

Draft Final Report, QA-2688:

An assessment of the potential hazards of anticoagulant rodenticides to salamanders

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We conducted an assessment of the hazards of anticoagulants (diphacinone and brodifacoum) to salamanders. This was done in anticipation of an attempt to eradicate the invasive house mice from the Farallon Islands National Wildlife Refuge. Live-captured salamanders of three species were exposed to the anticoagulant rodenticides by both oral and dermal exposure. There were some deaths and it appears that dermal exposure posed the greatest hazard. Little sub-lethal effects were noted. We concluded that while anticoagulant rodenticide pose some hazard to salamanders, the level appears to be relatively low, especially given the very high exposure rates in this study.

Introduction

House mice cause many types of damage and when introduced to islands, house mice can cause significant damage to natural resources, including both flora and fauna (Witmer and Jojola 2007). For example, on Gough Island in the South Atlantic, house mice fed on nestling albatross chicks (Cuthbert and Hilton, 2004). Additionally, Witmer et al. (2012) documented seedling damage by house mice in a pen study. House mice are omnivores, yet their diet is largely dominated by insects, some of which are likely plant pollinators (Shiels et al., 2013; Shiels and Pitt, 2014). House mice are subordinate to introduced rats so the impacts of mice may go unnoticed when rats are also present on the island (Angel et al., 2009). This phenomenon was demonstrated by the large increase in mice abundance on Buck Island, U.S. Virgin Islands, after invasive roof rats were eradicated (Witmer et al., 2007a). In very dry habitats on islands, house mice may numerically dominate over introduced rats.

There have been numerous successful eradications of invasive rodents on islands (Howald et al. 2007, Witmer et al. 2011) and these projects have relied upon rodenticides for their completion (Witmer et al. 2007b). APHIS maintains the registrations for two rodenticide active ingredients for invasive rodent eradication: diphacinone and brodifacoum. However, rodenticides can pose hazards to non-target animals so careful considerations and measures must be taken to reduce those risks (Witmer et al. 2007b).

Invasive house mice are present on the Farallon Islands National Wildlife Refuge (NWR) and are causing damage to seabirds, the endemic arboreal salamander (*Aneides lugubris farallonensis*), terrestrial invertebrates, native plants, and may be dispersing weed seeds (Farallon National Wildlife Refuge 2006, Island Conservation Undated). Hence, the USFWS would like to eradicate the invasive mice from the refuge (Farallon National Wildlife Refuge 2006, Island Conservation Undated). For inclusion in their EIS document, the USFWS would like an assessment of the potential hazards of anticoagulants to salamanders. They have requested that NWRC conduct the assessment based on our extensive animal research facilities and staff and our previous experience of assessing hazards of anticoagulants to reptiles (Witmer and Mauldin 2012).

The objective of this study was to assess the potential hazards of the rodenticides brodifacoum and diphacinone to salamanders. We exposed the salamanders to the rodenticides through two routes: 1) secondary oral exposure by allowing the salamanders to consume crickets that have fed upon anticoagulant pellets, and 2) direct external exposure by allowing salamanders to be exposed to crushed pellets and water that has been used to soak anticoagulant pellets thus allowing dermal absorption. We hypothesized that the rodenticide exposure will cause some mortality or other sub-lethal effects (decline in food consumption and/or loss of weight).

Methods

The salamanders used in this study were live-captured in California and shipped to NWRC, Fort Collins, CO, by faculty and graduate students of San Francisco State University. These persons have considerable experience in capturing and maintaining salamanders for research purposes.

They also have the permits required to capture, maintain, and transport salamanders. They were under a separate agreement with the USFWS to conduct those activities.

We originally planned to use two species of salamanders in this study. The first is *Ensatina eschscholzii xanthoptica* which is fairly widespread and common on the mainland California, but does not occur on the Farallon NWR. However, it is closely related to the second species, *Aneides lugubris farallonensis*, a subspecies of the arboreal salamander, which is endemic to the Farallon NWR. This was the species of interest, but is rare and protected on the Farallon NWR. Hence, we also used arboreal salamanders, *Aneides lugubris*, from the mainland of California because they are somewhat more common and thus somewhat more readily available. We also note that although the species of interest, the arboreal salamander, is named “arboreal” because of its climbing ability, it mainly uses the ground surface and spends much time under the ground or duff where it hides and rests in moist substrates. This is very similar to the habits of the *Ensatina eschscholzii xanthoptica* salamander. For purposes of this study, we considered the *Ensatina eschscholzii xanthoptica* to be a surrogate species and it was being used because it is more readily available (allowing adequate sample sizes for the treatment groups) and yet is closely related to the species of interest. Because we did not receive an adequate number of those two species of salamanders, we amended the study protocol to include slender salamanders (*Batrachoseps attenuates*).

Salamanders were individually maintained in plastic mouse shoebox cages and were fed small crickets. The cages contained wet paper towels on the floor of cages and a plastic hide tube. Salamanders were maintained as per the university-approved SOP on salamander maintenance that was provided by San Francisco State University.

Two anticoagulant rodenticides (diphacinone and brodifacoum) were tested for their potential hazards to salamanders. There were to be two treatment groups for each of these two rodenticides with each providing a different route of exposure (secondary oral exposure and direct dermal exposure). However, because we did not enough of the first two species of salamanders, we combined the two routes of exposure and had some of each species (*Aneides* and *Ensatina*) in each group. This was called Trial 1. There was also a control group which had no rodenticide exposure. Because we had enough of the third species of salamander (*Batrachoseps*), we were able to have separate treatment groups for each route of exposure (Trial 2).

Treatment 1 Procedures; secondary oral exposure. Ten salamanders of the third species of salamanders (*Batrachoseps*) were to be used in this treatment group for each rodenticide. However, group size varied somewhat because of the number of salamanders available at the start of the study. In this trial, the salamanders were to be fed crickets that had been exposed to the rodenticide by only allowing them to feed on crushed rodenticide pellets for about 10 days. However, when we first fed rodenticides to the crickets, they all died shortly thereafter. Consequently, we again amended the study protocol to state that we would sprinkle powdered rodenticide on the crickets just before putting them in with the salamanders. Some crickets were fed to salamanders twice weekly. The treated crickets were fed to the salamanders for 10 days. At the end of the 10-day exposure period, salamanders were placed in clean cages and observed

for a nother 14 days (post-exposure period). During this period, they were fed clean crickets that have not been exposed to the rodenticide.

Treatment 2 Procedures; direct dermal exposure. Ten salamanders of the third species of salamanders (*Batrachoseps*) were to be used in this treatment group for each rodenticide. However, group size varied somewhat because of the number of salamanders available at the start of the study. In this trial, the salamanders were exposed to external dermal exposure from crushed pellets being sprinkled on the ground cover material and by spraying the ground cover paper towels with water in which crushed pellets were allowed to dissolve for 7 days. With this treatment group, there may also have been some direct oral exposure if the salamanders chose to eat some of the crushed pellets. As in the other treatment group, the salamanders were exposed to the crushed pellets and treated water for 10 days. At the end of the 10-day exposure period, salamanders were placed in clean cages and observed for the 14-day post-exposure period. During this entire treatment, the salamanders were fed clean crickets that had not been exposed to the rodenticide.

The control group of about 10 salamanders was maintained with no rodenticide exposure during the two trials.

Staff monitored cricket consumption over the course of the trials to determine if there was a decline in food consumption as the trial progressed. Additionally, salamanders were weighed at the start and end of the trials to determine if a decline in weight occurred. These data provided us with a measure of sub-lethal effects. Generally, birds and mammals that have consumed anticoagulants will stop feeding and lose weight as the symptoms of toxicosis advance.

Salamanders were examined twice daily by the study director and/or study staff and their condition and any mortalities were recorded. Animals were examined more frequently as symptoms progressed, but how frequently depended on how quickly the symptoms progressed. If any animal was observed, in the opinion of research or animal care staff, to be experiencing more than momentary pain or distress, they contacted the Study Director and/or the Attending Veterinarian to have the animal examined and possibly euthanized. Signs of severe pain and distress and of a moribund condition that was used as criteria for humane killing of study animals listed by OECD (2000) included abnormal vocalization, persistent difficult labored breathing, prolonged impaired ambulation preventing the animal from reaching or water, persistent convulsions, and significant blood loss. Dead salamanders were weighed and placed in individual, labeled zip-lock bags and frozen for later rodenticide residue determination by the Analytical Chemistry Unit (ACU) staff. All surviving salamanders were euthanized at the end of the study using MS222 for later submission to ACU staff. Aniedes and Ensatina salamanders were necropsied at the end of the study to check for signs of internal hemorrhaging (Stone et al. 1999). Additionally, some crickets were used for residue analyses along with samples of the water that had been exposed to the crushed pellets. We also had some of rodenticide pellets analyzed for the concentration of active ingredients in them.

Results

There were 2 trials conducted. The Trial 1 used Aneides (n= 12) and Ensatina (n= 8) salamanders. These were divided into 3 groups: brodifacoum exposure group (n= 7),

diphacinone exposure group (n= 7), and a control group (no rodenticide exposure (n= 6) and each group contained some of both species,

Both routes of exposure to the rodenticides were used with the 2 treatment groups: oral exposure (fed crickets dusted with powdered rodenticide) and dermal exposure (paper towels in the cage wetted with water that had been soaked with crushed/powdered rodenticide pellets and then sprinkled with powered and crushed rodenticide pellets).

In the brodifacoum group, 2 (both *Aneides*) of the 7 salamanders died (28.6% mortality). We noted a sloughing of skin in some animals (57.1%) and sores (mainly on the underside of animals; 14.3%). One of our chemists noted that the pellets for both brodifacoum and diphacinone are rather acidic so this may be responsible for much of the skin sloughing and sores. There were no deaths in the control group and we did not note any sloughing of skin or sores. There was a considerable difference in cricket consumption by the salamanders in all 3 groups. During the brodifacoum exposure period, salamanders consumed 3-14 crickets, while in the post exposure period they consumed 1-32 crickets. There was an increase in cricket consumption in the post-exposure period in 3 of 4 salamanders. Additionally, skin sloughing and sores seemed to decrease in the post-exposure period. Over the course of the study, there was a small loss of weight in the salamanders (0.4-3.4g). Upon necropsy of the two dead *Aneides* salamanders, internal hemorrhaging was noted. After euthanasia of the surviving salamanders, necropsy revealed no internal bleeding. Brodifacoum residues in salamanders were quite variable, but low: *Aneides* 42.7-226 ppb (parts per billion); *Ensatina* 48.3-101 ppb.

In the diphacinone group, 1 (*Aneides*) of the 7 salamanders died (14.3% mortality). This salamander was bleeding externally and was euthanized. We noted a sloughing of skin in some animals (42.7%) and sores (mainly on the underside of animals; 28.6%). There were no deaths in the control group and we did not note any sloughing of skin or sores. There was a considerable difference in cricket consumption by the salamanders in all 3 groups. During the diphacinone exposure period, salamanders consumed 3-24 crickets, while in the post exposure period they consumed 5-38 crickets. There was an increase in cricket consumption in the post-exposure period in 4 of 6 salamanders. Additionally, skin sloughing and sores seemed to decrease in the post-exposure period. Over the course of the study, there was a small loss of weight in the salamanders (0.7-3.4g). Upon necropsy of the dead *Aneides* salamander, internal hemorrhaging was noted. After euthanasia of the surviving salamanders, necropsy revealed no internal bleeding. Diphacinone residues in salamanders were quite variable, but low: *Aneides* 10.8-174 ppb (parts per billion); however, no residues were detected in the *Ensatina*s.

In both rodenticide groups, we did not observe sub-lethal effects as there was no external bleeding, little or no loss of body weight, and little or no drop in food (cricket) consumption. The one exception with the one *Aneides* in the diphacinone group that was euthanized because of external bleeding. Table 1 summarizes the results of Trial 1.

In trial 2, we used *Batrachoseps* salamanders. Because we had considerably more salamanders in trial 2 than in trial 1, we were able to divide the exposure routes. One brodifacoum group (n= 7) received oral exposure (dusted crickets) only, while the second brodifacoum group (n= 8) received dermal exposure ((paper towels in the cage wetted with water that had been soaked with

crushed/powdered rodenticide pellets and then sprinkled with powdered and crushed rodenticide pellets) only. Similarly, one diphacinone group (n= 8) received oral exposure only, while the second diphacinone group (n= 8) received dermal exposure. This was done to assess which exposure route caused more deaths/problems if there was a difference. The control group (n= 7) received no rodenticide exposure.

In the brodifacoum oral exposure group, no animals died. There was no skin sloughing or sores noted. Salamanders mostly maintained the same weight with the most change only 0.1g. There was one death (14.3% mortality) in the control group, and interestingly, 14.3% of the control animals had sloughing skin and sores. Again, cricket consumption was quite variable: 13-70 in the exposure period and 4-59 in the post-exposure period. Cricket consumption was also variable in the control group: 18-229. Control animals also showed only a small change in weights: -0.02-0.43g. Brodifacoum residues in the oral exposed salamanders were variable: 51.3-91.1 ppb.

In the brodifacoum dermal exposure group, 5 of 8 animals died (62.5%). There was no skin sloughing or sores noted. Salamanders mostly lost a small amount of weight: -0.21-0.0g. Again, cricket consumption was somewhat variable: 9-27 in the exposure period, but increased in the two surviving crickets (44-55). The results of the control group are the same as presented in the previous paragraph. Brodifacoum residues in the dermal exposed salamanders were quite variable: 16.5-95.1 ppb.

In the diphacinone oral exposure group, no animals died. There was no skin sloughing or sores noted. Salamanders mostly maintained weight: 0.02-0.15g. Again, cricket consumption was somewhat variable: 6-68 in the exposure period, but stayed about the same in the post-exposure period: 4-66. The results of the control group are the same as presented in a previous paragraph. Interestingly, there were no diphacinone residues detected in the oral exposed salamanders.

In the diphacinone dermal exposure group, no animals died, but 50% of animals had some skin sloughing. Salamander weights were mostly stable: -0.11-0.11g. Again, cricket consumption was variable: 6-57 in the exposure period, but stayed about the same in the post-exposure period: 5-59. The results of the control group are the same as presented in a previous paragraph. Again, there were no diphacinone residues detected in the dermal exposed salamanders.

Again, in Trial 2, we did not observe sub-lethal effects as there was no external bleeding, little or no loss of body weight, and little or no drop in food (cricket) consumption. These salamanders were not necropsied because of their very small size. Table 2 summarizes the results of Trial 2.

Brodifacoum residues in crickets fed brodifacoum pellets were quite variable (296-688 ppb), while crickets dusted with powdered brodifacoum were much higher and somewhat less variable (2887-3340 ppb).

Diphacinone residues in crickets fed diphacinone pellets were quite variable (954-2930 ppb), as were crickets dusted with powdered diphacinone (1823-3980 ppb).

Residues in water used to soak crushed and powder rodenticide pellets were very low probably because of the low solubility of anticoagulants. Brodifacoum residues varied from 5.75-29.7 ppb. Diphacinone residues were similar and varied from 0.08-17.7 ppb.

Because of the low residue levels in the salamanders (i.e., ppb), we tested the brodifacoum and diphacinone pellets for rodenticide concentrations. These were very close to the label concentrations. For the diphacinone pellets, it was 46.4 ug/g (= ppm) which is 93% of the desired 50 ug/g. For the brodifacoum pellets, it was 26.3 ug/g (= ppm) which is 105% of the desired 25 ug/g.

We are sending the residue report of the Analytical Chemistry Unit to the USFWS and DOI as a separate document.

Discussion

A search of the scientific literature revealed no publications on anticoagulants and amphibians. As stated in some reviews, little is known about the risk of anticoagulants to amphibians, but it is generally considered to be low (Eason, 1995; Chris et al., 2010). Studies have focused on risks to mammals, birds, invertebrates, and to a much lesser focus, on reptiles as these are thought to be the most sensitive taxonomic groups and they are also the groups most likely to consume baits (primary exposure) or animals that have consumed baits (secondary exposure). Additionally, relatively few native amphibians occur on islands and many islands don't have any.

As such, we have little to compare our results with salamanders to with the exception of the taxonomic groups listed above. This information and residue levels comes from eradication projects with non-target monitoring before and after rodenticide application.

Witmer and Mauldin (2012) reported levels of diphacinone and brodifacoum residues in whole bodies of captive snakes, turtles, and lizards that had been twice orally gavaged with solutions containing those anticoagulants. These ranged from lows of 0.07 µg/g (= ppm) to highs of 1.58 µg/g (= ppm). Note that the levels in our salamanders were much lower than those in these reptiles as our residue levels were reported in ng/g (= ppb). They also noted that 5 of 37 (13%) *Ameiva* lizards died during the study with one showing external hemorrhaging. One of 38 (0.03%) green iguanas died and it had external hemorrhaging.

Pitt et al. (2015) also reported levels of brodifacoum residues in various taxonomic groups and in environmental substrates after the eradication project on Palmyra Island in the Pacific. While the levels were higher than they expected, they note that there was very high applications rates of the rodenticide in that project (6 times higher than the EPA recommended label rate). Using whole body carcasses found after the baiting operation, they reported levels of 0.10-0.76 µg/g (= ppm) in birds, 0.34-0.44 µg/g (= ppm) in fish, and below the detection level to 0.97 µg/g (= ppm) in crabs. These levels are much lower than those found in dead rats, 3.75 µg/g (= ppm). Again, note that the levels in our salamanders were much lower than those in these animals as our residue levels were reported in ng/g (= ppb). Pitt et al. (2015) also reported that only one fresh water sample had a residue level (0.05 µg/g (= ppm) above the detection level and none was

detected in the salt water samples. They also reported soil residue levels of 0.007-0.018 µg/g (= ppm).

Shiels et al. (2017) reported levels of brodifacoum residues in various taxonomic groups and in environmental substrates after the eradication project on Desecheo Island in the Caribbean. Most carcasses found from various taxonomic groups had residues. They also live-harvested various lizard species about 3 weeks after the baiting operation. While all these animals appeared healthy, 65-100% had detectable residue levels ranging from 12.2-1100 ng/g (= ppb). Additionally, some insect and crabs had detectable residue levels ranging from 10.3-1580 ng/g (= ppb). These are similar levels to those we found in the salamanders.

From our Trial 1 results, it appears that rodenticide exposure poses some hazard to salamanders, but that hazard appears to be relatively low and they can begin recovery after some exposure. One must also realize that in this trial there was a relatively high exposure rate which combined oral and dermal exposure. The high exposure rates were from the feeding of dusted crickets instead of crickets that had fed on the rodenticides; the former had much higher levels of rodenticide residues. Additionally, the level of dermal exposure was much higher than it would be in an eradication project (see Figure 1). Hence, this trial presents, in essence, a worst case scenario.

The Trial 2 results basically confirm the results from Trial 1. However, Trial 2 seems to suggest that the higher hazard to salamanders from anticoagulants is from dermal exposure versus oral exposure. This could be determined because we had enough slender salamanders to separate the two types of exposure into separate groups. It is cautioned, however, that we gave very high exposure rates to the salamanders in this study (Figure 1). In an aerial broadcast baiting in an invasive rodent eradication project would result in much lower dermal exposure to all animals. Trial 2 also presents a worst case scenario.

The residue levels in this study were so low that our Analytical Chemistry Unit had to modify the normal method of detection. Normally they use High Performance Liquid Chromatography (HPLC) or the more sensitive mass spectrometer. In the case of this study, they combined those methods which greatly increasing the sensitivity and gave a much greater probability of detecting residues.

With regard to the residues levels in crickets fed rodenticides, we need to clarify an early assumption that we made. When we first tried to feed rodenticides to crickets, all the crickets died shortly thereafter. We assumed crickets might be sensitive to anticoagulants even though most invertebrates are known to not be sensitive to anticoagulants. Because of the early result, for the study we chose to dust crickets with powdered anticoagulants before feeding them to the salamanders. However, when we later fed rodenticides to crickets, all the crickets survived. We now surmise that we got a bad batch of crickets early on in the study. Later batches of crickets did just fine and were used in the study without problems. This is consistent with the scientific literature which has shown little or no impacts to invertebrates from anticoagulants even though some have been found to have substantial residues in them.

Acknowledgments

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Figure 1. Salamander in its plastic cage showing the high level of dermal exposure in this study.



Table 1. Summary of the Aneides and Ensatina trial (Trial 1). Animals coded QO are Aneides; those coded QP are Ensatina.

Treatment	ID #	Initial Weight (g)	Final Weight (g)	Weight Change (g)	Comments	% Sloughing Skin	% Sores	% Mortality
Brodifacoum /crickets & dermal	QO1	9.4	6.1	-3.3	Died	57.14%	14.29%	28.57%
	QO4	9.0	7.8	-1.2	Euthanized at end of trial			
	QO7	9.7	7.5	-2.2	Euthanized at end of trial			
	QO10	9.4	6.0	-3.4	Died			
	QP1	7.7	6.8	rickets	Euthanized at end of trial			
	QP4	7.3	6.9	-0.4	Euthanized at end of trial			
	QP7	13.0	10.5	-2.5	Euthanized at end of trial			
Diphacinone /crickets & dermal	QO2	10.5	7.7	-2.8	Euthanized due to condition	42.86%	28.57%	14.29%
	QO5	17.3	15.8	-1.5	Euthanized at end of trial			
	QO8	12.9	12.2	-0.7	Euthanized at end of trial			
	QO11	20.7	17.3	-3.4	Euthanized at end of trial			
	QP2	9.6	8.6	-1.0	Euthanized at end of trial			
	QP5	9.3	8.1	-1.2	Euthanized at end of trial			
	QP8	8.0	6.8	-1.2	Euthanized at end of trial			
Control	QO3	19.4	18.5	-0.9	Euthanized at end of trial	0.00%	0.00%	0.00%
	QO6	10.8	10.4	-0.4	Euthanized at end of trial			
	QO9	20.3	18.2	-2.1	Euthanized at end of trial			
	QO14	10.4	10.0	-0.4	Euthanized at end of trial			

QP3	6.0	4.8	-1.2	Euthanized at end of trial
QP6	15.4	13.3	-2.1	Euthanized at end of trial

Table 2. Summary of the Batrachoseps trial (Trial 2).

Treatment	Animal ID	Initial Weight (g)	Final Weight (g)	Weight Change (g)	Days Until Death	% Sloughing Skin	% Sores	% Mortality
Brodifacoum /Crickets	QS5	0.73	0.73	0.00		0.00%	0.00%	0.00%
	QS10	0.45	0.55	0.10				
	QS19	0.84	0.94	0.10				
	QS27	0.52		-0.52				
	QS35	0.46	0.54	0.08				
	QS42	1.17	1.21	0.04				
	QS56	0.78	0.83	0.05				
Brodifacoum /Dermal	QS6	0.52	0.42	-0.10	2	0.00%	0.00%	75.00%
	QS11	1.03	0.97	-0.06	9			
	QS30	0.81	0.60	-0.21	14			
	QS36	0.41	0.34	-0.07	10			
	QS38	0.30	0.23	-0.07	10			
	QS43	0.52	0.52	0.00				
	QS51	0.80	0.67	-0.13	10			
	QS57	0.58	0.57	-0.01				
Diphacinone /Crickets	QS7	0.50	0.64	0.14		0.00%	0.00%	0.00%
	QS13	0.69	0.79	0.10				
	QS23	0.56	0.70	0.14				
	QS31	1.15	1.27	0.12				
	QS39	0.30	0.32	0.02				
	QS44	0.89	1.04	0.15				
	QS52	0.29	0.34	0.05				
	QS58	0.56	0.61	0.05				
Diphacinone /Dermal	QS8	0.31	0.36	0.05		50.00%	0.00%	0.00%
	QS14	0.39	0.48	0.09				
	QS24	0.88	0.88	0.00				
	QS33	0.88	0.92	0.04				
	QS40	0.83	0.89	0.06				
	QS48	0.86	0.97	0.11				
	QS53	0.82	0.71	-0.11				
	QS55	0.93	0.89	-0.04				
Control	QS9	0.45	0.55	0.10		14.29%	14.29%	14.29%
	QS17	0.75	0.81	0.06				
	QS22	0.54	0.52	-0.02	6			
	QS26	0.90	0.94	0.04				

	QS34	0.38	0.40	0.02				
	QO12	1.41	1.83	0.42				
	QO13	1.43	1.86	0.43				