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# 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

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## 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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## Contents

<b>Introduction</b>	<b>1</b>
<b>1 Material and methods</b>	<b>2</b>
1.1 Chemicals . . . . .	2
1.2 Animals . . . . .	2
1.3 Pharmacokinetic study . . . . .	2
1.4 Dosages . . . . .	3
1.5 Data Analysis . . . . .	3
<b>2 Results</b>	<b>3</b>
2.1 General pharmacokinetics of each stereoisomer and effect of sex on pharmacokinetic parameters . . .	3
2.2 Non-compartmental analysis of the four individual stereoisomers of difethialone according to species	5
<b>3 Discussion</b>	<b>6</b>
<b>4 Author contributions</b>	<b>8</b>
<b>References</b>	<b>8</b>

## 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)<sup>1-6</sup>. 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<sup>7,8</sup>. 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<sup>9–11</sup>. 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<sup>12,13</sup>.

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<sup>14,15</sup>. 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 mixtures<sup>16</sup>.

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 species<sup>17</sup>. 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<sup>18–21</sup>, 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  $20 \pm 1$  °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.

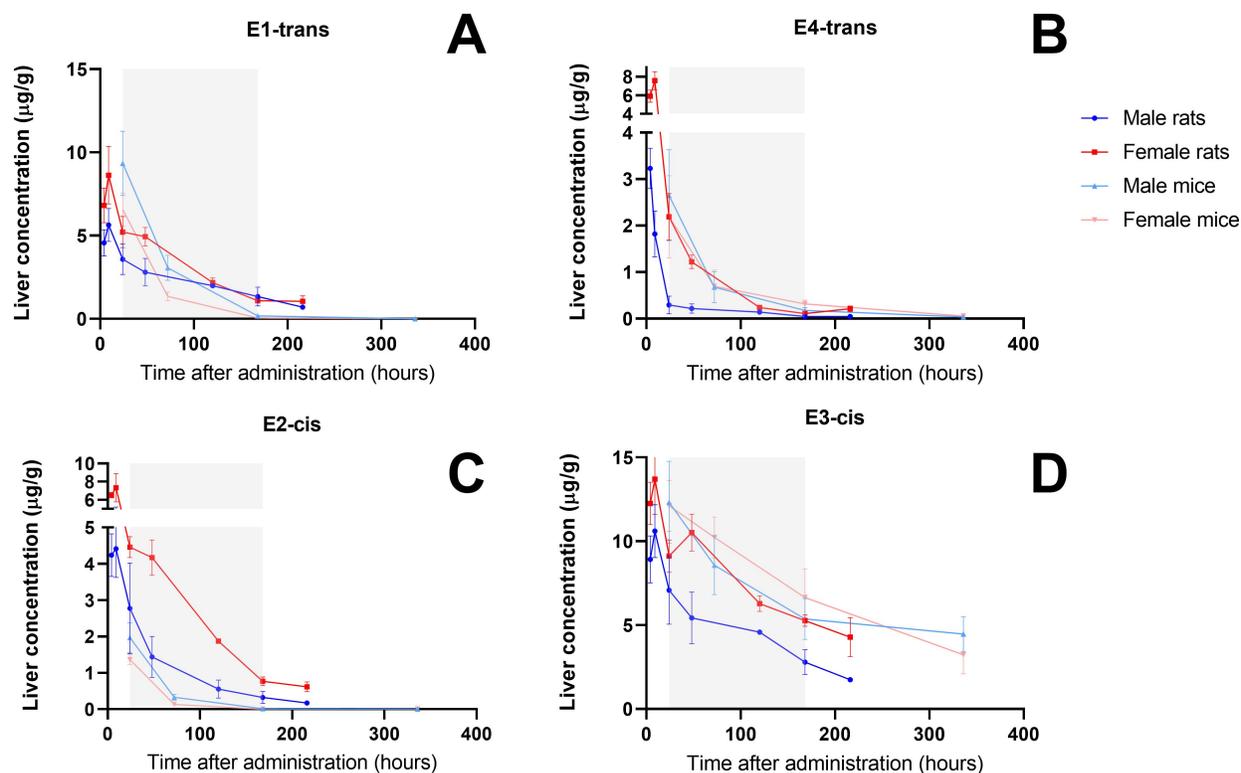
### 1.3 Pharmacokinetic study

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<sup>-1</sup>). 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 CO<sub>2</sub> 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. 2020<sup>16</sup>.

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<sup>-1</sup>). 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 CO<sub>2</sub> 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 2020<sup>16</sup>

## 1.4 Dosages

Difethialone stereoisomers were dosed as described previously in Fourel et al.<sup>22</sup>.

## 1.5 Data Analysis

Data handling and statistical analyses were performed with R 4.0.5<sup>23</sup> and the following package PK (1.3.5)<sup>24</sup>. The non-compartmental analysis was performed by applying the Bailer's method for sparse sampling as described in Nedelman<sup>25</sup>. 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  $AUC_{CAh-infinite}$  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 <sub>4-216h</sub> (µg.h/g)		AUC <sub>4-Infinite</sub> (µg.h/g)		MRT <sub>4-216h</sub> (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	1558±50,3*	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. 2020<sup>16</sup>. \*Statistical significant between male and female < 0,05

	Cmax (µg/g)		Tmax observed (h)		AUC <sub>24-336h</sub> (µg.h/g)		AUC <sub>24-Infinite</sub> (µg.h/g)		MRT <sub>4-216h</sub> (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±2,03*	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

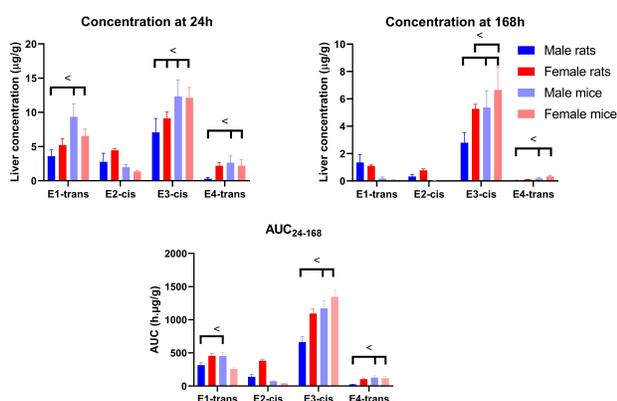
**Table 2.** Pharmacokinetic parameters of the non-compartmental analysis of each difethialone stereoisomer in the liver of mice. Data are presented with standard error.\*Statistical significant between male and female < 0,05

	Male Rat				Female Rat				Male Mice				Female Mice			
	MRT <sub>24-168h</sub> (h)	Half-life (h)	λ(h <sup>-1</sup> )	R <sup>2</sup>	MRT <sub>24-168h</sub> (h)	Half-life (h)	λ(h <sup>-1</sup> )	R <sup>2</sup>	MRT <sub>24-168h</sub> (h)	Half-life (h)	λ(h <sup>-1</sup> )	R <sup>2</sup>	MRT <sub>24-168h</sub> (h)	Half-life (h)	λ(h <sup>-1</sup> )	R <sup>2</sup>
E1-trans	82.7 <sup>a</sup>	107,6 <sup>a</sup>	0,0064	0,711	72.3 <sup>a</sup>	66,09 <sup>a</sup>	0,01049	0,9316	50.1 <sup>b</sup>	27.53 <sup>b</sup>	0,02518	0,9512	43.8 <sup>b</sup>	21.13 <sup>b</sup>	0,03280	0,9784
	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 <sup>a</sup>	44,5 <sup>a</sup>	0,01558	0,7731	71.4 <sup>a</sup>	61,93 <sup>a</sup>	0,01119	0,7731	41.4 <sup>b</sup>	19.15 <sup>b</sup>	0,03620	0,9606	35.4 <sup>b</sup>	14.57 <sup>b</sup>	0,04756	0,9802
	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	0,6776	83.5	152,7	0,004538	0,798	81.0	119,6	0,005796	0,7519	84.4	165,7	0,004182	0,7392
	81.1-90.6	78,14 – 259,2	0,002675 - 0,008871		81.5-85.4	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	0,6451	55.1	31,44	0,02205	0,964	53.6	31,92 c	0,02171	0,8159	63.0	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 µg/g, respectively) had higher liver concentration than male rats (3.6 µg/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 µg/g for males and 1.1 µg/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 AUC<sub>24–168h</sub> of male mice was higher than that in male rats (453 µg.h/g versus 314 µg.h/g respectively).

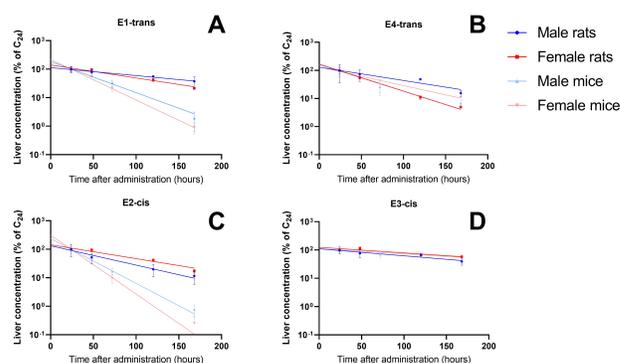
For the E3-cis stereoisomer, the liver concentrations 24 hours after the administration were significantly greater in mice (both sexes, 12.3 µg/g in males and 12.1 µg/g in females) than in rats (both sexes, 7.1 µg/g in males and 9.1 µg/g in females). However, at 168 hours if male and female mice liver concentrations (5.4 µg/g and 6.6 µg/g respectively) were significantly higher than that of male rats (1.7 µg/g), only female mice concentration was greater than that of female rats

(4.3 µg/g). AUC<sub>24–168h</sub> of male and female mice (1170 µg.h/g and 1346 µg.h/g respectively) were twice as high as that of male rats (662 µg.h/g), however no significant difference was highlighted with female rats (1093 µg.h/g).

Considering E2-cis stereoisomer, concentration at 24 hours and 168 hours and AUC<sub>24–168h</sub> 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 µg/g and 0.61 µg/g respectively), versus a few nanograms for both male and female mice (0.015 µg/g and 0.003 µg/g respectively).

Finally, considering E4-trans stereoisomer, the liver concentrations at 24 hours of both male and female mice (2.7 µg/g and 2.2 µg/g respectively) were significantly higher than that of male rats (0.29 µg/g). The same kind of differences were observed for the concentration at 168 hours with values of 0.18 µg/g for male mice and 0.32 µg/g for female mice that were both significantly higher than the value of 0.046 µg/g obtained for male rats. The AUC<sub>24–168h</sub> with values of 127 µg.h/g for male mice and 117 µg.h/g for female mice were also both significantly higher than the 23 µg.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 criteria<sup>16</sup>. 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)<sup>26</sup> 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-

$$C_{d,f_{1...4}}(t) = \frac{d}{D} \sum_{i=1}^4 \frac{f_i}{F_i} * C_{D,i,24} * e^{-\lambda_i(t-24)}$$

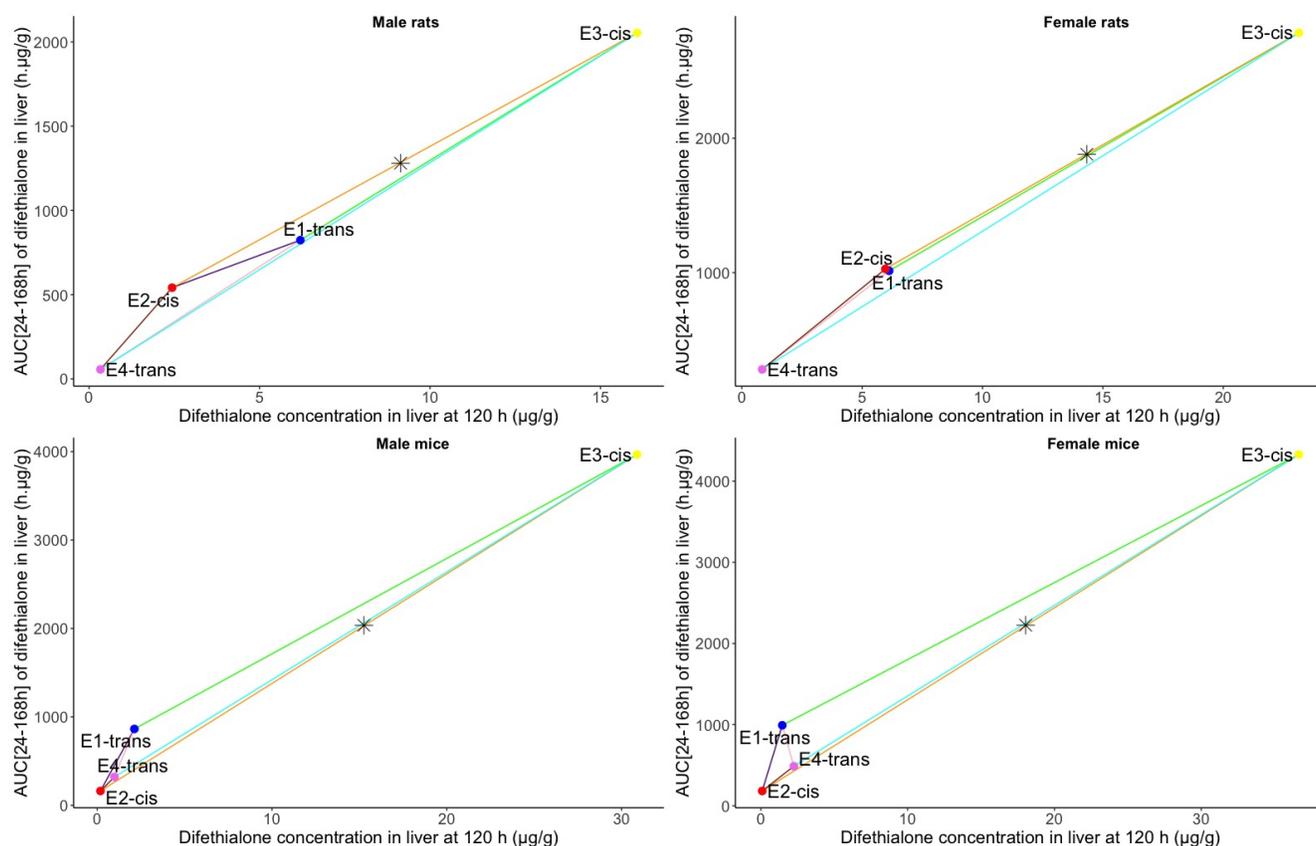
1. Equation used to model the liver concentration evolution in time of different mixes of difethialone stereoisomers. *i* is the index of each stereoisomer; *f* the fraction of each stereoisomer in the modeled mix; *F* the fraction of each stereoisomer in the experimental mix (*F*<sub>1</sub> = 0.275, *F*<sub>2</sub> = 0.225, *F*<sub>3</sub> = 0.225, *F*<sub>4</sub> = 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*<sub>(*D*,*i*,24)</sub> is the average concentration obtained experimentally for the respective stereoisomer at 24h and with the *D* dose of difethialone; *λ*<sub>*i*</sub> 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*<sub>24</sub> concentration of each stereoisomer and included the notion of the administered dose in the model. For the administered dose and its relationship with *C*<sub>24</sub>, 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 study<sup>27</sup>. Then, equation 1 is used to model the pharmacokinetic of difethialone stereoisomeric mixture. For each stereoisomer *i*, *C*<sub>*D*,*i*,24</sub> is defined with *C*<sub>24</sub> concentration from figure 2 and *λ*<sub>*i*</sub> 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 prey<sup>28</sup>? 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<sup>29</sup>, 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<sup>11,30</sup>, 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



**Figure 4.**  $\text{AUC}_{24-168}$  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<sup>31-34</sup> 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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