Anticoagulant Resistance in Meadow Voles (*Microtus californicus*)

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ABSTRACT: The California meadow vole, *Microtus californicus*, is a major vertebrate pest in artichoke fields of Castroville, California. Complaints from growers about the effectiveness of the only available rodenticide, chlorophacinone treated artichoke bracts, led researchers to the test the bait and baiting strategies. When laboratory trials were conducted in 2001, the poor dose-response correlation and apparent low sensitivity to chlorophacinone in some animals suggested the possibility of anticoagulant resistance. The current study was initiated to examine potential resistant of voles from artichoke fields in the Castroville area. Baseline blood coagulation data were obtained from wild, anticoagulant-susceptible voles trapped in Yolo County, California and compared to data from Castroville voles. Results indicate a significant difference in clotting times 24 hours after dosing with anticoagulant between voles from Castroville artichoke fields and voles from the Yolo population. This supports the hypothesis that voles from Castroville artichoke fields are resistant to anticoagulants.

KEY WORDS: anticoagulant resistance, blood clotting, chlorophacinone, meadow voles, *Microtus*, prothrombin time

Proc. 22nd Vertebr. Pest Conf. (R. M. Timm and J. M. O'Brien, Eds.)
Published at Univ. of Calif., Davis. 2006. Pp. 156-160.

INTRODUCTION

Artichoke growers in Castroville, California use the anticoagulant chlorophacinone (0.01% Rozol oil artichoke bract bait), to control their primary vertebrate pest, the California meadow vole (*Microtus californicus*). Due to complaints from artichoke growers of poor efficacy of chlorophacinone, we conducted a study to determine the dose needed for effective control (Salmon and Gibson 2003). Results showed a poor dose-response correlation and apparent low susceptibility to chlorophacinone, which is a possible indication of anticoagulant resistance.

Anticoagulant resistance has been found in a number of commensal rodent species where some portion of the target population does not respond to the treatment. Greaves provides an industry accepted definition: *"Anticoagulant resistance is a major loss of efficacy in practical conditions where the anticoagulant 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."* (Greaves 1994)

When resistance is due to a genetically inheritable trait, there is the potential for the rodenticide to quickly become ineffective in controlling a pest as the resistance builds throughout the population. To compound the problem, resistance to a first-generation anticoagulant such as chlorophacinone often confers cross-resistance to other first-generation and sometimes, though to a lesser extent, to second-generation anticoagulants (Greaves 1994).

Anticoagulant rodenticides are by far the most extensively used rodent control method in temperate countries, including in agricultural crops (Hadler and Buckle 1992). If these toxicants become ineffective, it would create an ominous situation for managing rodents, especially since there are few alternative toxicants available. In agriculture, the loss of use (or effectiveness) of rodenticides has the potential to create a serious economic loss (Salmon 1987). Anticoagulant rodenticides affect rodents by interrupting the vitamin K cycle, which in turn inhibits the synthesis of normal blood clotting factors (MacNicoll 1986). If anticoagulant levels increase in a rodent's body, the clotting factors decrease until a fatal hemorrhage results. Resistant animals are able to synthesize clotting factors in the presence of the anticoagulant, which keep them from hemorrhaging.

In artichokes, there are significant losses from meadow vole burrowing under and feeding on the plant. Artichokes are perennial plants with an average production life of 9 years. A damaged or dead plant not only looses its value the year it is damaged, but also for the remaining years of the rotation.

BLOOD CLOTTING RESPONSE TESTS

To determine potential resistance in the vole population, we conducted a study that measured the blood clotting response time to a measured amount of chlorophacinone. Blood clotting response (BCR) tests have increasingly taken the place of lethal feeding period (LFP) tests for determining anticoagulant resistance in rodents. Advantages of the BCR test over the LFP test are its shorter time to get results, independence from the feeding nature of the animal, and greater sensitivity in identifying resistance.

METHODS AND MATERIALS

Selection of "Susceptible" Strain (Yolo Voles)

According to European Plant Protection Organization (EPPO) guidelines, which follow the principles of the World Health Organization, wild animals used to establish blood clotting baseline data should be taken from

different locations that have received as little anticoagulant exposure as possible (OEPP/EPPO 1995). In our previous meadow vole projects, we used voles from fields near U.C. Davis, Yolo County, because they had not been exposed to anticoagulant treatments. To satisfy the EPPO guidelines, voles were collected from 4 areas in Yolo County to represent the susceptible population for this study. Breeding was also conducted in the lab by crossing voles from different Yolo County locations. Voles used for testing were randomly chosen from any of the 5 groups. With one exception, the locations were known to have had no exposure to anticoagulants in the previous 5 years and in most cases much longer, if ever: our research laboratory performed a chlorophacinone baiting trial for pocket gophers (*Thomomys bottae*) approximately 7 months prior to animal collection from one of the collection sites. Since this test used underground baiting and was in a different area of the field from where the voles were captured, we believe anticoagulant exposure to voles was extremely unlikely. All animals were trapped using metal, live-catch Sherman® traps baited with rolled oats or fresh apples. Traps were checked daily and closed during the hottest part of the day to avoid trap death.

Selection of "Unknown" or "Resistant" Strain (Castroville Voles)

To test the potential resistance of voles from the Castroville area, animals were captured from 3 different artichoke fields near Castroville, California during May 2005. Field selection was based on grower identification of infested fields with a history of anticoagulant use. Animals were collected after the harvest period when the artichoke plants were being cut to soil level. This was at least one month after the most recent chlorophacinone treatment. Voles were collected by digging up active vole burrows and hand capturing the animals.

Animals Maintenance

Animals were housed in groups (based on capture location) at the U.C. Davis research facility (TB-1) in 10 × 20-foot cement-bottom outdoor pens. During testing, animals were individually caged in 9 × 12-inch stainless steel wire bottom cages and given a portion of a 2-inch PVC tube and cotton batting for environmental enrichment. The room was temperature controlled at 68-72°F. and had a 12-hour light/dark cycle. The voles were given Purina Laboratory Rodent Chow and water *ad libitum* and a piece of potato daily to supplement water intake. Voles were allowed to acclimate to the lab for a minimum of 7 days prior to testing. All voles were sexually mature and appeared healthy. Females were separated from males for a minimum of 3 weeks (the gestation period for meadow voles) to assure they were not pregnant at the time of the trial.

BCR Procedure

Prothrombin Time (PT) and *Proteins Induced by Vitamin K Absence or antagonists* (PIVKA) clotting times are two measures of the time it takes for a blood-plasma sample to clot. When normal (non-resistant) animals receive a dose of anticoagulant, their blood takes longer to clot. If they receive enough anticoagulant, they hemorrhage and die. Anticoagulant resistant animals continue to clot normally (or very close to normal) when given a dose of anticoagulant.

The dose administered to the animal to determine whether it is susceptible is called the discriminating dose. Determining the level at which an animal is a responder is not generally defined (OEPP/EPPO 1995). It depends on the researchers' classification of "response." In our study, "response" is characterized as a clotting time that is greater than 2 standard deviations from the mean normal clotting time when a set dose of anticoagulant is administered. Therefore, when an animal receives the discriminating dose of anticoagulant and has a PT greater than 2 standard deviations from normal, it is considered "susceptible" to chlorophacinone. Animals with PT times within 2 standard deviations of normal PT are considered "resistant".

Gavage and Blood Collection

Voles were weighed and gavaged with an 18-gauge stainless steel ball-tipped needle at 0.5 ml per 100 g of body weight with a solution of polyethylene glycol (PEG 400) and technical grade chlorophacinone powder to get the appropriate dose. (Doses will be discussed below). Twenty-four hours after gavaging (±30 minutes), voles were weighed and anesthetized with carbon dioxide gas. A 0.45-ml blood sample was drawn via cardiac puncture into a syringe containing 0.05 ml 3.8% w/v sodium citrate. The blood and citrate were dispensed into a test tube, agitated, and placed on ice. Within 20 minutes, the samples were spun down in a micro-centrifuge for at least 2 minutes. The plasma was removed into a separate tube and placed directly into a -21°F. freezer. The frozen plasma samples were taken to the U.C. Davis Veterinary Medicine Teaching Hospital (VMTH) clinical chemistry laboratory and analyzed for PT and in some cases also for PIVKA time, according to their standard laboratory procedures. PT is the most commonly used measurement, but PIVKA is somewhat more sensitive to changes in clotting time. To be consistent with other resistant rodent BCR tests, we used PT for our analysis. The clotting time results were recorded to the nearest tenth of a second. In addition to laboratory controls (samples from animals that were not dosed), samples were taken from Yolo voles dosed with PEG 400 only. These samples were taken with each dose group to monitor for possible effects of day or PEG 400. The 0.45-ml blood sample was considered non-survivable, and the voles were euthanized immediately after the sample was taken.

Normal Clotting Times

Because this procedure has not been reported on voles, it was necessary to obtain the normal clotting time on un-dosed voles. Approximately 30 voles of each sex (42 from Yolo County and 22 from a Castroville population maintained at lab) were tested to establish normal PT for voles from each area. These animals were not gavaged prior to blood collection.

Effective Dose 99

The effective dose 99 (ED_{99}) is the dose in which we

would expect 99% of the susceptible animals to respond. We identified a responder as any animal that had a PT clotting time that was greater than 2 standard deviations from the normal mean clotting time of non-anticoagulant exposed animals. EPPO guidelines require testing both sexes at 5 different dose levels, with responders and non-responders in each group (OEPP/EPPO 1995). We chose dose levels based on previous anticoagulant studies of 0.03, 0.05, 0.07, 0.09, 0.11 mg/kg of body weight. Twelve voles of each sex were tested at each dose level. Doses were administered and blood drawn as described above. For the males, responders had a PT time greater than 11.68 seconds. For females, PT times greater than 10.35 seconds were considered responders. Voles with PTs equal to or less than these values were considered non-responders, i.e., resistant.

Resistance Testing

The ED_{99} was administered to three groups of Castroville voles (n = 44) with unknown susceptibility and one group of Yolo voles (n = 17). Approximately 9 males and 7 females from each group were tested and PT and PIVKA times were determined.

RESULTS

Normal Clotting Times

The normal clotting times for voles are listed in Table 1. Although normal clotting times did not vary significantly between sexes (p = 0.4975), we analyzed the data separately by sex as suggested by EPPO guidelines. Additionally, there was no significant difference in normal clotting times between voles from the Yolo and Castroville locations (p = 0.2109), so we pooled the data to obtain normal mean clotting time for meadow voles (Table 1). To test for potential affects of PEG 400, we compared clotting times of our PEG 400-dosed control voles to the clotting times of non PEG 400-dosed voles and found no significant difference in PT (p = 0.1960 for females, p = 0.2803 for males).

Effective Dose 99

We conducted probit analysis to determine the ED_{99} (the dose at which we would expect most susceptible animals to respond). This gave us a discriminating dose for males of 0.105 mg/kg (0.077-0.218), and females 0.16 mg/kg (0.108-1.186).

Table 1. Normal blood clotting times for meadow voles from Castroville and Yolo County.

Location	N	Mean PT* clotting time (seconds)	Standard deviation	Number to determine responder
Castroville males	13	7.90	0.35	
Castroville females	9	7.94	0.38	
Yolo males	22	8.60	2.06	
Yolo females	20	8.16	1.35	
Pooled males	35	8.34	1.67	**≥11.68**
Pooled females	29	8.09	1.13	**≥10.35**

* PT = Prothrombin Time

Resistance Testing

A resistant population can have significant variability in susceptibility. Table 2 shows the statistical information we obtained from the resistance testing. Using the cutoff point of 2 standard deviations from normal to classify animals as non-resistant, we found that only 10% of the males and none of the females in the Yolo group would be classified as resistant to chlorophacinone. Castroville groups showed much higher resistance with 89%, 57%, and 50% of the males in each group classified as resistant. For the female group, 43%, 17%, and 71% showed resistance, respectively. Figures 1 and 2 show the average clotting times by group and the percent resistant, respectively.

Locations (or groups) were evaluated for differences using ANOVA, followed up with a Tukey Kramer test for differences between means. The means followed by different letters are significantly different at the 0.05 level (Table 3).

DISCUSSION

There is a clear distinction between the Yolo males and the 3 Castroville male groups, with the Castroville groups being classified as resistant to chlorophacinone under our criteria. While this distinction is less dramatic for females, the Castroville groups all trend toward resistance. We know the Yolo voles come from areas with no anticoagulant exposure, and this suggests that the cause for the significant difference between the Castroville and Yolo voles is due to resistance to chlorophacinone.

Confirmatory feeding tests would give additional information about the resistance status of voles in the

Table 2. Blood clotting response of meadow voles dosed with chlorophacinone at the ED_{99} dose.

Sex	Group	N	Number of resistant	Median PT*	Avg. PT* (std. dev.)	Avg. PIVKA** (std. dev.)	Median PIVKA**
Male	Yolo	10	1	20.55	21.14 (6.94)	138.53 (71.63)	127.9
	Castroville 1	9	8	9.20	9.87 (1.63)	28.67 (12.42)	24.8
	Castroville 2	7	4	11.50	12.63 (4.08)	45.61 (35.87)	27.3
	Castroville 3	8	4	11.20	11.55 (2.30)	42.91 (20.57)	40.3
Female	Yolo	7	0	15.00	15.36 (2.76)	69.77 (23.93)	65.3
	Castroville 1	7	3	11.50	11.26 (2.76)	41.54 (22.90)	39.5
	Castroville 2	6	1	12.85	12.70 (2.72)	51.68 (19.67)	54.1
	Castroville 3	7	5	9.00	9.54 (1.69)	30.03 (14.25)	25.3

* PT = Prothrombin Time, ** PIKVA = Proteins Induced by Vitamin K Absence or antagonists

Table 3. Differences between mean clotting times of test groups of meadow voles by location.

Sex	Location	PT*			PIVKA**		
		Mean	N	Non-significant ranges	Mean	N	Non-significant ranges
Male	Yolo	21.14	10	A	138.3	10	A
	Castroville 2	12.628	7	B	45.6143	7	B
	Castroville 3	11.55	8	B	42.9125	8	B
	Castroville 1	9.866	9	B	28.667	9	B
Female	Yolo	15.3571	7	A	69.7714	7	A
	Castroville 2	12.7	6	AB	51.6833	6	AB
	Castroville 1	11.2571	7	B	41.5429	7	AB
	Castroville 3	9.542	7	B	30.0286	7	B

* PT = Prothrombin Time, ** PIKVA = Proteins Induced by Vitamin K Absence or antagonists

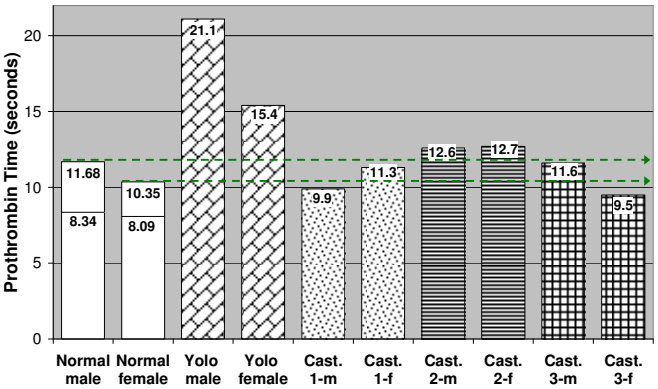

Figure 1. Average PT (prothrombin time) by meadow vole group. For "normal" voles, the stacked bars represent pooled value and responder level (see Table 1)**.**

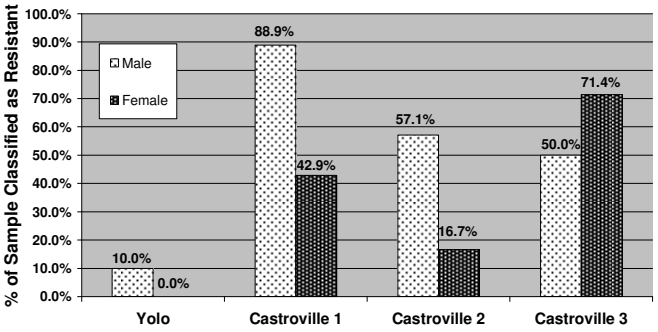

Figure 2. Percent of voles from test groups classified as resistant.

artichoke fields. We conducted terminal BCR tests, so follow-up studies on these voles were not possible. We can use information from a previous study, however, to support our findings. In studies conducted at U.C. Davis on baiting strategies for meadow voles in artichoke fields, laboratory feeding trials of chlorophacinone baits showed significantly different efficacies between Yolo and Castroville voles. The Yolo voles had 90-100% mortality during the feeding tests, while the Castroville voles experienced 25-80% mortality (Salmon and Gibson 2003). If we assume the voles tested as "responders" in our BCR test would die and that "non-responders" would survive similar feeding tests, we would get overall averages that are very similar to the previous studies. Specifically, 3.3 % of the Yolo voles and 55% of the Castroville voles survived the feeding tests, and 5.9% of the Yolo voles and 56.8% of the Castroville BCR voles were classified as resistant (Table 4).

WHY CASTROVILLE VOLES?

Anticoagulant resistance is not a new phenomenon, though as best we can determine, it has only been documented in commensal rodents. Several factors are likely contributors to resistance to in our artichoke field voles:

A) Voles are present year round. Artichokes are a perennial crop and provide fairly constant food and shelter throughout the year.

B) Artichoke growers bait with chlorophacinone on a continuous cycle. Baiting practices generally include baiting each field 1-3 times per year. Thus, it is reasonable to assume that almost every generation is exposed to the same toxicant (chlorophacinone). No significant alternate control methods are used.

C) Voles have high fecundity. Litter sizes are usually 3-9 pups, and because of post-partum estuation, females can have a new litter every 3 weeks (Marsh *et al.* 1985). This means the resistant genes can be exposed and presumably selected for multiple times each year.

D) There is no significant source of susceptible voles for immigration into the artichoke fields. Castroville artichoke fields are bordered in some places by natural areas where voles could live, but these areas probably do not harbor a large enough population to significantly dilute the resistant genes in the artichoke field population. Many ditch banks and other areas adjoining the fields are kept clear of vegetation and are therefore not suitable habitat for voles.

We believe meadow voles in Castroville artichoke fields were particularly vulnerable to developing anticoagulant resistance because of these factors.

MANAGEMENT IMPLICATIONS

It is clear that continued use of chlorophacinone in artichoke fields will only further compound the resistance problem. However, with a high value crop such as artichokes, even 50% control gives growers incentive to use chlorophacinone, since it is the only rodenticide registered. Aluminum phosphide burrow fumigant is

Table 4. Comparison of bait feeding trial mortality and BCR (blood clotting response) tests for anticoagulant resistance in meadow voles from Castroville and Yolo County.

	% of Voles Resistant			
	Castroville	N	Yolo	N
Feeding Test (all tests compared)	47.2%	220	3.3%	30
Feeding Test (equal treatments compared)	55.0%	80	3.3%	30
BCR Test	56.8%	44	5.6%	17

used, but the labor required and difficulty in use when the plants are mature make this approach of limited value. Vole populations naturally decline in the summer months but rapidly rebuild through fall and winter.

It is important that artichoke growers implement some sort of resistance monitoring and management program so that they may weigh the costs and benefits of additional chlorophacinone and other alternative treatments. This could be accomplished through BCR tests, lethal feeding tests, or field indexing measures pre and post-baiting. While this study indicates approximately 50% resistance, we do not know the affects of additional anticoagulant treatments without continual monitoring.

Effective control must incorporate several methods so that animals that escape toxicant treatment are controlled in an alternate way. Studies have shown that altering the concentration or application rate of a toxicant does not provide much long term benefit and often can make the situation worse (Roush 1989). Even stopping the use of anticoagulants will not decrease the prevalence of resistance (Heiberg *et al.* 2002). A good non-anticoagulant bait is needed to help artichoke growers deal with this serious pest. Fortunately, zinc phosphide-treated artichoke bracts have proven to be effective bait for voles in artichoke fields. Once registered, this material, if used properly and in conjunction with chlorophacinone, will be a key part of an effect resistance management program.

ACKNOWLEDGMENTS

We appreciate the help of Ocean Mist Farms and associated artichoke growers in Monterey County, California for access to Castroville artichoke fields. Special thanks to Dale Huss, Adrian Zendejas, and Chris Drew for their willingness to manage these areas to accommodate our studies. Thank you to Mike Scattini from Scattini and Sons for his participation as a grower. We also thank Thomas Schmitt from Liphatech for supplying us with technical grade chlorophacinone. In addition, we thank Dr. Mary Christopher of U.C. Davis for her advice on adapting the blood clotting response test to our specific study and for loaning us lab equipment. Thank you to the Cache Creek Nature Preserve, Putah Creek Nature Preserve, and Rachael Long for providing access to areas with susceptible voles. Thank you to Jessica Quinn for her support in statistics and Tracy Ellis for her participation in implementing and analyzing the BCR portion of the study. This study was conducted under the UC Davis Animal Use Protocol #10530, approved March 27, 2003.

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