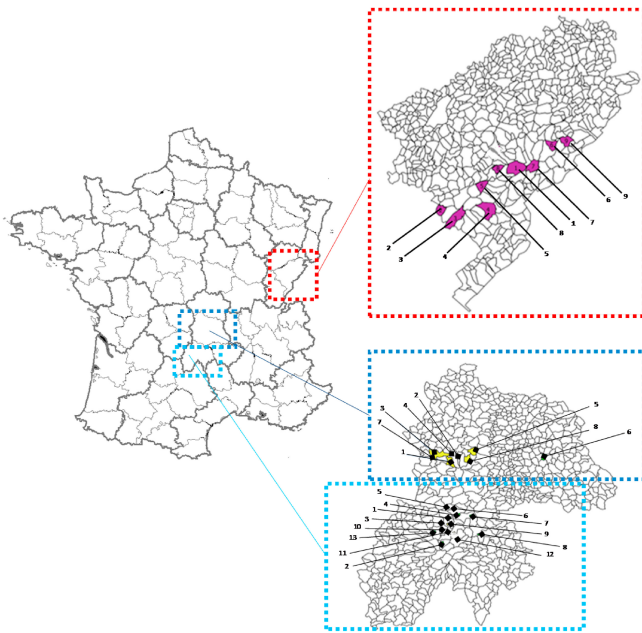


Figure 1. French regions where water voles were trapped. In red, the 9 sites of trapping in Jura Mountain; in dark blue, the 8 sites of trapping in Puy-de-Dôme in Massif central mountain; in light blue, the 13 sites of trapping in Cantal in Massif central mountain. (For interpretation of the references 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'-TAATCCCAGCACTTGGAAGG-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'-AGGGGGATTGGAATGTAAGG-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].

Yeasts 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 Laborotechnick® (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 at 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).

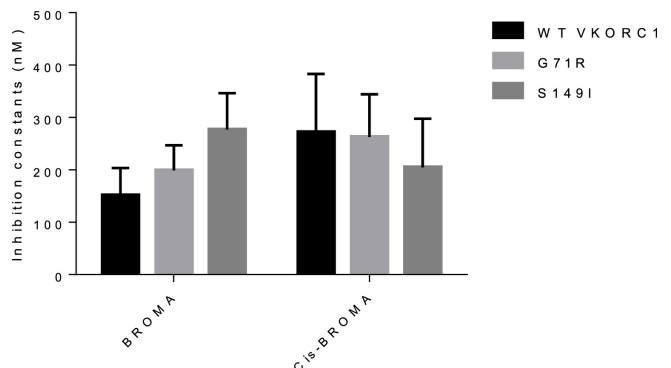


Figure 2. Constant of inhibition of wild type VKORC1 or its mutants G71R or S149I towards bromadiolone (composed of 85% Trans- and 15% Cis-isomers) or Cis-bromadiolone (composed of 4% Trans- and 96% Cis-isomers). Values are presented as mean ± SD. ANOVA analysis was used to compare results. $P < 0.05$ was the accepted level of significance.

2.2 Vitamin K concentrations in liver in *Arvicola amphibius* compared to *Rattus norvegicus*

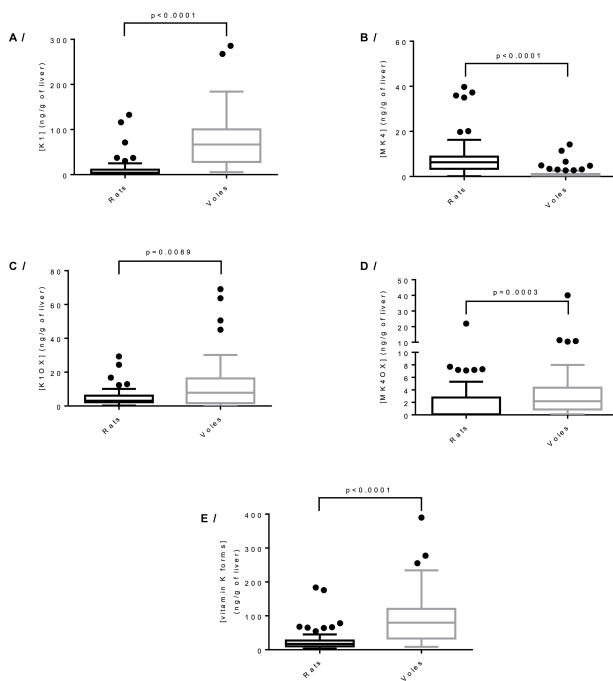
Vitamins K concentrations in liver - i.e., phylloquinone or vitamin K1 (K1) and menaquinone 4 or vitamin K2 (MK4), and their quinone or epoxide forms-, were determined in fossorial water voles, but also in urban brown rats as references. Sixty-five urban rats - 30 males and 35 females - captured in Vienna, 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					
Haplotype numbers		1/2	1/3	1/4	1/5	1/6	1/7	1/8	1/9
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).



	[K1] (ng/g)	[MK4] (ng/g)	[K1OX] (ng/g)	[MK4OX] (ng/g)	Vitamins K forms (ng/g)
Urban rats	11.57 [5.89-17.24]	8.17 [6.08-10.26]	4.80 [3.55-6.06]	1.90 [1.08-2.71]	26.44 [18.53-34.34]
Fossorial water voles	73.50 [60.23-86.77]	1.347 [0.81-1.88]	11.32 [8.16-14.48]	3.32 [2.15-4.50]	88.50 [72.36-104.6]

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.

Empty Cell	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.

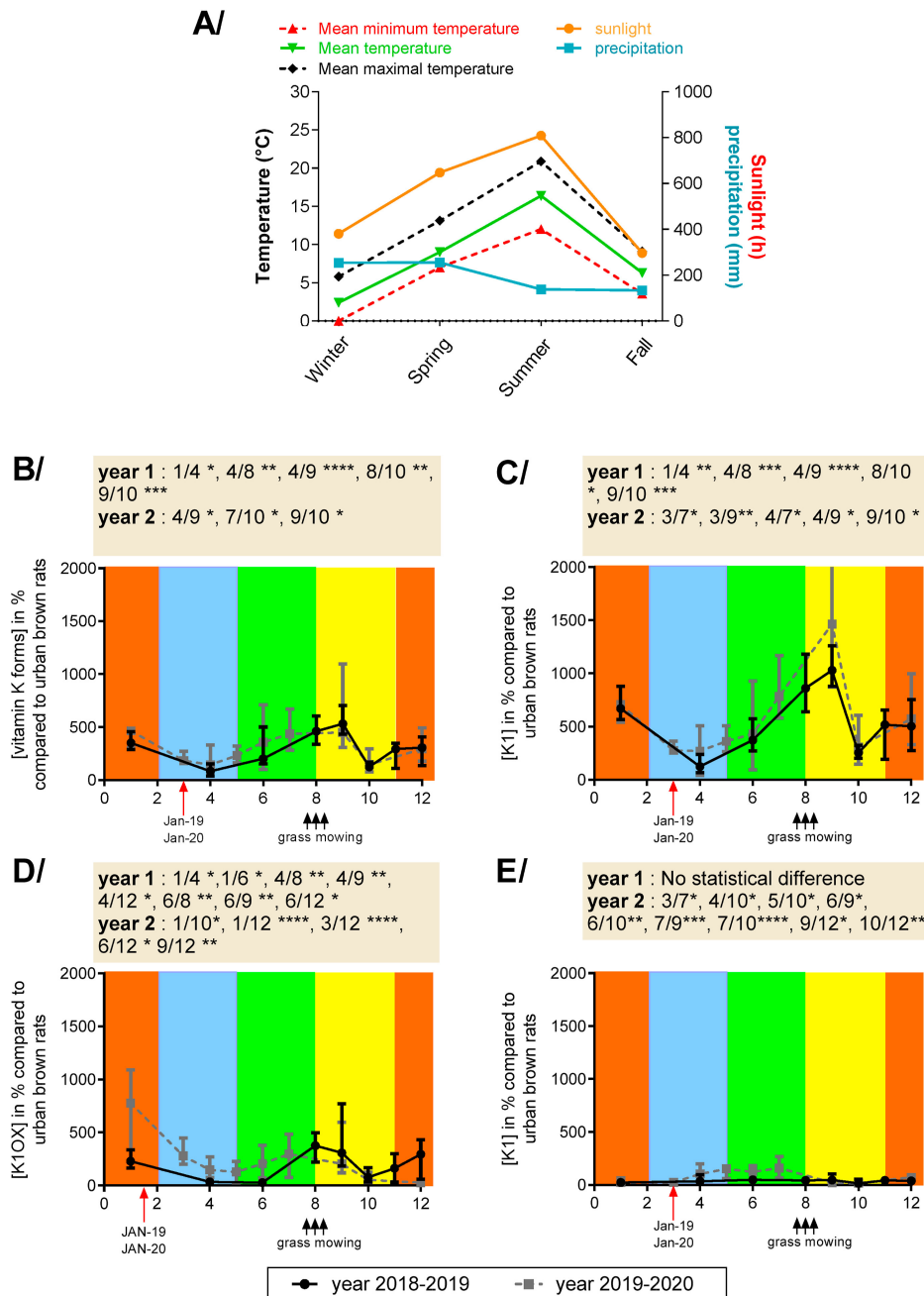


Figure 4. Seasonal variation of concentrations of vitamins K in the liver of voles. In A/Meteorological parameters for the four seasons, in B/Total of the different K forms, in C/Vitamin K1 in its quinone form, in D/Vitamin K1 in its epoxide form, in E/Menaquinone-4 in its quinone + epoxide forms. Results 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 (Boitet et al., 2018)[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 factors 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 interspecies 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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