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Increased diphacinone and chlorophacinone metabolism in previously exposed wild caught voles, *Microtus californicus*

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ABSTRACT

Rodents have been noteworthy pests in agricultural areas for decades. Because rodents impact diverse ecosystems, anticoagulant rodenticides have been heavily used throughout the world to control rodent populations. This continued use has led to the development of resistance to anticoagulant rodenticides in some populations of targeted rodents. Although many studies have investigated the genetic and molecular basis of anticoagulant resistance, few have focused on potential changes in metabolic function of resistant animals. In this study, vole (*Microtus californicus*, Peale) liver microsome preparations were made from unexposed animals living in areas that had never used anticoagulant rodenticides for either crop protection or for the control of commensal rodents and exposed voles living in artichoke fields that have used anticoagulant rodenticides since the mid-1990s. Using these microsome preparations, the metabolism of diphacinone and chlorophacinone was tested. Microsomes from both male and female voles from exposed areas metabolized significantly more anticoagulant than unexposed animals. Also, both exposed and unexposed animals metabolized more diphacinone than chlorophacinone. These findings suggest that alterations in metabolic function may play a role in anticoagulant resistance.

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1. Introduction

Anticoagulant rodenticides have been used to control pest rodent species for decades. All have the same mechanism of action, inhibiting the vitamin K epoxide reductase enzyme and therefore blocking the formation of necessary blood clotting factors (Gill et al., 1993; Heiberg, 2009). Intensive use of anticoagulants has led to the development of resistance in rodent populations from some areas; a reduction in efficacy was first observed in warfarin resistant rats from Scotland in 1958 (Boyle, 1960). There are reports of resistance worldwide and in rats in the United States of America as early as 1972 (Jackson and Kaukeinen, 1972). Although resistance is a widespread historic problem, the exact biochemical mechanism for all cases is still unknown. Alterations in the VKOR enzyme have been cited as possible causes for some cases resistance (Markussen et al., 2008; Thijssen et al., 1989; Zimmermann and Matschiner, 1974). Pelz and colleagues showed that when expressed in HEK293 cells, 5 of the 6 VKORC1 mutations tested showed reduced activity, indicating a possible role in anticoagulant resistance (Pelz et al., 2005).

Voles, small rodents belonging to the genus *Microtus*, cause serious damage to agricultural crops in the US, specifically California (Clark, 1984, 1994; Edge et al., 1995). They are a pest species in orchards, nurseries, and numerous field crops. Voles, specifically *Microtus californicus* (Peale), are considered a serious threat to the production of globe artichokes (Koehler et al., 1989). Because of the extent of damage and subsequent economic impact, growers have historically relied heavily on anticoagulant rodenticides to manage vole populations. Although once quite useful, anticoagulant rodenticides have been reported to be losing their efficacy; there are now reports of resistant voles in California (Salmon and Lawrence, 2006).

Although many studies support the hypothesis that mutations in the VKOR gene are responsible for anticoagulant resistance, other mechanisms have been reported and may vary among species. Reduced expression of the cytochrome p450 3A (cyp3A) isoform has been found in resistant rats (*Rattus rattus*, Linnaeus) (Sugano et al., 2001). Moreover, Ishizuka and colleagues did not find the VKORC1 mutation in anticoagulant resistant roof rats. In their study, resistant rats had increased gene expression of cyp3A2.

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Also, resistant rats showed an increase in the formation of warfarin metabolites when compared to warfarin sensitive rats (Ishizuka et al., 2007). This variation in metabolic function associated with anticoagulant resistance may be influenced by the anticoagulants to which the test animals had been exposed. This discrepancy in potential mechanisms of resistance may also be species specific. Therefore, studies on the exact animals that are displaying anticoagulant resistant must be performed to elucidate the mechanisms of the resistance. Also, these previous studies did not find genetic mutations in resistant rodents but did find potentially genetically linked changes such as increased gene expression. The current study was designed to determine if voles from agricultural areas treated with anticoagulant rodenticides exhibit increased metabolic activity toward the anticoagulants diphacinone and chlorophacinone when compared to voles from areas with no anticoagulant exposure.

2. Materials and methods

2.1. Study areas and trapping methodologies

Voles, *M. californicus*, were trapped in two separate areas in May 2008. Control animals in areas with no known previous exposure to anticoagulant rodenticides were trapped in Woodland, CA. Voles were also trapped in artichoke fields surrounding Castroville, CA. This location had been exposed to anticoagulant rodenticides, both diphacinone and chlorophacinone, for at least 20 years as part of the pest management plan of the agricultural producer. Baiting occurred throughout the year to control outbreaks and decrease total vole populations. Areas with resistant vole populations were determined based on interviews with agricultural producers and bait applicators.

Voles were live-trapped using Sherman traps (H.B. Sherman Traps, Inc., 3 × 3.5 × 9″ Folding 0.025 Aluminum Heavy Duty trap) for six consecutive days in May 2008. Traps were placed along runways and at burrow openings in order to maximize trapping success. Peanut-butter/oat balls were used as bait in all traps. In addition to peanut butter/oat balls, untreated artichoke bracts were also placed in traps set in the artichoke fields to increase trapping success. Polyester cotton was added to the traps to provide warmth and dryness to the animals. Vegetation was placed over traps for cover, shade, and insulation. Traps were checked twice daily, in the morning (0700–0830) and in the evening (1700–1830). Any non-target animals that were trapped were released near the trap site. Every trap that was placed in the field was considered one trap night. If traps were found triggered but did not catch an animal, the trap was reset and still used in the calculation of trap nights. Trap success was calculated by dividing the number of animals trapped by the number trap nights. Voles were euthanized at the site of capture using CO_2. Livers were immediately removed, weighed, and frozen in liquid nitrogen. Livers were stored at −80 °C until further processing. All animal handling and experiments were approved by IACUC (QA#1570).

2.2. Liver microsome preparation

Liver microsomes were isolated using differential centrifugation according to Pelkonen et al. with minor alterations (Pelkonen et al., 1998). Frozen liver samples were minced using a scalpel to approximately 50 mm × 50 mm × 50 mm, keeping the sample as cold as possible. Then 10 g of minced sample was weighed and transferred to a Teflon pestle/glass homogenizer with 2 volumes (w/v) homogenization buffer (sucrose 250 mM, HEPES, N-(2-Hydroxyethyl)piperazine-N′-(2-Ethanesulfonic Acid) Sodium Salt 50 mM, 0.010 M KCl, potassium chloride 25 mM, 0.010 M $MgCl_2$, magnesium chloride 5 mM, EDTA, Ethylenediaminetetraacetic acid 0.1 mM, adjust pH to 7.4) with the addition of phenylmethanesulfonyl fluoride (Sigma–Aldrich, St. Louis, NJ USA) 2.5 μL/mL homogenization buffer. The tissue was homogenized with six passes of the Teflon pestle homogenizer (Wheaton Overhead Stirrer, Millville, NJ USA) while on ice. The homogenates were centrifuged at 10,000 g for 10 min at 4 °C (Avanti J-301, Beckman Coulter, Brea, CA USA). The supernatants were transferred to a clean centrifuge tube and spun at 15,000 g for 20 min at 4 °C. The supernatant was then transferred to ultracentrifuge tubes and spun at 105,000 g for 60 min at 4 °C. The pellets were then washed four times with approximately 1 mL homogenization buffer and transferred to the Teflon pestle/glass homogenizer. While on ice, these pellets were resuspended in homogenization buffer (1 g w/0.8 mL vol) by four passes of the Teflon pestle, and spun at 105,000 g for 60 min at 4 °C. The supernatants were discarded and the remaining pellets were resuspended in homogenization buffer using Teflon pestle/glass homogenizer (1:1, w:v) and frozen at −80 °C for further analysis. Total protein in the liver microsome preparations was assayed and determined to not be significantly different between samples (data not shown).

2.3. Microsome incubation

Microsome incubations were performed using 50 μL microsome extract, 50 μL cofactor solution (11.5 mg NADP sodium salt, 5.2 mg glucose-6-phosphate and 50 μL glucose-6-phosphate dehydrogenase, 950 μL 0.01 M MgCl in phosphate buffer, Sigma–Aldrich, St. Louis, NJ USA), the analyte (Sigma–Aldrich, St. Louis, NJ USA) (in 0.01 M phosphate buffer), and 0.01 M phosphate buffer added to bring volume to 500 μL. These incubations contained 2.4 ppm of either diphacinone or chlorophacinone (Sigma–Aldrich, St. Louis, NJ USA). Samples were incubated in a 37 °C water bath for 60 min. To quench the reaction, 0.40 mL of the incubation solution was mixed with 0.60 mL of ice cold methanol containing 5 mM tetrabutylammonium phosphate (TBAP) (Sigma–Aldrich, St. Louis, NJ USA) and vortex mixed. These samples were filtered with 0.45 μm Teflon syringe filters prior to analysis.

2.4. Residue determination

The quenched microsome incubations were analyzed using high performance liquid chromatography (HPLC) (Agilent 1100 series; Agilent Technologies, Santa Clara, CA USA) using a C18 column (Luna, 3.0 × 50 mm, 3 μm; Phenomenex, Torrance, CA USA). The mobile phase was 5 mM TBAP in a pH 8.5 6 mM phosphate buffered solution of water:methanol. The percent methanol was held at 55% for 2 min and subsequently increased at 3%/min to 85%. Standards and samples (20 μL) were chromatographed at 0.60 mL/min (32 °C) and detected at 325 nm. The calculated LOD for diphacinone and chlorophacinone were 0.016 and 0.015 ppm, respectively. Similarly LOQ for diphacinone and chlorophacinone were 0.053 and 0.049 ppm, respectively.

2.5. Statistical methods

After method development and HPLC quality control experiments, there were seven liver microsome samples left per group (male or female, unexposed or exposed). Therefore, data are representative of seven individual voles per group and are expressed as mean ± standard error of the mean. Data were tested for homogeneity of variance and normality. The differences between groups were analyzed using the Student's t-test ($p \leq 0.05$ significant). Also, physiological data were analyzed using ANOVA in R 2.15.2 using a linear model of weight and liver weight as a function of trap location (in exposed or unexposed area) and sex and their interaction.

3. Results

3.1. Outcomes of trapping

Over all the trap nights, 14 male and 11 female voles were trapped from the exposed area (Castroville, CA). At the unexposed area (Davis, CA) a total of 21 male and 25 female voles were trapped. The trapping success between the two test areas, exposed (Castroville, CA) and unexposed, (Davis, CA) in both males and females was quite similar (Table 1). In the unexposed area, an average of 3.65 male and 2.41 female voles were trapped per 100 trap nights. This was comparable to the average 3.87 male and 1.77 female voles that were trapped per 100 trap nights in the exposed area. Results from the ANVOA analyses indicate that there was no difference in mean body weight between exposed and unexposed areas ($F = 1.47$, $p = 0.230$) and sex ($F = 2.62$, $p = 0.110$) in either male or female voles. The weight of the liver was not significantly difference between male and female voles within the exposed or unexposed areas. However, mean liver weight was significantly greater in males from exposed areas compared to unexposed areas ($F = 4.17$, $p = 0.045$).

3.2. Effects of anticoagulant exposure on metabolism

The metabolic activity of captured voles was examined using liver microsome preparations (Fig. 1). Male voles from exposed areas metabolized significantly more chlorophacinone than unexposed male animals (1.7% and 14.8% respectively, $p < 0.001$). This increase in chlorophacinone metabolism in voles from anticoagulant exposed areas when compared to unexposed areas was also found in female voles (3.8% and 28.9% respectively, $p < 0.0001$). Animals from anticoagulant exposed areas also exhibited increased diphacinone metabolism. Female voles from exposed areas metabolized 57.8% more diphacinone than female animals from unexposed areas (65.1% and 7.3% respectively, $p < 0.0001$). Male voles exhibited this increase as well with exposed voles metabolizing 33.3% of diphacinone and unexposed metabolizing 5.8% ($p < 0.0001$). Both male and female voles from both capture locations metabolized significantly more diphacinone than chlorophacinone. This increased diphacinone metabolism was significant for all comparisons ($p < 0.005$).

4. Discussion

Agricultural producers have been using anticoagulants to control vole populations for decades. The present study demonstrates the presence of increased metabolic activity of anticoagulants diphacinone and chlorophacinone in liver microsomes of voles from areas with previous anticoagulant exposure. These *in-vitro* data support previous findings of anticoagulant resistance in both male and female voles from agricultural areas in California (Salmon and Lawrence, 2006). To design the most effective vole control strategy, more information about the resistant animals is needed, including potential biochemical pathways of resistance.

In previous studies of laboratory rats, warfarin resistant animals weighed less than susceptible conspecifics (Smith et al., 1991). Additional studies using rats with wild origins showed similar results with homozygous warfarin resistant rats having decreased growth rates (Smith et al., 1993). Body weight measurements were included in the current study due to the findings of Smith et al. that in offspring of wild-caught warfarin resistant rats weight conveyed social dominance (Smith et al., 1994). Differences in the weight of bromadiolone-resistant Norway rats have been shown to effect reproductive success and therefore the spread of resistance (Jacob et al., 2012). Exploiting this "selective disadvantage" has been cited as one way to manage anticoagulant resistance (Greaves, 1986). In our study, voles from anticoagulant exposed areas did not show significant differences in body weight compared to those from unexposed areas. Also, there was no difference in body weight between male and female animals from either area. Liver weights of animals from both exposed and unexposed areas were recorded because changes in metabolic activity are often associated with differences in organ weight. Livers of male voles from exposed areas weighed significantly more than those of animals from unexposed areas. However, there were no significant differences in liver weight in female voles. This is an interesting finding when taken in the context of previous work that has shown that male Norway rats are more susceptible to diphacinone and chlorophacinone than their female conspecifics. This same study found that male rats from both Welsh and Hampshire warfarin resistant colonies were more susceptible to diphacinone and chlorophacinone than the female members (Prescott and Buckle, 2000), This gender specific susceptibility to diphacinone is also reported in house mice (reviewed in (Fisher, 2005)). When examined in the context of metabolic activity data from this study, these findings suggest that further research should to be done to gain a better understanding of possible phenotypic differences between animals from exposed and unexposed areas.

Liver microsome incubations from wild caught voles from exposed areas showed an increase in both diphacinone and chlorophacinone metabolism when compared to voles from unexposed areas. This finding is similar to other studies that found expression of cyp 2B, 1A1, 3A, and 2E1 increased in wild voles exposed to agrochemicals (Fujita et al., 2001). Ishizhuka and colleagues found a two-fold increase in warfarin metabolism in liver microsomes from warfarin resistant rats when compared to controls. Moreover, the expression of major cytochrome p450 genes increased in resistant animals (Ishizuka et al., 2007). We found that voles from both exposed and unexposed areas metabolized significantly more diphacinone than chlorophacinone. It is possible that this increased metabolism, and therefore clearance of the active form of these rodenticides from the body, could account for the differences in their toxicities. The difference in the efficacy of diphacinone and chlorophacinone has also been shown to be related to differences in their ability to inhibit the VKORC enzyme (Lasseur et al., 2007). This difference in toxicity has been previously reported in pine voles (*Microtus pinetorum*) where the LD50 for diphacinone (57.0 mg/kg) is significantly greater than that for chlorophacinone (14.2 mg/kg) (Byers, 1978).

Microsome incubations were performed with livers from female

Table 1
Summary of *M. californicus* trapped in May 2008 (average ± SEM).

Trap location	Gender	Captures per 100 trap nights	Mean body wt (g)	Mean liver wt (g)
Unexposed (Davis, CA)	M	3.65	39.1 ± 2.5	1.9 ± 0.1*
Unexposed (Davis, CA)	F	2.41	39.4 ± 3.6	2.1 ± 0.2
Exposed (Castroville, CA)	M	3.87	46.1 ± 3.0	2.4 ± 0.1
Exposed (Castroville, CA)	F	1.77	35.2 ± 4.6	2.0 ± 0.3

*$p < 0.01$ vs exposed male.

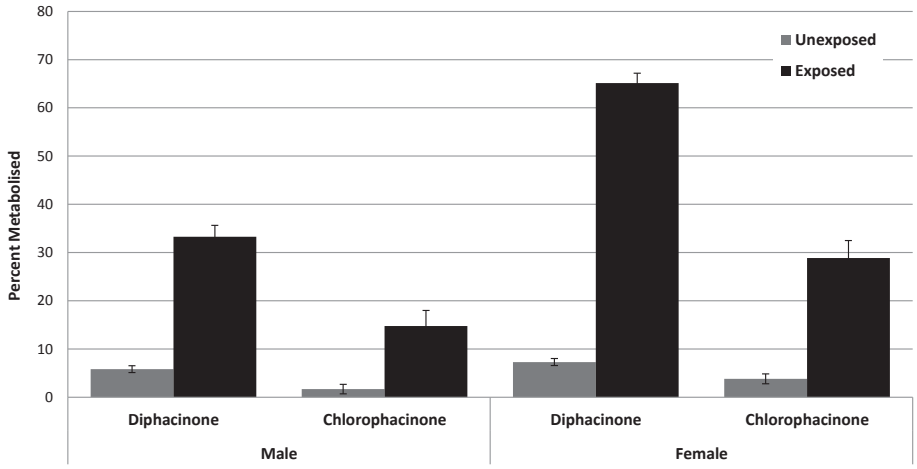

Fig. 1. Percent of chlorophacinone or diphacinone metabolized by liver microsome preparations from unexposed and exposed voles. Microsomes were incubated at 37 °C for 60 min. Percent metabolized was calculated by dividing the concentration of the metabolite by the amount of parent compound added.

and male samples separately. In these incubations, female voles from exposed areas metabolized 57.8% more diphacinone and 25.1% more chlorophacinone than animals from unexposed areas. Although the same phenomenon is true in male voles, the difference is not as pronounced. Incubations of microsomes from male voles showed that animals from exposed areas metabolized 27.5% and 13.1% more diphacinone and chlorophacinone, respectively. Gender differences were also found by Pelz and colleagues in a study of suspected anticoagulant resistant brown rats in Germany (Pelz, 1995). They found that male and female rats had different clotting times in response to anticoagulant exposure. In another study, when compared to susceptible rats, bromadiolone resistant Norway rats were not only found to have changes in cyp gene expression but these changes were sex-linked (Markussen et al., 2007).

This is one of the first studies investigating potential metabolic changes vole populations with a history of exposure to anticoagulants. More research should be done to determine if changes in cyp genes or other metabolic pathways, such as phase two enzymes, are involved in anticoagulant resistance in this species. The increased metabolism could be used to better inform the development of future rodenticides and the optimization of baiting strategies using currently available rodenticides. If resistant animals are known to be high metabolizers of anticoagulants then other rodenticides metabolized by the same enzymes may not have the desired efficacy predicted when susceptible animals are used in development.

In conclusion, the findings in this study show that anticoagulant resistance in voles may be a result of increased metabolic function. Voles from areas previously exposed to anticoagulant rodenticides metabolized significantly more diphacinone and chlorophacinone than voles from unexposed areas. Female voles from exposed areas metabolized 57.8% more diphacinone and 25.1% more chlorophacinone while males from exposed areas metabolized 27.5% and 13.1% more diphacinone and chlorophacinone than conspecifics from unexposed areas. When considered in combination with results from other studies, there is significant evidence that resistance to anticoagulant rodenticides is the result of changes to numerous different biochemical pathways. These changes must be considered in future rodenticide development to ensure that new rodenticides are effective against resistant animals.

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