January 2023

Environmental Toxicity Of Isoxazoline Drugs On Non-Target Species

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ENVIRONMENTAL TOXICITY OF ISOXAZOLINE DRUGS ON NON-TARGET SPECIES

by

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Bachelor of Science, Valley City State University, 2019

A Thesis

Submitted to the Graduate Faculty

of the

University of North Dakota

in partial fulfillment of the requirements

for the degree of

Master of Science

Grand Forks, North Dakota

August

2023

This thesis, submitted by Haley Cooper in partial fulfillment of the requirements for the Degree of Master of Science from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

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This thesis is being submitted by the appointed advisory committee as having met all of the requirements of the School of Graduate Studies at the University of North Dakota and is hereby approved.

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Haley Marie Cooper

06/29/2023

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ACKNOWLEDGMENTS

I would like to thank my committee members, my advisor, Dr. Jefferson Vaughan, Emily Gisi for her help with this project, and everyone else who helped.

I would like to thank my husband, Mason, and my kids, Nora and Murphy, for their support and encouragement. I would also like to thank my parents, Joe and Darlene, for everything they do for my family and me. I also want to thank my brother, Adam, and all my friends who have supported me on this journey. Without all of you, I would not have been able to do it.

ABSTRACT

Lyme disease affects tens of thousands of people every year in the United States, causing serious illness and even death. It is caused by the bacterium, *Borrelia burgdorferi,* through infected black-legged tick bites. The range of these ticks is expanding, causing Lyme disease cases to increase. Considerable effort has been put toward research and eradication of Lyme disease, with limited success. Isoxazoline drugs, commonly used to prevent flea and tick infestations in dogs, could be dispatched in the field to keep black-legged ticks off the reservoir host of Lyme disease, the White-footed mouse. Little research has been done on these drugs, so their environmental impact is unknown.

This study aimed to test how isoxazoline drugs (lotilaner, fluralaner and afoxolaner) affect non-target species. Lotilaner-treated mouse carcasses were left outside for 27 days to observe decomposition and infestation. Carcass invertebrates and soil samples were collected. Neurotoxic effects of fecal pellets from isoxazoline-treated white-footed mice on *Culex pipiens* mosquito larvae were also tested. Mosquito larvae were exposed to lotilaner, fluralaner, and afoxolaner-treated mouse fecal pellets to find the percent mortality. Fathead minnows were exposed to lotilaner contaminated water to see if the drug could leech out of the fecal pellet, pass through the gills and be toxic to the fish.

The results showed that treated mouse carcasses decompose slightly slower than control carcasses, but there is no effect on the invertebrates on or under the carcasses. Lotilaner is toxic to mosquito larvae for almost two months. Afoxolaner is toxic for about a week while fluralaner

is not toxic whatsoever to *Culex pipiens* mosquito larvae. Finally, lotilaner had no effect on fathead minnows, suggesting that lotilaner could be used around aquatic habitats and pose little to no danger to fish.

CHAPTER 1

Introduction

Lyme disease is one of the most common vector-borne diseases in the United States, with tens of thousands of people diagnosed each year. It can cause a variety of symptoms, but if the disease is left untreated, it can lead to death (Lyme Disease Data and Surveillance, 2022).

Lyme disease is caused by the bacterium, *Borrelia burgdorferi* and is transmitted through infected black-legged tick (*Ixodes scapularis*) bites. The ticks become infected when they feed on a reservoir host such as white-footed mice (*Peromyscus leucopus*). The bacteria then migrate to the salivary glands and are injected into humans when the ticks attach (Shapiro, 2014).

Lyme disease is most prevalent in the upper Midwest and northeastern and mid-Atlantic states, but recent studies have shown that the range of the black-legged tick is expanding, leading to an increase in Lyme disease cases (Lyme Disease, 2022).

With the range of black-legged ticks expanding and Lyme disease cases increasing, it is critical to find new ways to control the spread of the disease. Targeting the host directly, the white-footed mouse, could be one successful method. Oral and topical acaricide treatments have been used on white-footed mice in the past. Acaracides are pesticides used to kill mites and ticks, by affecting the central nervous system.

One family of acaricides that could be used is the isoxazolines (afoxolaner, lotilaner, fluralaner, sarolaner), a class of veterinary drugs used to prevent flea and tick infestations in dogs. Isoxazolines are a newer group, so little research has been done on them. An important factor to look at with these drugs, is the environmental impact they may have on non-target species such as invertebrates, fish, etc. In dogs, these drugs are ingested into the stomach. Some

of the drug is absorbed into the blood and adipose tissue. This allows the blood to come into contact with an attached tick and hopefully kill it. The rest is metabolized in the liver and excreted into the intestines. Finally, the drugs and metabolites are shed in the feces. This process would most likely be the same in mice, so this is where the environmental impact is important. If these drugs are dispatched out into the field to be used as tick control, there will be isoxazoline-treated mouse fecal pellets everywhere, as well as treated dead mouse carcasses. The question then is how isoxazoline drugs and/or the metabolites in fecal pellets and carcasses affect the non-target species.

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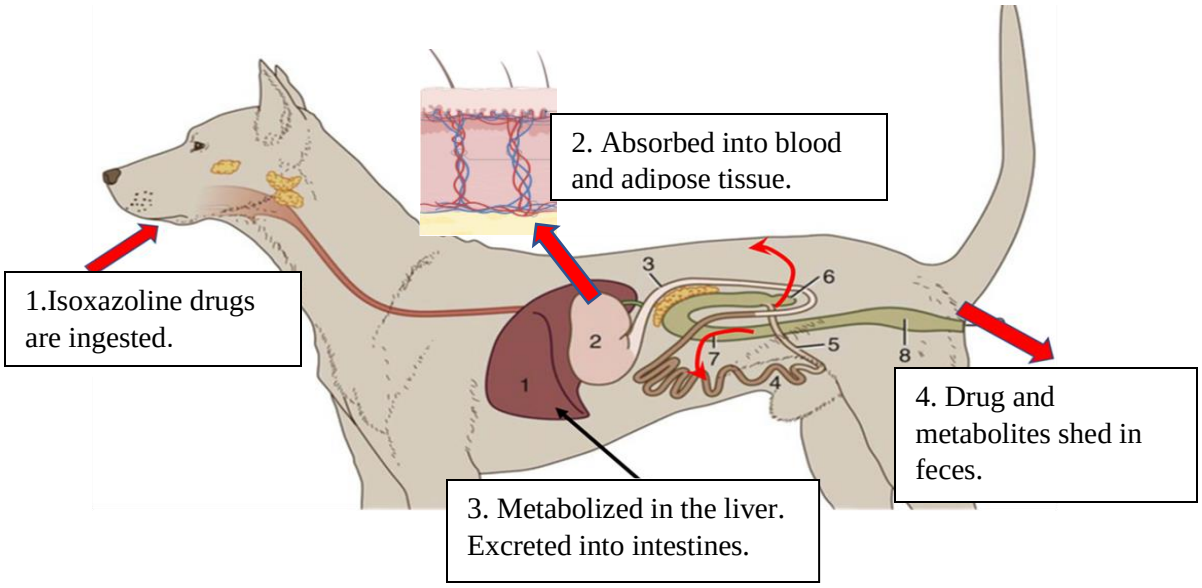

Figure 1. Schematic diagram illustrating the fate of ingested isoxazoline drugs.

CHAPTER 2

Toxicity and decomposition of lotilaner-treated White-footed mice (*Peromyscus leucopus*), a Lyme disease host

Abstract

Lyme disease affects tens of thousands of people every year in the United States, causing serious illness and even death. Considerable effort has been put toward research and eradication of Lyme disease, with limited success. Isoxazoline drugs, commonly used to prevent flea and tick infestations in dogs, could be used to keep black-legged ticks off the reservoir host of Lyme disease, the White-footed mouse. This study aimed to test how lotilaner affects the decomposition process of mouse carcasses and their toxicity towards organisms on and around them.

Eleven white-footed mice were treated with lotilaner (Credelio™) and euthanized 8, 15, 22 or 30 days after treatment. The mouse carcasses were put outside in Reynolds, ND to observe decomposition and arthropod infestation over time. Soil samples and arthropod specimens from the mice were collected. Percent weight loss was calculated at 16 and 27 days, with significant difference between treated and control carcasses. Invertebrates from soil samples were identified and counted as well as invertebrates from the carcasses. There was no significant difference between the number and density of invertebrates under the treated or control carcasses or the number of arthropods on the carcasses. The findings suggest that lotilaner is not toxic to the arthropods that are part of the decomposition process and does not inhibit decomposition, making it an option to stop the spread of Lyme disease through white-footed mice and ticks.

Introduction

Lyme disease is one of the most common vector-borne diseases in the United States, with approximately 30,000 people diagnosed each year. People diagnosed with Lyme disease can be asymptomatic, while others can have a wide range of symptoms including fever, rash, facial paralysis, and arthritis. If the disease is left untreated, it can even lead to death (Lyme Disease Data and Surveillance, 2022).

Lyme disease is caused by the bacterium, *Borrelia burgdorferi* and is transmitted through infected black-legged tick (*Ixodes scapularis*) bites. The ticks become infected when they feed on a reservoir host such as white-footed mice (*Peromyscus leucopus*). The bacteria then migrate to the salivary glands and are injected into humans when the ticks attach (Shapiro, 2014). Ticks must be attached for 36 to 48 hours in order to transmit the bacteria, so it is important to recognize a tick bite as soon as possible to reduce the chances of Lyme disease transmission (Transmission of Lyme Disease, 2023).

Lyme disease is most prevalent in the upper Midwest and northeastern and mid-Atlantic states, but recent studies have shown that the range of the black-legged tick is expanding, leading to an increase in Lyme disease cases (Lyme Disease, 2022).

With the range of black-legged ticks expanding and Lyme disease cases increasing, it is critical to find new ways to control the spread of the disease. Targeting the host directly, the white-footed mouse, could be one successful method. Oral and topical acaricide treatments have been used on white-footed mice in the past. Acaracides, pesticides used to kill mites and ticks, affect the central nervous system through various mechanisms. They affect gamma-aminobutyric acid (GABA)-gated chloride channels, octopamine tyramine receptors, voltage-gated sodium

channels, glutamate gated chloride channels (Glu-Cl), and can inhibit acetylcholine-esterase (AChEs). Each acaricide has a different target and mode of action that affects growth, reproduction, and survival of different tick species (Obaid et al., 2021). Acaricides also have selective toxicity. This means that they kill ticks and mites, but they do not do any harm to the mammals the acaricide is given to. This could be due to the differential sensitivity of tick and mite GABA receptors compared to mammalian GABA receptors (Capinera, 2008).

One acaricide that has been studied to prevent Lyme disease is fipronil. In the northeast United States, bait boxes that apply the drug topically to mice have been used (Schulze et al., 2007). This method works for short time periods but has not shown any significant reduction in *I. scapularis* ticks or Lyme disease (Wormser, 2004).

Another family of acaricides that could be tested is the isoxazolines (afoxolaner, lotilaner, fluralaner, sarolaner), a class of veterinary drugs used to prevent flea and tick infestations in dogs. In dogs, these drugs are ingested and absorbed into the blood and adipose tissue. They are then metabolized in the liver and excreted into the intestines. Finally, the drugs and metabolites are shed in the feces. They are long lasting with a single dose lasting one month and sometimes more (Snyder at al., 2016). They are also highly effective at killing ticks and have a fast rate of kill, which is important to prevent the transmission of *B. burgdorferi* (Murphy et al., 2017). These characteristics make the isoxazolines a very good candidate to control Lyme disease in rodent hosts.

Isoxazolines are a newer group of acaricides, so little research has been done on them. So far, fluralaner is the only one that has been tested on white-footed mice, but only protected against ticks for a short amount of time. Of the four isoxazoline drugs, lotilaner (Credelio™) is the newest and could potentially be used to control Lyme disease in white-footed mice.

The environmental toxicity of isoxazolines remains unstudied. It could be important to know how much of the drug is passed on through feces or how much is left in the body once they have died. The amount left in the feces and carcasses could potentially be harmful or deadly to other organisms. To know if lotilaner is toxic to non-target species, decomposition could play an important role.

Decomposition has many stages and requires a variety of arthropods to fully decompose a carcass. Each stage attracts different species due to the physical and chemical conditions of the carcass. The different species are also attracted in waves, some coming in first and then others following. The pattern of when species come to the carcass can depend on many variables. These include geographical region, type of death, size of the corpse or carcass, temperature, and other environmental conditions (Horenstein et al., 2010)

There are four main categories of arthropods found in decomposition: necrophagous species feeding on the corpse or carcass, predators and parasites feeding on the necrophagous species, omnivores and species like spiders or springtails that use the corpse as their living environment (Joseph et al., 2011). Stage one is known as the fresh stage and includes insects in the Calliphoridae and Sarcophagidae families including blow flies, flesh flies and house flies whose eggs and larvae need fresh flesh to develop. The second stage is known as the bloated stage and is dominated by maggot masses. In the post-bloated stage, members of the Staphylinidae and Histeridae families can be found. At this point, adult dermestid beetles come in and feed on the remaining skin and ligaments. When the carcass is very well decayed and dried out, young dermestids finish the decomposition cycle (von Hoermann et al., 2012).

This study will look at the toxicity and decomposition of lotilaner treated white-footed mouse carcasses. We hypothesized that treated carcasses will decompose slower than control carcasses and will be toxic to arthropods and non-target species on and around the carcasses.

Methods

Treatment

Eleven white-footed mice from the University of North Dakota (UND) breeding colony were treated with lotilaner (Elanco US Inc, 2018) via a peanut butter bait at UND on May 30, 2022. The mice ranged in weight from nineteen to forty-one grams. The bait was one part rat chow (ground to a coarse powder with a mortar and pestle) and one-part creamy peanut butter. Warm tap water was added to bring the mixture to a dough-like consistency. A Credelio™ tablet for puppies weighing 4.4-6 pounds (56.25 mg lotilaner per tablet) was ground into a powder and added to the bait at a concentration of 1 mg lotilaner per 500 mg of bait. Once the bait was mixed, 500 mg was applied to a baby carrot slice and given to the mice. Ten control mice were also given the bait on a carrot, without the lotilaner. Mice were caged individually and checked the next day to ensure all bait was consumed.

The mice were part of another study in the lab looking at the efficacy of isoxazoline drugs against ticks attached to the mice and if that could be an option to eradicate Lyme disease. They were euthanized when those tests were completed. They were anesthetized using a pentobarbital injection and blood was collected via cardiac puncture, killing them. Their ears were also removed for further testing in that same study. The mice were euthanized at various times, from 8-30 days post treatment.

Set-up and specimen collection

The mice were marked with different color zip ties (red, blue, yellow, grey, and black) to differentiate between control and treated mice and the date they were euthanized. At site 1, three control mice (black) and three treated mice (red) were set out to observe decomposition. All six mice were euthanized eight days after treatment. At site 2, three control mice (black) and three treated mice (blue) were set out. All six mice were euthanized fifteen days after treatment. At site 3, three mice were set out, one control (black) and two treated (blue, yellow). The control mouse and blue treated mouse were euthanized fifteen days after treatment and the yellow treated mouse was euthanized 22 days after treatment. At site 4, three control mice (black) and three treated mice (grey) were set out. All six mice were euthanized thirty days after treatment. At each site, the control and treated mice were kept separate from each other to eliminate contamination between them. The mice were set on the ground and covered with a rat cage top to prevent them from getting taken or eaten by bigger animals such as raccoons, cats, birds, etc. Flies, beetles, and other arthropods could still access the mice in order to observe the decomposition process.

Carcass weights were recorded before the mice were put outside. They were also recorded on day sixteen and again at the end of the experiment on day 27. Carcasses were checked every two to three days for infestation and to check on decomposition. Specimens such as maggots, flies, beetles, etc. were collected using forceps and were stored in vials containing 70% ethanol. The vials were kept in the UND laboratory until the specimens could be counted and identified at a later time.

Experimental Sites

The experiment took place at 417 2nd Ave, Reynolds, ND, twenty miles south of the University of North Dakota (UND) campus for 27 days in August and September 2022. Four sites were chosen, each with distinct characteristics. Site 1 was an area with mowed Kentucky bluegrass (*Poa pratensis*), shade for part of the morning and full, direct sun the remainder of the day. Site 2 was an area in the trees with long Kentucky bluegrass, Canada Thistle (*Cirsium arvense*), sticks, leaves, and other vegetation. It was shady and did not get any direct sunlight. Site 3 was an area of soil surrounded by Kentucky bluegrass. There was also a rhubarb *(Rheum rhabarbarum*) plant on one side of the soil. The area received direct sunlight most of the day. Site 4 was an area with a combination of mowed and longer Kentucky bluegrass, next to an apricot tree (*Prunus armeniaca*). It received shade in the morning with part shade and sun in the afternoon.

Soil Sample Collection

When the experiment was completed, soil samples were taken from under the carcasses. A small garden shovel was used to collect 2-3 scoops of soil which were placed in plastic Ziploc® bags. The soil was brought to UND, where it was stored in the plastic bags at room temperature in the laboratory until further testing could be done.

The samples were weighed and put into Berlese funnels, which were used to separate and extract the small arthropods from the soil samples. The device consisted of a funnel with wire mesh at the bottom to hold the sample. Below the funnel was a reservoir that contained 70% ethanol. Above the funnel was a light bulb that dried the samples. This forced any specimens

such as beetles, ants, mites, etc. to move down through the soil and into the reservoir of alcohol (Capinera). When all specimens were collected, they were counted and identified.

Data Analysis

Percent weight loss for treated and control carcasses at each site were examined using analysis of variance on the arcsine transformed percentages (Zar, 1999), using the variables 'treatment' (lotilaner vs. control), 'day' (*i.e*., days 16 & 27), and 'site' (no. 1, 2, 3, 4) as the main effects and individual mice as the covariate to test for individual random variation among mice (Statistix 9, Tallahassee, FL). The least significant difference test was used to define statistical differences among means. The two-sample t-test in Statistix 9 was also used to look at the difference in on-carcass invertebrate specimens between control and treated as well as number of taxa and invertebrate density in the soil samples.

Results

Weight Loss

Biomass reduction of mouse carcasses are presented in Table 1. An initial analysis of variance indicated that there was a highly significant effect ($p<0.0001$) of 'site' on carcass decomposition. The overall biomass reduction at Site 2 was significantly less than at the other three sites (alpha = 0.05, df=29). Thus, site 2 was excluded from subsequent analysis of variance to test for the effect of 'treatment' and 'day'. Without site 2 in the analysis, there was no longer a significant effect of 'site' ($p=0.482$). However, there were significant effects of both 'treatment' ($F=5.6$, df=1, $p=0.03$) and 'day' ($F=6.3$, df=1, $p=0.02$). Biomass loss in treated carcasses was significantly different between treated and untreated mice on both days 16 and 27 post-mortem (Table 2). However, biomass loss in treated carcasses at day 27 post-mortem did

not differ statistically from that of untreated carcasses at day sixteen post-mortem, indicating that decomposition of treated carcasses was delayed but not altogether inhibited. For control carcasses, there was no significant difference in biomass lost between days 16 and 27, indicating that the decomposition process was beginning to plateau.

Carcass Specimens

Over 27 days, there was a total of 97 specimens collected off the carcasses. Table 3 shows the different taxa from the treated and control carcasses. The treated carcasses had a total of 42 specimens and the control carcasses had 55 specimens. Treated carcasses had a total of eight taxa compared to thirteen taxa on the control carcasses. A two-sample t-test to compare the total number of taxa between the two was run, resulting in a p-value of 0.74. This means there is no significant difference in the number of taxa that were on the treated versus control carcasses.

Soil Samples

Total number of taxa and density of invertebrates per 100 grams of soil are shown in Table 4. There was no difference in the total number of invertebrate taxa recovered from beneath the carcasses of treated mice (15 taxa) versus untreated mice (14 taxa) ($p=0.89$). Similarly, there was no difference in the density of soil invertebrates recovered from beneath the carcasses of treated mice (73 ± 32.9) versus that from the untreated mice (62.3 ± 42.0) ($t=0.43$, $df=6$, $p=0.681$).

Discussion

The results of this study suggest that lotilaner treated white-footed mouse carcasses are not toxic to arthropods and do not inhibit the decomposition process enough to cause problems

for the environment. Thus, lotilaner bait could potentially be used in the field to prevent the spread of Lyme disease through white-footed mice and *I. scapularis* ticks.

Before the start of this study, there were a few unanswered questions about how the use of lotilaner in the field would affect the environment. One question was if there will be a significant difference in biomass loss between treated and control carcasses of white-footed mice. This study showed that on day 0, 16 and 27, there was a significant difference in biomass loss. Although there were only two sample periods examined during this study, the results clearly indicate that, although lotilaner-treated carcasses decomposed more slowly, the decomposition process was not halted. With adequate time, decomposition of lotilaner-treated carcasses would most likely catch up with that of control carcasses, suggesting that the use of lotilaner in the field as a tick control strategy, would not unduly disrupt the turn-over of treated animals.

Another question was would the specimens that help decompose the carcasses be affected. The results showed that lotilaner does not affect the specimens on the carcasses, since there was about the same number on the treated versus the control carcasses. This is important to keep the life cycle going. The decomposing carcasses provide essential nutrients for plants and can help with the growth and development of new organisms.

Another question was does the soil invertebrate fauna change underneath the treated carcasses versus control carcasses. The answer was no. The number of taxa beneath the carcasses was almost the same with a p-value of 0.89. The abundance of invertebrates per 100 grams of soil was also similar between treated and control. This is important because it means that the lotilaner does not leech out from the carcass into the soil and is not toxic to the invertebrates living under the carcasses.

In summary, this study shows that lotilaner is not toxic to soil invertebrates or arthropods that help in decomposition. It slows decomposition but still allows carcasses to decompose. Lotilaner bait could be put out into the field and could potentially decrease the spread of Lyme disease, *B. burgdorferi*, from white-footed mice to *I. scapularis* ticks and people.

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Figure 2. Mouse carcasses with zip ties used to differentiate the mice. The mice were set on the ground and covered with a rat cage top.

Figure 3. A: Experimental site 1 B: Experimental site 2 C: Experimental site 3 D: Experimental site 4.

Figure 4. Berlese funnel used to separate invertebrates from soil samples.

Table 3. Biomass reduction, measured as percentage of body weight loss, between carcasses of *Peromyscus leucopus* that had received oral lotilaner (Treated) versus a placebo (Control).

	Original Weight (gm)		% Weight Loss – Day 16		% Weight Loss – Day 27	
Site	Control (n)	Treated (n)	Control	Treated	Control	Treated
1	24.7 (3) *	28.2 (3) *	70.7 %	61.5 %	73.1 %	69.5 %
2	24.7 (3) *	33.7 $\pm$ 12.7 (3)	47.4 %	44.3 %	58.7 %	55.4 %
3	24.7 (1)	25.1 $\pm$ 4.4 (2)	67.2 %	57.4 %	74.5 %	71.0 %
4	23.3 $\pm$ 2.3 (3)	24.6 $\pm$ 4.7 (3)	82.2 %	56.3 %	84.0 %	70.1 %

*estimated

Table 4. Overall loss in biomass over time for *Peromyscus leucopus* that had received oral lotilaner (Treated) versus a placebo (Control). Sites 1, 3, and 4 combined.

	Total Weight (gm) of Fresh *Peromyscus leucopus* Carcasses (n)	Total % Biomass Loss	
		16 Days Post-mortem	27 Days Post-mortem
Control	168.6 (7)	75.0 % AB	77.8 % A
Treated	208.7 (8)	58.2 % C	70.0 % B

[1] Percentages followed by the same letter are not significantly different from one another at alpha=0.05

(Least Significant Difference test, t=2.110, df=17)

Table 3. Treated and control carcass specimens collected throughout the study.

Treated Carcass Specimens	
Ant	6
Acalyptrate Fly	3
Maggot	25
Orbatic Mite	1
Rove Beetle	4
Carrion Beetle	1
Dermestid Beetle	1
Hydrophilid Beetle	1
Total	42

Control Carcass Specimens	
Ant	2
Acalyptrate Fly	2
Millipede	1
Maggot	31
Mesostigmatid Mite	2
Other Mite	9
Rove Beetle	1
Carrion Beetle	1
Slug	1
Sap Beetle	1
Dermestid Beetle	2
Blow Fly	1
Miridae	1
Total	55

Table 4. Invertebrate density per 100 g of soil and number of taxa in the treated and control soil samples.

	Treated	Control	P-value
Density per 100 g of Soil	73.8 ± 32.9 (n=4)	62.3 ± 42.0 (n=4)	0.68
Number of Taxa	15	14	0.89

CHAPTER 3

Neurotoxic effects of fecal pellets from Isoxazoline treated White-Footed mice on *Culex pipiens* mosquito larvae

Abstract

Lyme disease is a growing concern in the United States. Tens of thousands of people are diagnosed with the disease every year and some even die if not treated properly. Lyme disease is caused by a bacterium, *Borrelia burgdorferi*, and is spread by black-legged ticks when they feed on rodents like the white-footed mouse, a Lyme disease host. The range of the black-legged tick is growing, so finding a way to stop the spread of the disease is critical. Isoxazoline drugs, commonly used to prevent flea and tick infestations in dogs, could be one option to stop the spread. This study aimed to test the neurotoxic effects fecal pellets from isoxazoline treated white-footed mice have on *Culex pipiens* mosquito larvae.

Fecal pellets were collected from treated white-footed mice as well as untreated mice. Mosquito bioassays were set up using 25 mL glass vials. The vials were filled approximately half full with dechlorinated water along with 10-20 mosquito larvae and 1-2 fecal pellets. The vials sat for 24 hours after which the larvae were counted as alive or dead. Three isoxazoline drugs were tested: Afoxolaner (NexGard®), Fluralaner (Bravecto®), and Lotilaner (Credelio®). Lotilaner was the longest-lasting and most effective at killing mosquito larvae, fluralaner did not kill any larvae and afoxolaner was effective through day seven. These findings suggest that lotilaner is the most toxic to invertebrates and lasts the longest. Because it is toxic for so long in the feces, that means it is still active in the mouse as well and could be an option to stop the spread of Lyme disease.

Introduction

Lyme disease is one of the most common vector-borne diseases in the United States with tens of thousands of people diagnosed each year. Lyme disease can cause a wide range of symptoms including fever, rash, facial paralysis, and arthritis while other people show no symptoms. If the disease is left untreated, it could ultimately lead to death (Lyme Disease Data and Surveillance, 2022).

Lyme disease is caused by the bacterium, *Borrelia burgdorferi* and is transmitted through infected black-legged tick (*Ixodes scapularis*) bites. When the ticks feed on a reservoir host of the disease such as a white-footed mouse (*Peromyscus leucopus*), *B. burgdorferi* is passed on to the tick. The bacteria then migrate to the salivary glands and are injected into humans when the ticks attach (Shapiro, 2014). Ticks must be attached for 36 to 48 hours to transmit the bacteria, so removing a tick as soon as possible is important to reduce the chances of Lyme disease transmission (Transmission of Lyme disease, 2023).

Lyme disease is most prevalent in the upper Midwest and northeastern and mid-Atlantic states, but recent studies have shown that the range of the black-legged tick is expanding, leading to an increase in Lyme disease cases (Lyme Disease, 2022).

With the range expanding and an increase in Lyme disease cases, it is critical to find a way to stop the spread. One way to stop or lessen the spread of Lyme disease, would be to target the host directly, the white-footed mouse. This could be accomplished using isoxazoline drugs (afoxolaner, lotilaner, fluralaner, sarolaner), a class of veterinary drugs used to prevent flea and tick infestations in dogs, also known as acaricides. Isoxazoline drugs are long lasting with a single dose lasting one month or more (Snyder et al., 2016). They are also highly effective at

killing ticks and have a fast rate of kill (Murphy et al., 2017). Oral and topical acaricide treatments have been used on white-footed mice in the past. Acaracides, pesticides used to kill mites and ticks, affect the central nervous system through various mechanisms. They affect gamma-aminobutyric acid (GABA)-gated chloride channels, octopamine tyramine receptors, voltage-gated sodium channels, glutamate gated chloride channels (Glu-Cl), and can inhibit acetylcholine-esterase (AChEs). Each acaricide has a different target and mode of action that affects growth, reproduction, and survival of different tick species (Obaid et al., 2021). Acaricides also have selective toxicity. This means that they kill ticks and mites, but they do not do any harm to the mammals the acaricide is given to. This could be due to the differential sensitivity of tick and mite GABA receptors compared to mammalian GABA receptors (Credelio (Lotilaner) for Dogs, 2023).

Some research has been done on isoxazoline drugs, but an important aspect that needs to be studied is the environmental impact that isoxazoline baits could have on non-target organisms. Studies with fluralaner in dogs indicate the primary route of drug clearance is through the liver and into bile secretions – meaning the drugs are metabolized and then shed through the feces. Thus, if isoxazoline drugs and/or metabolites are being dispersed throughout the environment, it is important to examine the effects of ingesting feces from treated rodents on non-target species.

This study will look at the possible environmental and neurotoxic effects of fecal pellets from isoxazoline treated white-footed mice on *Culex pipiens* mosquito larvae. *Culex pipiens* are one of the most common mosquitos found throughout the northern hemisphere. They inhabit almost every type of water source including ponds with vegetation, river edges, road drains and ditches, water barrels, metal tanks or any type of container. *Culex pipiens* larvae have a high tolerance for fecal pollution and often breed in latrines (ECDC, 2020).

Since *Culex pipiens* can live almost anywhere, including latrines, they are a good test subject for this study. They are tolerant of polluted water, so they should be a good indicator if isoxazoline drug is leeched out of the fecal pellets and into the water. This study is not specifically looking at the possible interaction between mosquito larvae and excreted mouse feces, but rather using the larvae because they are a great model system to see if there is biologically active drug and/or metabolites excreted in the mouse feces.

Methods

Treatment and Collection

White-footed mice from the University of North Dakota (UND) breeding colony were treated with afoxolaner, fluralaner, and lotilaner. Fluralaner and lotilaner were administered via a peanut butter bait at UND. The bait was one part rat chow (ground to a coarse powder with a mortar and pestle) and one-part creamy peanut butter. Warm tap water was added to bring the mixture to a dough-like consistency. Drug tablets were ground into a powder and added to the bait at a concentration of 1 mg per 500 mg of bait. Once the bait was mixed, 500 mg was applied to a baby carrot slice and given to the mice. Control mice were also given the bait on a carrot slice, minus the drug. Mice were caged individually and checked the next day to ensure all bait was consumed.

Afoxolaner was administered via oral gavage. Ten mg of technical grade afoxolaner powder was dissolved in 400 µl of DMSO by vortex. 600 µl of sunflower oil was added to the mixture to bring the final volume of the stock solution to 1 ml. The concentration of the stock solution was 10mg/ml solvent. To administer the appropriate volume, the stock solution was

diluted 20-fold to a concentration of 0.5 mg/50 gm mouse. The mice were weighed and the correct amount of afoxolaner solution was pushed into the mouse stomach.

The mice were used in isoxazoline drug efficacy studies during which fecal pellets were collected from the bedding, plastic discs in the water and water trays under the cages of treated and untreated rodents. Pellets from the bedding and discs were considered dry and pellets from the water trays were considered wet. Pellets collected from individual rodents were placed into small centrifuge tubes, labeled with mouse ID, date, and wet or dry, and stored at -20^{o}c.

Bioassays

Mosquito bioassays were set up using 25mL glass vials labeled with mouse ID, wet/dry and the day the fecal pellets were collected. The vials were filled approximately halfway with dechlorinated water. Ten to twenty *Culex pipiens* mosquito larvae were added to each vial as well as 1-2 fecal pellets, depending on the number of pellets available. For each collection day, two vials were set up to have a larger sample size and make sure the outcome was repeated in each vial. The vials sat for 24 hours and were then examined for mosquito larvae mortality where they were counted as either dead or alive.

Data Analysis

Larval mortalities in treated groups were corrected for any mortalities that occurred in the corresponding control groups using Abbott's formula (Abbott, 1925). Corrected percent mortalities were then transformed to arcsine (Zar, 1999) before using analysis of variance to detect statistically significant effects of treatments (lotilaner vs. fluralaner), day post-treatment, and whether the fecal pellets had been recovered from the water beneath the wire-bottomed

cages and totally saturated ('wet' pellets) or collected from the bedding or floating discs in the water (i.e., 'dry' pellets).

Percent efficacy of lotilaner and afoxolaner on *Culex pipiens* larvae and *Ixodes scapularis* and *Dermacentor variabilis* ticks was calculated using the following formula:

$$\text{Efficacy (\%)} = 100 \times \frac{\text{(number of engorged ticks fed on control)} - \text{(number of engorged ticks fed on treated)}}{\text{number of engorged ticks fed on control}}$$

Efficacy is the ability of a treatment or drug to work under carefully controlled scientific testing conditions. The results are shown in Figures 8 and 9.

Results

There were significant differences in corrected percent larval mortalities between fluralaner and lotilaner (ANOVA, $F=939.3$, $df=1$, $p<0.0001$), day post-treatment (ANOVA, $F=40.5$, $df=17$, $p<0.0001$), and on the saturation state of the fecal pellets (ANOVA, $F=4.1$, $df=1$, $p=0.0414$). Larvae exposed to both wet and dry fecal pellets from fluralaner-treated mice had 0% mortality on all days tested (Day 4, 5, 7, 15 post-treatment). The total number of mosquito larvae studied for wet and dry fluralaner days 4-15 was 648 and 566, respectively. In contrast, the fecal pellets excreted from lotilaner-treated mice produced 100% mortality in *C. pipiens* larvae for nearly a month but became less toxic over the ensuing month. Toxicity of the fecal pellets were no longer toxic by day 82 (Figure 6).

There was a significant effect in mosquito mortality depending on the state of saturation of the fecal pellets at the time pellets were collected. Lotilaner in wet fecal pellets produced 100% mortality through day 36. On day 46, it dropped slightly to 97.53% and 67.44% on day 50. On day 60, percent mortality rose slightly to 81.25 but then decreased through day 86. The total

number of mosquito larvae studied for lotilaner wet days 4-86 was 1,529. Lotilaner in dry fecal pellets had a 100% mortality rate through day 22. On days 25, 29, and 36, the mortality rate dropped slightly into the 90's and then dropped steadily after until it reached 0% on day 86 (Figure 6). The total number of mosquito larvae studied for lotilaner dry days 4-86 was 1,459.

Afoxolaner had an 89.4% mortality rate on day 3, 23.9% on day 5, 1.3% on day 7 and was barely measurable after that (Figure 7). The total number of mosquito larvae studied for afoxolaner wet and dry days 3-7 was 881.

Discussion

The results of this study suggest that *Peromyscus leucopus* fecal excretion of lotilaner (and/or its metabolites) occurs over a period of almost two months, whereas excretion of afoxolaner (and/or its metabolites) occurs for less than a week. Excretion of fluralaner (and/or its metabolites) may not occur at all, at least not in a form that is toxic to *C. pipiens* larvae, which is unusual because a preliminary test of the commercial fluralaner product, Bravecto, was found to be extremely toxic to *C. pipiens* larvae when incubated for just a few hours.

Our results with fecal pellets from lotilaner-treated mice suggest that, as the toxic levels in the feces begin to diminish, the toxicity of feces is influenced by the saturation state of the feces. Fully saturated feces collected from water had significantly greater toxicity (ANOVA, $p=0.04$) than feces collected from the same animals but on a dry surface and allowed to dry overnight. The reason(s) for this is not known. It is doubtful that this result was due to the testing procedure because even dried pellets stored in the freezer, when placed in water and held for 24 hours, would become saturated and sink to the bottom of the test vials in a similar fashion as wet pellets. It is possible that the toxic properties of lotilaner in feces decrease as the feces

dry. Alternatively, it could be that water increases or synergizes the toxic properties of lotilaner in feces.

Figures 8 and 9 compare the toxic properties of the fecal pellets with the toxic properties of the mice that ingested lotilaner or afoxolaner. They show that the mosquitocidal activity in the pellets corresponds with the acaricidal activity in the mice. Interestingly, the mosquitocidal activity diminishes several weeks (lotilaner) or days (afoxolaner) before the acaricidal activity in the mice. This implies that even though the isoxazoline drug is metabolized and shed through the feces, the drug is still active in the mouse. The drugs are most likely stored in the adipose or skin where they come into contact with ticks as they attach, pass through their cuticle, and kill the tick shortly after.

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Figure 5. Glass vial used for bioassays with *Culex pipiens* mosquito larvae swimming around fecal pellets on the bottom.

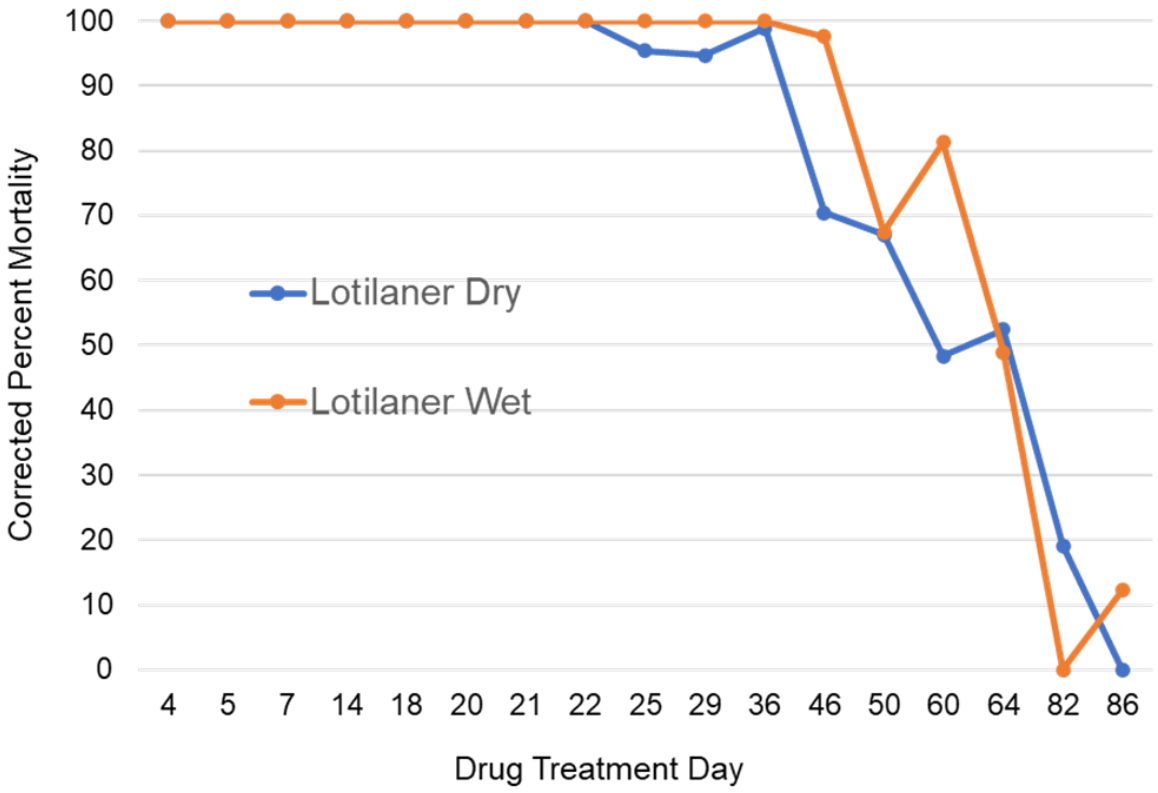

Figure 6. Toxicity of fecal pellets excreted by *Peromyscus leucopus* mice that ingested Credelio (lotilaner) to late instar *Culex pipiens* larvae. Mortality is expressed by corrected percent mortality.

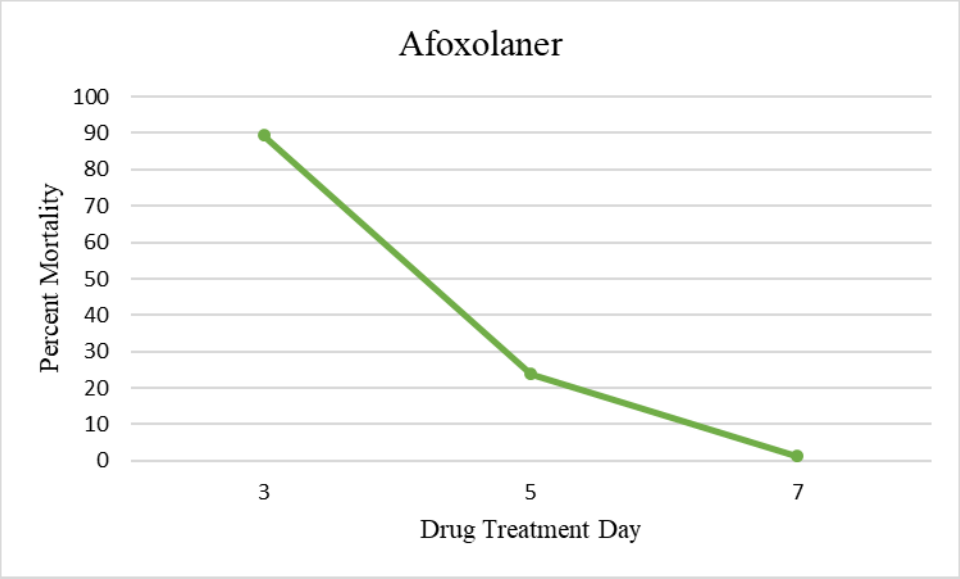

Figure 7. Toxicity of fecal pellets excreted by *Peromyscus leucopus* mice that ingested NexGard (afoxolaner) to late instar *Culex pipiens* larvae. Mortality is expressed by percent mortality.

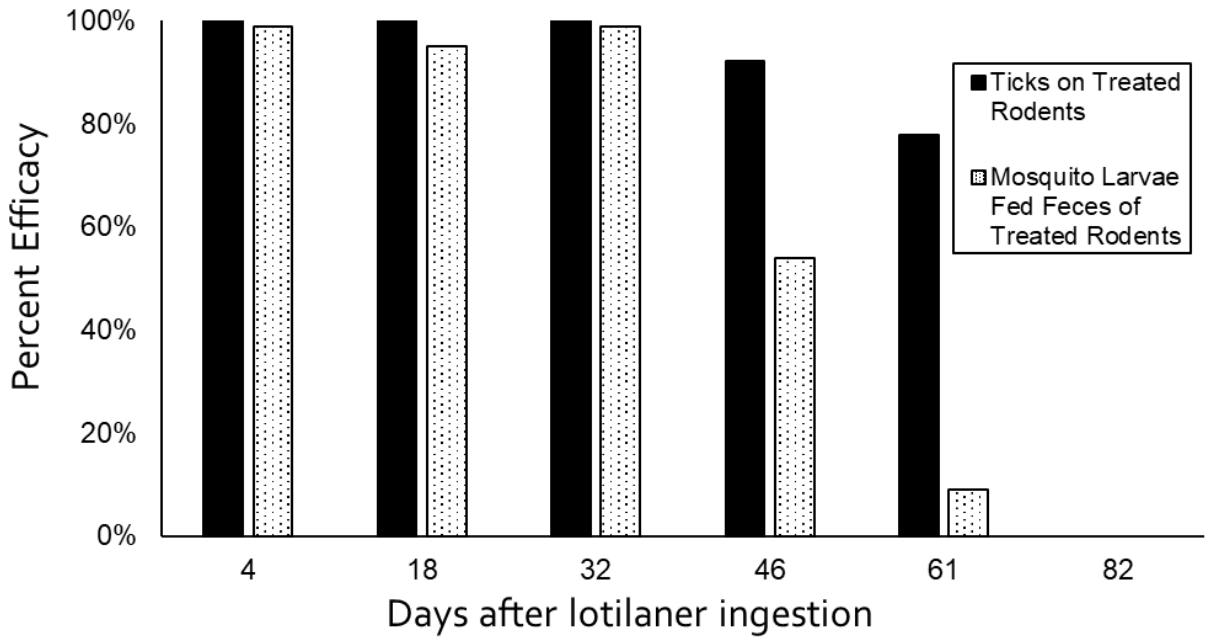

Figure 8. The toxicity of feces excreted by lotilaner-treated *Peromyscus leucopus* to *Culex pipiens* larvae decreases before the toxicity of the same mice to larval ticks (*Ixodes scapularis* and *Dermacentor variabilis* combined).

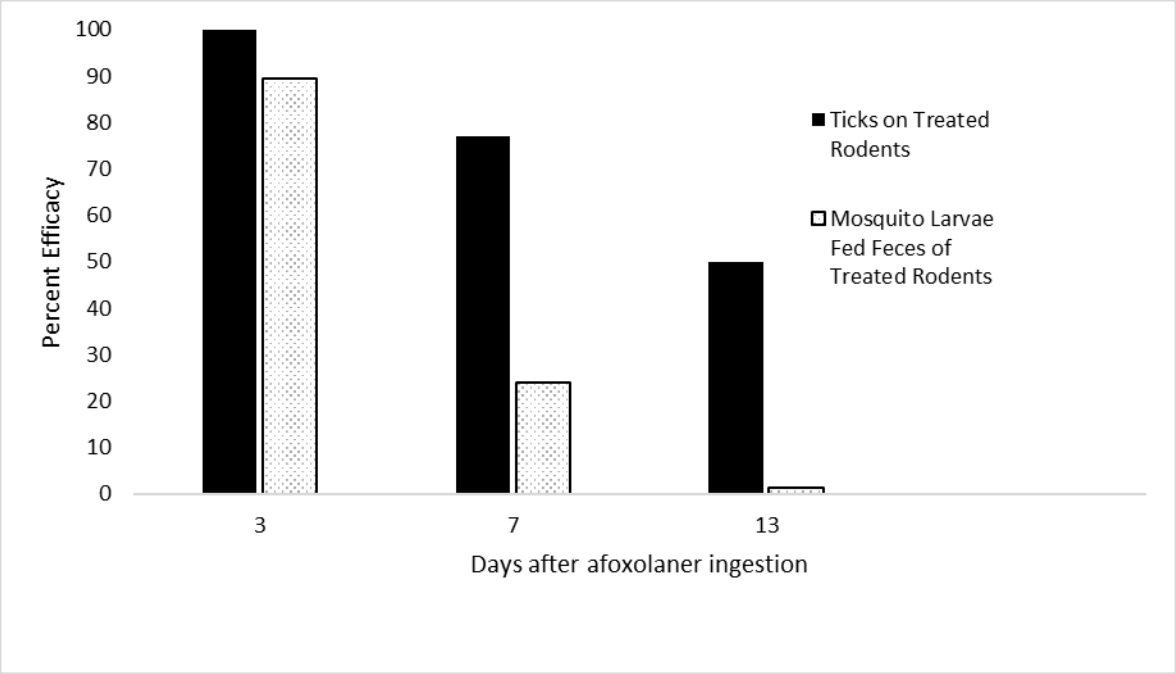

Figure 9. The toxicity of feces excreted by afoxolaner-treated *Peromyscus leucopus* to *Culex pipiens* larvae decreases before the toxicity of the same mice to larval ticks (*Ixodes scapularis* and *Dermacentor variabilis* combined).

CHAPTER 4

Assessing potential toxicity of isoxazoline drug metabolites shed in the feces of treated Peromyscus mice to fish

Abstract

Lyme disease is a growing concern in the United States. Every year it affects tens of thousands of people, and if not treated correctly, can be deadly. The disease is spread through black-legged ticks when they feed on rodents such as white-footed mice, a Lyme disease host. The range of the black-legged tick has been growing, so it is imperative to find a way to stop the spread of the disease. Isoxazoline drugs, commonly used to prevent flea and tick infestations in dogs, could be one solution to the problem. This study aimed to test if lotilaner, an isoxazoline drug, is toxic to fish when they are exposed to the drug and/or its metabolites shed in the feces.

Fecal pellets were collected from lotilaner (Credelio™) treated white-footed mice and soaked in water for 24 hours. The water was then filtered, and six fathead minnows were exposed to the water for 24 hours. *Culex pipiens* mosquito larvae were also exposed to the water because it is known from other studies that lotilaner is toxic to them. All six fathead minnows were alive after 24 hours, while all mosquito larvae were dead which was expected. These findings suggest that lotilaner shed through feces is not toxic to fish and could potentially be an option to stop the spread of Lyme disease.

Introduction

Lyme disease is one of the most common vector-borne diseases in the United States, with tens of thousands of people diagnosed each year. People diagnosed with Lyme disease can be asymptomatic, while others can have a wide range of symptoms. These could include fever, rash,

facial paralysis, and arthritis. If left untreated, Lyme disease can even lead to death (Lyme Disease Data and Surveillance, 2022).

Lyme disease is caused by the bacterium, *Borrelia burgdorferi* and is transmitted through infected black-legged tick (*Ixodes scapularis*) bites. When ticks feed on a reservoir host such as white-footed mice (*Peromyscus leucopus*), they become infected. The bacteria then migrate to the salivary glands and are injected into humans when the ticks attach (Shapiro, 2014). Ticks must be attached for 36 to 48 hours in order to transmit the bacteria, so it is critical to recognize a tick bite as soon as possible to reduce the chance of Lyme disease transmission (Transmission of Lyme Disease, 2023).

Lyme disease is most prevalent in the upper Midwest and northeastern and mid-Atlantic states. Recently, studies have shown that the range of the black-legged tick is expanding, leading to an increase in Lyme disease cases (Lyme Disease, 2022).

With the range of black-legged ticks expanding and Lyme disease cases increasing, it is critical to find new ways to control the spread of the disease. One potential way could be to target the host directly, the white-footed mouse. Oral and topical acaricide treatments have been used on white-footed mice in the past. Acaracides, pesticides used to kill mites and ticks, affect the central nervous system through various mechanisms. They affect gamma-aminobutyric acid (GABA)-gated chloride channels, octopamine tyramine receptors, voltage-gated sodium channels, glutamate gated chloride channels (Glu-Cl), and can inhibit acetylcholine-esterase (AChEs). Each acaricide has a different target and mode of action that affects growth, reproduction, and survival of different tick species (Obaid et al., 2021). Acaricides also have selective toxicity. This means that they kill ticks and mites, but they do not do any harm to the mammals the acaricide is given to. This could be due to the differential sensitivity of tick and

mite GABA receptors compared to mammalian GABA receptors (Credelio (lotilaner) for Dogs, 2023).

One family of acaricides that could be tested is the isoxazolines (afoxolaner, lotilaner, fluralaner, sarolaner), a class of veterinary drugs used to prevent flea and tick infestations in dogs. These drugs are long lasting with a single dose lasting one month and sometimes more in dogs (Snyder et al., 2016). When isoxazolines are given to dogs, they are absorbed into the blood and adipose tissue and then metabolized and excreted into the intestines. The drug and metabolites are then shed in the feces.

There has been very little research done on these drugs, so the environmental toxicity of isoxazolines remains unstudied. If these drugs are put out into the field to stop the spread of Lyme disease, there is always a possibility they could be toxic to other living organisms in the environment. This study will look at the toxicity of isoxazoline drug metabolites shed in the feces of treated Peromyscus mice to fish, specifically fathead minnows (*Pimephales promelas*).

Fathead minnows are part of the Cyprinidae family, an ecologically important family. They are tolerant of a wide range of water quality characteristics such as pH, turbidity, temperature, etc. The reproductive activities of this fish are very well known. They become mature 4-5 months after hatching and can spawn for several months continually. Because they can live in a variety of water types and can reproduce for a long period of time with many offspring, they make a great fish species for laboratory testing (Ankley and Villeneuve, 2006). It is important to note, this study is not specifically looking at the possible interaction between fathead minnows and excreted mouse feces, but rather using the minnows because they are a great model system to see if there is biologically active drug and/or metabolites excreted in the mouse feces.

Methods

Treatment

White-footed mice from the University of North Dakota (UND) breeding colony were treated with lotilaner (Elanco US Inc, 2018) via a peanut butter bait at UND on May 30, 2022. The bait was one part rat chow (ground to a coarse powder with a mortar and pestle) and one-part creamy peanut butter. Warm tap water was added to bring the mixture to a dough-like consistency. A Credelio™ tablet for puppies weighing 4.4-6 pounds (56.25 mg lotilaner per tablet) was ground into a powder and added to the bait at a concentration of 1 mg lotilaner per 500 mg of bait. Once the bait was mixed, 500 mg was applied to a baby carrot slice and given to the mice. Control mice were also given bait on a carrot, without the lotilaner. Mice were caged individually and checked the next day to ensure all bait was consumed.

The mice were used in isoxazoline drug efficacy studies during which fecal pellets were collected from the bedding, plastic discs in the water and water trays under the cages of treated and untreated rodents. Pellets from the bedding and discs were considered dry and pellets from the water trays were considered wet. Pellets collected from individual rodents were placed into small centrifuge tubes, labeled with mouse ID, date, and wet or dry, and stored at -20^{o}c.

Bioassays

Fathead minnow bioassays were conducted at the University of North Dakota (UND) laboratory in May and June 2023. Preliminary tests were run to find the correct variables. 3,000 mL of dechlorinated water and 300 mL of water the minnows came in, were added to nine containers. Three of the containers were fecal pellet only, three were minnow only and three were minnow plus pellet. Fathead minnows were obtained from Cabela's in East Grand Forks,

MN. One minnow was put in each minnow container along with five untreated fecal pellets in the minnow plus pellet containers. Five untreated pellets were also added to each of the pellet only containers. The water in the containers was aerated using pumps and air stones. The minnows and pellets then sat for 24 hours. After 24 hours, all six fish were dead, and all fecal pellets were intact and had not been eaten. A few rounds of preliminary tests were done before the correct variables were discovered.

The minnows would not eat the fecal pellets, so it was decided to make contaminated lotilaner water so the lotilaner would run over the minnow's gills and into the blood stream. Water from the coulee that runs through campus was collected to make Poisonous Peromyscus Pellet (PPP) water. Approximately 100 dry fecal pellets and 50 wet pellets were added to 14,000 mL of coulee water. The pellets were from days 5, 11, 12, 13, 14, 15 and 19. The water sat for 24 hours. After 24 hours, a Buchner funnel with filter paper connected to a vacuum pump was used to filter the PPP out of the water. 2000 mL of PPP water was put into each PPP fish container, along with one minnow. 2000 mL of control coulee water was put into the control containers along with one minnow. 1000 mL of PPP water was put into a PPP mosquito container and 1000 mL of control coulee water was put into the control mosquito container. Approximately 50-100 mosquitos were put into each mosquito container. Air stones were put into the fish containers. The fish and mosquitos were left to sit for 24 hours. The fish were left to sit for another 24 hours along with ten mosquito larvae per container. The fish were euthanized by rapid chilling for 10 minutes and then decapitated.

Data Analysis

Percent mortality was calculated using Microsoft Excel®.

Results and Discussion

Water contaminated with the feces from lotilaner-treated *P. leucopus* mice was toxic to *C. pipien*s mosquito larvae because all larvae died within 24 hours after exposure. However, the same water had no effect on fathead minnows swimming in the same water for 48 hours. At 48 hours, all fish were alive and swimming with a lot of energy. The fact that all fecal particulates were filtered from the water before use indicates that lotilaner and/or its metabolites leeched from the feces into the water and were able to pass through the cuticle of the mosquito larvae to affect the central nervous system and kill the insects. This suggests that lotilaner and/or its metabolites may have been able to also pass through the gills of the minnows, but without producing any observable acute neurotoxic effect.

Isoxazoline drugs have a very good safety profile for a wide range of vertebrate species and have been used as systemic ectoparasiticides in dogs and cats for the last decade (Zhou et al. 2022). More recently, isoxazoline drugs have been used to treat chickens (Durden et al., 2022) and snakes (Fuantos Gamez et al., 2018). The only study to-date that has examined isoxazoline toxicity in fish is the studies of Jia et al. 2018, who tested fluralaner in zebrafish, *Danio rerio*, and found that fluralaner was of low toxicity to this species. This study confirms that another isoxazoline drug, lotilaner, also exhibited no acute toxicity to the fathead minnow. This is encouraging for the use of lotilaner as tick control in the field, as it suggests that lotilaner can be used near aquatic habitats and pose little to no danger to fish.

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Table 5. Exposure of mosquito larvae (*Culex pipiens*) and fathead minnows (*Pimephales promelas*) to filtered water contaminated with the feces of lotilaner-treated mice (*Peromyscus leucopus*).

		Percent Mortality (n)	
		24 hour	48 hour
Lotilaner Contaminated water	Minnows	0 % (6)	0 % (6)
	Mosquito larvae	100 % (72)	N/A
Uncontaminated Control water	Minnows	0 % (6)	0 % (4)
	Mosquito larvae	0 % (94)	N/A