Acaricidal activity of the orally administered isoxazoline drugs (Lotilaner, Fluralaner, and Afoxolaner) in white-footed mice against larval ticks

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Abstract

Lyme disease, caused by *Borrelia burgdorferi*, is currently the most prevalent vector borne disease in North America. With the CDC estimating almost 500,000 new cases every year, methods are needed to control the vector, the black legged tick (*Ixodes scapularis*). Like other tick-borne diseases, Lyme disease is zoonotic and involves tick parasitism on rodent reservoirs in the wild. The main reservoir host of Lyme disease is the white-footed mouse (*Peromyscus leucopus*). This study aims to explore the effectiveness of a reservoir targeted control approach by the treatment of white-footed mice with oral isoxazoline class drugs. We aim to understand the effectiveness and length of protection of isoxazoline drugs in mice against both black-legged ticks and American dog ticks as well as any protection that may be passed from nursing mice to offspring through drug residue in milk.

A white-footed mouse colony was established from wild mice caught in Grand Forks, ND. Pathogen free larval black legged and American dog ticks were obtained from the CDC through BEI Resources. Mice were treated once orally with 50mg/kg bodyweight of a selected commercially available isoxazoline drug (afoxolaner, fluralaner, lotilaner). Control mice received no treatment. After treatment mice were infested with serial tick infestations at various time points to test for acaricidal activity. Mice were anesthetized with a pentobarbital injection and 20 larval ticks of both species were applied. Mice were placed in wire bottom cages suspended over a tray of water. Tick drop-off was recorded for five days. Mother mice were also treated with lotilaner to determine if acaricidal activity might be passed to their offspring. A high-dose treatment mother received three weekly treatments throughout her 23-day nursing period while a low-dose treatment mother received only one treatment on post-partum day 10. Control mothers did not receive treatment. After weaning, pups were exposed to serial larval tick infestations. The

number of successful engorged larval ticks that detach from control mice were compared with the number of engorged ticks that detached from treated animals.

All isoxazolines provided a decrease in engorged ticks in treated mice with lotilaner providing the longest duration of protection of up to 2 months. Lotilaner was transferred to nursing pups and provided pups with up to 2 weeks of protection against tick attachment.

Chapter 1. Introduction

Tick-borne illnesses are of increasing concern. The CDC currently classifies ticks as the most medically important group of arthropods. Around 75% of human vector-borne diseases in the USA are tick-borne, with the number of human cases steadily on the increase (Eisen et al 2017). This may be due to the expansion of the geographical range of many tick vectors (Eisen et al. 2016, Garner et al. 2020). In addition, there have been several new tick-borne pathogens that have emerged within the last few decades, including Powassan virus and Heartland virus, among others (Hermance & Thangamani 2017, Brault et al. 2018). With the rise of tick-borne illnesses, methods are needed to control tick populations to lower the risk of tick-borne illness.

Of particular interest for the development of tick control methods involves the prevention of Lyme disease. Lyme disease is the most prevalent vector borne disease in North America, with the CDC estimating that almost 500,000 infections every year (Kugeler et al. 2021). The disease is spread from the bite of infected black-legged ticks (*Ixodes scapularis*). These ticks pick up the infectious agent, the bacterium *Borrelia burgdorferi*, after feeding on an infected vertebrate reservoir (Nguyen et al. 2019, Johnson et al. 1984). The primary reservoir host for Lyme disease is the white-footed mouse (*Peromyscus leucopus*) (Rafinesque, 1818, Nguyen et al. 2019). Current strategies to keep blacklegged ticks off white footed mice and halt the transmission of the infectious bacteria to the tick make use of the acaricidal compound fipronil as a topical treatment. Results from this method have had varying success (Hinckley et al. 2016, Little et al. 2020). We believe that an approach with oral acaricides such as isoxazolines may provide better results.

Isoxazolines are prescribed veterinary flea and tick medications primarily in use in dogs. Four isoxazoline compounds are approved by the FDA for use in dogs and cats including fluralaner, afoxolaner, sarolaner, and lotilaner (Goncalves et al. 2021). Several characteristics suggest that these compounds are excellent candidates for the use in tick control. These compounds work by blocking arthropod GABA-gated chloride channels (GABACLs). Under normal function, GABA binds to the chloride channel and activates the influx of chloride ions into the neuron to activate an inhibitory action potential. Isoxazolines bind to these chloride channels, inhibiting the flow of chloride ions. This causes hyperexcitation and ultimately death in the arthropod (Shoop et al. 2014, Rufener et al. 2017). While isoxazolines target arthropod GABACLs, they do not target mammalian GABACLs (Goncalves et al. 2021). In addition, Isoxazoline compounds have a much lower oral toxicity to rats (rat oral LD50>1,000 mg/kg BW) than fipronil (rat oral LD50=95 mg/kg BW) (National Pesticide Information). Isoxazoline drugs have also been shown to kill ticks much faster (4-12 hours after attachment) than fipronil (24-48 hours) (Six et al. 2016, Wengenmayer et al. 2014, Murphy et al. 2017, Cruthers et al, 2001). Lastly, an oral isoxazoline formulation may be easier to use in comparison to topical application of fipronil. To apply fipronil to mice, special bait boxes must be used. Mice must pass under a wick to have the topical fipronil applied (Dolan et al. 2004, Schulze et al. 2017).

An important property to keep in mind is that isoxazolines are lipophilic. Drugs that are lipophilic are not approved for the use in lactating animals due to the possibility of drug secretion to offspring via the milk. Ivermectin, another lipophilic acaricide, was detected in nursing lambs after treatment of ewes (Cerkvenik et al. 2002). While not ideal for the use in lactating human mothers or commercial livestock, this property could be beneficial for a rodent targeted tick control. If the transfer of acaricidal drugs from the mother provides protection to the offspring,

the abundance of tick killing rodents in a reservoir population could be increased. This could increase the success of reservoir targeted approaches to control ticks.

While these characteristics make isoxazolines a promising choice for a rodent reservoir targeted tick control, first they must be studied for their effectiveness in white footed mice. The aim of this study is to compare the efficacy and duration of acaricidal activity of isoxazoline drugs in white footed mice as well as to test for any maternal passage of drugs to offspring. This study aims to compare the efficacy and duration of protection against larval ticks in white-footed mice after a single dose of one of three of the following isoxazoline drugs; afoxolaner, fluralaner or lotilaner. In addition, this study will determine if drug residue is passed from mother to nursing pups through the milk and provides tick protection. I hypothesize that all three isoxazoline drugs will provide protection to white-footed mice against larval tick attachment and that there will be drug passage through the milk to offspring that will provide a short amount of protection after weaning.

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Chapter 2. Efficacy of three orally administered isoxazoline acaricides against larval ticks on *Peromyscus leucopus*, a reservoir host of Lyme disease

Abstract

With the spread of Lyme disease across North America, solutions are needed to control the black-legged tick (*Ixodes scapularis*), the vector of the disease in the northeastern and upper Midwest U.S. White-footed mice are an important reservoir host for Lyme disease and infected individuals will transfer the bacterial agent (*Borrelia burgdorferi*) to early-stage ticks. Treating wild mice to control ticks could stop this transmission and reduce tick populations. In addition to the black-legged tick, this intervention could control tick species, such as the American Dog Tick (*Dermacentor variabilis*) that transmit other diseases. Little research exists that tests the newer isoxazoline class flea and tick medications, commonly used on dogs, for efficacy in mice against ticks. We tested the efficacy and protection longevity of afoxolaner, lotilaner (Credelio™) and fluralaner (Bravecto®) against ticks in mice. Fifty-six white-footed mice were allotted into treatment groups of control, afoxolaner (10 mg/kg), lotilaner (50 mg/kg) or fluralaner (50 mg/kg). At determined time points after treatment mice were infested with 20 larvae of both *I. scapularus* and *D. variabilis*. Engorged tick drop off was recorded for five days after infestation, at which point any remaining ticks were removed.

Lotilaner-treated mice were protected from larval *D. variabilis* attachment up to 46 days and larval *I. scapularus* attachment up to 61 days after treatment. Fluralaner was successful at preventing tick attachment at day 3, but not at day 11 after treatment. Afoxolaner was successful in reducing the survivability of ticks on mice for up to 6 days. These results indicate that lotilaner provides long-term protection from ticks in mice and would be an excellent option for further testing of field-based tick control methods.

Introduction

Between 2010 and 2018, an estimated 476,000 people were diagnosed annually with Lyme disease (Kugeler et al. 2021). The disease is transmitted through bites from black-legged ticks (*Ixodes scapularis*) (Say, 1821) that are infected with the spirochete bacterium *Borrelia burgdorferi* (Nguyen et al. 2019, Johnson et al. 1984). Over the past few decades, studies have noted the increase in range of the black-legged tick in the upper Midwest, northeast and the mid-Atlantic states as well as an increase in the areas that report cases of Lyme disease (Eisen et al. 2016, Garner et al. 2020). With the range of the black-legged tick forecasted to continue increasing (Gardner et al. 2020), new strategies are needed to control the spread of Lyme disease.

One avenue to controlling both the density of tick population and the transmission of *B. burgdorferi* to ticks is to target the reservoir host and vector interaction. One of the major reservoir hosts of Lyme disease is the white footed mouse (*Peromyscus leucopus*) (Rafinesque, 1818, Nguyen et al. 2019). Treatment of mice with acaricides, chemicals specifically formulated to target ticks and mites, could potentially lower the density of ticks and prevent the transmission of Lyme disease. Studies have found that oral and topical insecticide treatments of mice could slow the spread and prevalence of Lyme disease (Poché et al. 2020, Dolan et al. 2004, Brown et al. 2020, Little et al. 2020). Fipronil-baited boxes are implemented already in the Northeast United States, although with varying levels of success (Hinckley et al. 2016, Dolan et al. 2004, Little et al. 2020). Mouse treatment with acaricides could also help control the density of other tick species such as the American Dog tick (*Dermacentor variabilis)* (Say, 1821).

Another family of acaricides that could be a successful option is the isoxazolines, a class of veterinary flea and tick medication, prescribed primarily to dogs (*e.g.*, afoxolaner, fluralaner, sarolaner, lotilaner) (Gassel et al. 2014, Shoop et al. 2014). Isoxazolines work by interfering with the passage of chloride ions through the GABA-gated chloride channels in the nervous system of arthropods, causing hyperexcitation and death (Shoop et al. 2014, Simon-Delso et al. 2015, Rufener et al. 2017). Isoxazolines are highly effective at killing ticks on the target animal (*e.g.*, dogs and cats) and are long lasting with a single dose lasting one month or more (Cavalleri et al. 2018, Murphy et al. 2017, Cavalleri et al. 2017, Murphy et al, 2017, Six et al. 2016, Wengenmayer et al, 2014). To add to this, the rate of kill of isoxazolines is fast enough to kill the tick before *B. burgdorferi* transmission to dogs (Baker et al. 2016, Honsberger et al. 2016, Six et al. 2016). All these factors indicate that isoxazolines are excellent candidates for Lyme disease control.

Outside of the target species, little research has been done with these drugs and little is known about the drug's efficacy against ticks in white-footed mice. Of the four formulations of isoxazolines, only fluralaner has been tested in white-footed mice. While effective in protecting mice from tick attachment, the duration of protection that is seen in dogs was not achieved in mice; more testing is necessary to understand the duration of protection (Pelletier et al. 2020). A study of fluralaner in the field found the drug to be successful in lowering the number of attached ticks on mice (Pelletier et al. 2022). The other isoxazoline drugs (afoxolaner, lotilaner and saurolaner) remain untested in white footed mice.

Our study will provide a more fine-tuned look at the efficacy of fluralaner in mice and, to our knowledge, will provide the first look at the efficacy and duration of protection against both *I. scapularis* and *D. variabilis* of lotilaner, afoxolaner, and fluralaner in white-footed mice.

Materials & Methods

2.1 Animal models and safety

A white footed mouse (*Peromyscus leucopus*) colony was established from wild individuals (n=12) caught in spring 2021 using live traps placed around residential homes in Grand Forks, ND. All collections and handling were done in accordance with University of North Dakota Institutional Animal Care and Use Committee policies (IACUC # 2103-7) and Federal requirements for the appropriate use of animals in research. Genomic DNA from the mice was collected from blood samples and the species confirmed using multiplex primers targeting the cytochrome oxidase subunit III (COIII) (Tessier et al. 2004, Larson et al. 2018). Mice were treated for endo and ectoparasites on arrival with an oral gavage of moxidectin praziquantel solution and a topical application of DuMOR® Equine Fly Spray (Tractor Supply Co. Brentwood, TN). Mice were screened for Hantavirus by Direct ELISA and for intestinal worms by fecal examination. All mice used for this study were born in captivity and generation F1 or F2 of the breeding colony with no previous exposure to ectoparasites. Pathogen free larval *Ixodes scapularis* and *Dermacentor variabilis* were supplied from BEI Resources (Manassas, VA).

2.2 Direct Enzyme-linked Immunosorbent Assays.

The capture antigen for hantavirus was recombinant protein of the nucleocapsid from Sin Nombre virus (ATCC No. NR-9670, BEI Resources, Manassas, VA). The ELISAs were started by adding a 50 ml of a capture antigen (0.025 µg/ml) in phosphate-buffered saline (PBS) solution to each well of 96-well microtiter plate (Corning® Costar®, Corning, NY). Plates were covered and incubated overnight at 3°C.

Wells were emptied and incubated for one hour at room temperature with 0.5% boiled casein blocking buffer. Boiled casein was prepared by suspending 5 gm casein in 100 ml 0.1 N NaOH and bringing the solution to a boil. After the casein had dissolved, 900 ml of PBS was added, and the solution was allowed to cool. The pH was then adjusted to 7.4 with hydrochloric acid (1 M HCL) and 0.1 gm of thimerosal plus 0.2 gm of phenol red were added. Samples and commercial reagents were diluted in 0.5% boiled casein; 50µl per well volumes were used throughout, and plates were covered during incubations. Assays were conducted at room temperature. Test sera from captured Peromyscus rodents were diluted serially 1:200 to 1:1,000. In addition to test sera, each plate included positive and negative control wells. Positive and negative control rodent sera were diluted serially 1:1000 to 1:32,000 and tested. Positive control serum was high-tittered polyclonal antiserum to the nucleocapsid protein of Sin Nombre virus produced by immunization of deer mice (*Peromyscus maniculatus*) with the recombinant Sin Nombre nucleocapsid protein (ATCC No NR-9676, BEI Resources, Manassas, VA). Negative control sera were collected from non-immune laboratory mice (*Mus musculus)*.

After an hour incubation at room temperature, wells were emptied and washed 3 times with PBS plus 0.05% Tween 20. The detection system consisted of affinity-purified polyclonal antibody to whole *P. leucopus* IgG, H+L chains, made in goat, and labeled with horseradish peroxidase (SeraCare, Milford, MA). This was diluted 1:4000 in boiled casein buffer and incubated for 1 hr. Wells were emptied and washed 6 times with PBS plus 0.05% Tween 20 and the enzyme substrate added (SureBlue TMB 1-Component Microwell Peroxidase Substrate, SeraCare, Milford, MA). Absorbance values (414nm) were recorded after 30 minutes using an ELISA plate reader. Samples were tested in triplicate and the mean and standard deviation (SD)

calculated for each dilution. The cutoff value determining the ELISA endpoint titer of a sample was defined as the mean absorbance value of the appropriate negative control + 3 SD.

2.3 Afoxolaner Treatment

Twenty mice were assigned to two treatment groups; afoxolaner (10 ml/kg BW; n = 12) and control (n = 8), administered as a single oral treatment via oral gavage. Technical grade afoxolaner was dissolved into DMSO (10 ml/ml), then diluted with sunflower oil to create a working solution. Afoxolaner solution (10 mg/kg BW) was administered by gavage at volumes 0.2 to 0.4 cc depending on body weight. Control mice received 0.2 cc untreated sunflower oil via gavage. Mice were infested with ticks on days 2, 6 and 13 after treatment to check for acaricidal activity.

2.4 Lotilaner and Fluralaner Treatment

Forty-six mice were assigned to three treatment groups (1 mg lotilaner; n = 20, 1 mg fluralaner; n = 7, or untreated control; n = 19) administered as a single oral treatment via a bait ball. For lotilaner, one Credelio™ tablet for dogs and puppies (56.25 mg lotilaner per chewable tablet) was ground and vortexed in 1.0m hot water. The mixture was added to 1.5 g of 1:1 ground rat chow and peanut butter. For fluralaner, 250 mg was cut from a Bravecto® tablet for dogs 88-123 lb. (1400 mg fluralaner per chewable tablet) and was ground and vortexed with 500 µm hot water and added to 1.5 g of 1:1 ground rat chow and peanut butter. The amount of active ingredient for both treatments was 1 mg/0.5 g of bait. Treated bait (0.5 g) was placed on a carrot slice and offered as the only food source to the mouse overnight. Control mice received 0.5 g bait of rat chow and peanut butter mixture on a carrot slice. Cages were inspected the following day to check for bait consumption. All bait was consumed.

2.5 Tick infestation

Mice were anesthetized with pentobarbital (Sigma St. Louis, MO) via intraperitoneal injection to allow for effective tick attachment (Levin & Fish, 1998). Pentobarbital doses were determined by weight class, with each mouse receiving approximately 60 mg/kg body weight. Fur was trimmed around the neck to allow ease of tick application. Forty larval ticks (20 *Ix. scapularis* + 20 *D. variabilis*) were applied with a paint brush to the head, shoulders, and ears. Afterwards, mice were rolled in a paper towel to keep the mouse warm, for use of bedding, and importantly, to keep ticks from falling off the mouse prematurely. Mice were placed in a wire bottom cage over a tray of water and monitored every several hours until they recovered from anesthesia. No mice died from anesthesia overdose. At each infestation period, 3 to 4 mice from each treatment group were infested (Table 1 & 2). Infestations of fluralaner-treated mice were conducted on days 3 and 11 after treatment; infestations of afoxolaner-treated mice were conducted on days 2, 6, and 12 after treatment; and infestations of lotilaner-treated mice infestations were conducted on days 3, 11, 18, 25, 32, 46, 61, and 82 after treatment.

2.6 Tick counting

On days 1-5 of the infestation, water trays and bedding were inspected daily for unattached flat and engorged ticks. All ticks were assessed as alive, dead, or moribund. Tick species were determined by anatomical characteristics identifiable in both flat and engorged ticks. Infestations concluded on day 5 at which point mice were anesthetized with a pentobarbital injection and any remaining attached ticks were removed and counted. Selected individuals were euthanized to collect samples of plasma, skin and subcutaneous adipose for later analyses.

2.7 Tick infestation 5-8

Due to a shortage of mice and the longer duration of efficacy than expected, starting at infestation 5 and for all subsequent infestations, only one treated mouse and no control mice were euthanized at the conclusion of each infestation. On day 5 after tick infestation, mice were again anesthetized with a pentobarbital injection to remove any remaining attached ticks and returned to their normal holding cages until infested again at the next time point. The time duration of tick attachment infestation 8 was extended to 6 days because attached ticks appeared to be completing their engorgement at a slower rate than was observed in previous infestations.

2.8 Data analysis

The primary outcome variable for these trials is the number of larval ticks that successfully completed a blood meal and detached from their host – hereafter referred to as 'replete ticks'. The average number of replete ticks of each species was calculated for each treatment and infestation. The average number of replete ticks per treatment group was compared with that of corresponding control groups using two sample t-tests tests in R (R Team, 2022) using the *t.test()* package.

Drug efficacy was determined by the percent reduction in the number of replete ticks on treated mice compared to replete ticks from control mice. Efficacies were calculated according to the formula as follows:

$$\text{Efficacy (\%)} = 100 \times (MC - MT) / MC$$

"MC" is the mean number of replete ticks produced on control mice, and "MT" is the mean number of replete ticks produced on mice in the treated groups.

The effect that the independent variables 'tick species' and 'number of times a mouse had been infested' had on the amount of recovered replete ticks was tested by ANOVA using the *lm()*

function in R. Residuals were plotted against fitted values to confirm that the assumption of normality was reasonable.

To determine if mice exhibited behavioral or immunological resistance to tick infestation over the course of these trials, differences between naïve mice and mice previously exposed to ticks were examined in the untreated control group. Replete ticks in control mice was modeled as a function of the number of times a mouse had been exposed to ticks with a linear regression using the *lm()* function in R and graphed with 95% confidence intervals using *ggplot2* (Wickham, 2016). Residuals were plotted against fitted values to confirm that the assumption of normality was reasonable.

Results

The number of engorged *Ixodes scapularis* was not significantly different in afoxolaner treated mice at any time point ($p > 0.05$) (*Table 1*). Efficacy was 100% on day 3 after treatment, and dropped to 0% for both day 6 and 13 (*Table 3*).Number of engorged *I. scapularis* was significantly lower on lotilaner treated mice than control on days 3, 11, 18, 25, 32, and 46 ($p < 0.05$) but not on days 61 and 82 ($p > 0.05$) (*Table 2*) Efficacy was over 95% on days 3-32, and dropped to 81.9%, 85.7% and 0% on days 46, 61 and 82 respectively (*Table 3*). Number of engorged *I. scapularis* was significantly lower on fluralaner treated mice than control on day 3 ($T = 5.19$, $df = 4$, $p = 0.0065$) but not on day 11 ($p = 0.1966$) (*Table 2*). Efficacy was at 100% on day 3, and 0% on day 11 (*Table 3*).

The number of engorged *Dermacentor variabilis* was significantly lower on afoxolaner-treated mice on day 2 after treatment ($p = 0.0064$) but not on day 6 or 13 ($p > 0.05$) (*Table 4*). Efficacy was 100% on day 3, 81.8% on day 6, and 23.1% on day 13 (*Table 3*). Number of

engorged *D. variabilis* was significantly lower on lotilaner-treated mice than control on days 3, 11, 18, 32, and 46 ($p < 0.05$), but not on days 25, 61 and 82 ($p > 0.05$) (*Table 4*). Lotilaner efficacy was over 98% through day 46, and dropped to 50% on day 61 and 0% on day 82 (*Table 3*). Number of engorged *D. variabilis* was significantly lower in fluralaner treated mice than control on day 3 ($T = 6.20$, $df = 4$, $p = 0.0034$) but not on day 11 ($T = 0.93$, $df = 6$, $p = 0.389$) (*Table 4*). Fluralaner had 72.7% efficacy on day 3, and 11.8% on day 11 (*Table 3*).

The number of replete ticks recovered from each mouse was not affected by the number of times a mouse had been exposed to ticks, tick species, or interactions between number of exposures and species ($p = 0.131$).

The number of replete ticks produced by control mice was inversely proportional to the number of times a mouse had been infested with larval ticks (Figure 3). This relationship was significant for both tick species *I. scapularis* ($F_{1,23} = 10.91$, $p = 0.003$, $R^2 = 0.3217$) and *D. variabilis* ($F(1:23) = 27.93$, $p < 0.0001$, $R2 = 0.548$).

Discussion

The three isoxazoline compounds tested had variable success at reducing larval *I. scapularis* and *D. variabilis* tick attachment and engorgement on treat mice. Lotilaner provided nearly 100% protection against attachment and engorgement of both tick species for up to 46 days after treatment and continued to provide substantially reduced tick attachment and engorgement up to 61 days as compared to that in untreated control mice. Afoxolaner was successful in reducing the attachment of *D. variabilis* for up to 6 days. Due to poor *I. scapularis* attachment rates during these trials, it is unclear if afoxolaner reduced *I. scapularis* attachment on days 2 and 6, but the drug was no longer effective on day 13. Fluralaner was successful at

completely protecting mice from *I. scapularis* attachment on day 3 after treatment, but less effective against *D. variabilis*. At 11 days fluralaner was no longer effective in protecting mice from either tick species. This is consistent with a previous laboratory study that found orally administered fluralaner lost its acaricidal efficacy against larval from *I. scapularis* somewhere between 3 and 28 days after treatment (Pelletier et al, 2020). Fluralaner is the first isoxazoline compound to have been tested in the field for tick control. Weekly deployments of fluralaner-treated baits over a 3-year period were shown by Pelletier et al. (2022) to significantly decrease the number of juvenile *I. scapularis* ticks per mouse. Due to its longer protective duration, lotilaner may be an even better isoxazoline compound than fluralaner to use for the control of ticks on white-footed mice. If similar results are seen in the field, lotilaner may also be more cost-effective, with fewer doses needing to be deployed over the course of the tick season.

While lotilaner provided up to 2 months of protection in white footed mice from larval tick attachment, protection was shorter than that seen in dogs. Lotilaner provides dogs with acaricidal protection for at least 3 months against multiple species of tick (Cavalleri et al. 2017). This difference in the duration of acaricidal protection between mammal species is most likely due to higher metabolism in mice compared to dogs (Kleiber 1932).

A decrease in tick attachment was observed in the control mice with an increasing number of exposures to ticks. Because of factors affecting attachment at later time points, data from infestation 8 may not be a reliable end point for the efficacy of lotilaner. Ticks used at later infestations were older and mice may have become more adept at grooming after multiple exposures to ticks. Grooming in mice is a major contributor to larval tick mortality (Shaw et al. 2003). Initial methods eliminated learned grooming behaviors by using new mice at each infestation. All mice used were lab reared and had not encountered ectoparasites. Because of the

need to reinfest mice, the poor attachment seen in later infestations could be attributed to decreased tick fitness or learned mouse grooming behaviors. Although ticks used in later infestations were older, we do not believe that decreased fitness was affecting tick attachment rates. Ticks were only applied to mice if they were actively crawling or questing. Lethargic or immobile ticks were not selected. Instead, I believe poor tick attachment was more likely attributed to learned grooming behaviors. Mice were often observed grooming themselves.

Higher tick attachment was seen on naïve mice compared to tick-experienced mice. Previous studies have seen ticks less successful on experienced mice versus naïve mice (Hazler & Ostfeld, 1995; Davidar et al, 1989). Despite previous exposure to tick infestations, treated mice experienced higher tick attachment once the protection from lotilaner ceased compared to their control counterparts. It is possible that control mice learned grooming behaviors in response to repeated high tick load while treated mice did not.

Of the isoxazoline drugs tested, lotilaner is not only highly effective but also provides longer protection against ticks at 50 mg/kg body weight. These traits make lotilaner a good candidate for further field trials on the control of tick density and incidence of *B. burgdorferi* in ticks. Because of our small sample size, further study of the efficacy of lotilaner in white-footed mice may be necessary to understand differences between mice. Further testing in the laboratory may be necessary to optimize the dosage to create an effective treatment while also considering the cost of future deployment. Future studies should also consider any negative effects that may arise from deploying treatments into the environment as other acaricides such as fipronil have been found to have negative effects on a variety of non-target organisms, particularly arthropods (Miller et al, 2020; Al-Badran et al, 2019; Overmyer et al, 2007; Peveling et al, 2003; Waite et al, 2019). Isoxazoline class drugs remain untested for any adverse effects to the environment.

Before deployment, they should be tested for non-target side effects, particularly with non-target arthropods that may be sensitive to acaricide pollution from treated animals.

Table 1. Production of engorged larvae successfully engorging and detaching (=*repletes*) from untreated *Peromyscus leucopus* mice versus *Peromyscus leucopus* mice given a single oral dose (50mg/kg BW) of technical grade afoxolaner mixed in sunflower oil.

Infest No.	Days after treatment when ticks were applied	Tick Species	Control		Afoxolaner		
			% Mice yielding repletes (N)	Mean ± SD repletes	% Mice yielding repletes (N)	Mean ± SD repletes	T-test comparison of means
1	2	*Ixodes scapularis*	50% (8)	0.875 ± 1.36	0% (8)	0	P=0.0894 T=1.8249
		Dermacentor variabilis	100%	8.25 ± 4.03	0% (4)	0	P=0.0064 T=4.09
2	6	*Ixodes scapularis*	50% (8)	0.875 ± 1.36	25% (4)	0.25 ± 0.5	P=0.4024 T=0.8744
		Dermacentor variabilis	100% (4)	8.25 ± 4.03	75% (4)	1.75 ± 2.22	P=0.0301 T=2.826
3	12	*Ixodes scapularis*	87.5% (8)	5.75 ± 5.23	75% (8)	5.88 ± 6.13	P=0.9684 T=0.0404
		Dermacentor variabilis	100% (4)	3.25 ± 2.63	100% (4)	2.5 ± 6.24	P=0.8952 T=0.1374

Table 2. *Ixodes scapularis.* Production of engorged larvae successfully engorging and detaching (='*repletes*') from untreated control *Peromyscus leucopus* mice versus *Peromyscus leucopus* mice given a single oral dose (50mg/kg BW) of fluralaner (Bravecto®) or lotilaner (Credelio™).

Infest No.	Days after treatment when ticks were applied	Control		Fluralaner			Lotilaner		
		% Mice yielding repletes (N)	Mean ± SD repletes	% Mice yielding repletes (N)	Mean ± SD repletes	T-test comparison of means; fluralaner vs control	% Mice yielding repletes (N)	Mean ± SD repletes	T-test comparison of means; lotilaner vs control
1	3	100% (3)	10.3 ± 3.1	0% (3)	0	P=0.006 T=5.2	0% (3)	0	P=0.006 T=5.2
2	11	100% (4)	7.0 ± 3.6	100% (4)	11.0 ± 4.5	P=0.197 T=-1.4	0% (4)	0	P=0.008 T=3.9
3	18	100% (3)	14.0 ± 3.6	-	-	-	0% (3)	0	P=0.002 T=6.7
4	25	100% (3)	13.3 ± 3.1	-	-	-	33% (3)	0.3 ± 0.6	P=0.003 T=6.7
5	32	100% (3)	17.3 ± 0.6	-	-	-	0% (4)	0	P<0.001 T=62.1
6	46	100% (3)	7.0 ± 1.7	-	-	-	33% (3)	1.0 ± 1.7	P=0.020 T=3.7
7	61	100% (3)	7.0 ± 4.4	-	-	-	33% (3)	1.0 ± 1.7	P=0.091 T=2.2
8	82	100% (3)	2.7 ± 0.6	-	-	-	75% (4)	5.2 ± 4.1	P=0.301 T=-1.1

Table 3. Efficacy of a single oral dose of afoxolaner (10 mg/kg BW), fluralaner (50 mg/kg BW), and lotilaner (50 mg/kg BW) ingested by *Peromyscus leucopus* mice in preventing the successful engorgement of infesting larval ticks; *Ixodes scapularis* and *Dermacentor variabilis*. Numbers in parentheses indicate numbers of mice.

Days after treatment when ticks were applied	*Dermacentor variabilis*			*Ixodes scapularis*		
	Afoxolaner	Fluralaner	Lotilaner	Afoxolaner	Fluralaner	Lotilaner
3	100 % (4)	100 % (3)	100 % (3)	100 % (4)	100 % (3)	100 % (3)
11	23% (4)	11% (4)	100 % (4)	0% (4)	0 % (4)	100 % (4)
18	-	-	100 % (3)	-	-	100 % (3)
25	-	-	100 % (3)	-	-	98 % (3)
32	-	-	100 % (3)	-	-	100 % (3)
46	-	-	100 % (3)	-	-	86 % (3)
61	-	-	67 % (3)	-	-	86 % (3)
82	-	-	0 % (4)	-	-	0 % (4)

Table 4. *Dermacentor variabilis.* Production of engorged larvae successfully engorging and detaching (='*repletes*') from untreated control *Peromyscus leucopus* mice versus *Peromyscus leucopus* mice given a single oral dose (50mg/kg BW) of fluralaner (Bravecto®) or lotilaner (Credelio™).

Infest No.	Days after treatment when ticks were applied	Control		Fluralaner			Lotilaner		
		% Mice yielding repletes (N)	Mean ± SD repletes	% Mice yielding repletes (N)	Mean ± SD repletes	T-test comparison of means; fluralaner vs control	% Mice yielding repletes (N)	Mean ± SD repletes	T-test comparison of means; lotilaner vs control
1	3	100% (3)	11 ± 2	0% (3)	3 ± 1	P=0.003 T=6.20	0% (3)	0	P<0.001 T=9.53
2	11	100% (4)	12.75 ± 1.73	100% (4)	11.25 ±	P=0.389 T=0.93	25% (4)	0.25 ± 0.5	P<0.001 T=13.59
3	18	100% (3)	15 ± 1.73	-	-	-	0% (3)	0	P<0.001 T=15
4	25	100% (3)	7.67 ± 1.15	-	-	-	0% (3)	0	P=0.083 T=2.3
5	32	100% (3)	9.33 ± 4.04	-	-	-	0% (4)	0	P=0.003 T=5.29
6	46	100% (3)	6 ± 4.36	-	-	-	0% (3)	0	P=0.076 T=2.38
7	61	100% (3)	6 ± 1.73	-	-	-	33% (3)	3 ± 5.20	P=0.397 T=0.95
8	82	66.7% (3)	0.67 ± 0.58	-	-	-	75 % (4)	1 ± 0.82	P=0.576 T=-0.6

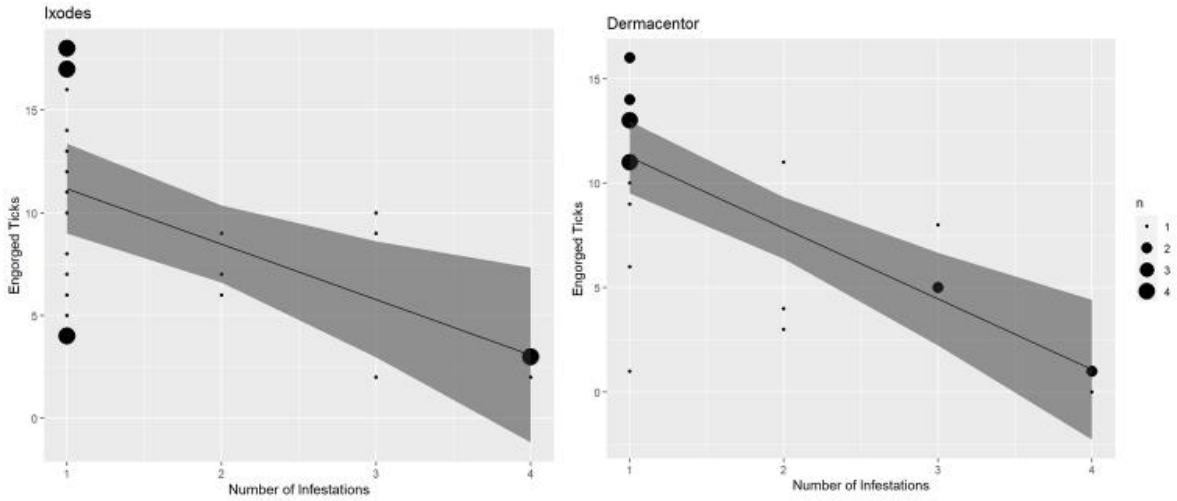

Figure 1. The number of successfully engorged ticks decreased in control mice as the number of infestations a mouse had experienced increased. Engorged *I. scapularis* = 13.88 + -2.70* Number of Infestations (linear regression, $F_{1,23} = 10.91$, $p = 0.003$, $R^2 = 0.3217$). Engorged *D. variabilis* = 14.63 + -3.39* Number of Infestations (linear regression, F (1:23) = 27.93, p < 0.0001, R2 = 0.548).

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Chapter 3. Credelio™ (lotilaner) ingested by nursing *Peromyscus leucopus* mice confers residual acaricidal activity to pups after weaning

Abstract

Treatment of mice with lotilaner has the potential to lower the density of ticks in a treated area and lower the transfer of Lyme disease. The transfer of drugs from mother to offspring through the placenta or milk is often seen as a negative outcome for both human and animal health; however, this transfer may be advantageous for controlling ticks in wild species. If drug protection passes from mother to offspring, our ability to control tick density and the spread of disease increases.

Lotilaner (Credelio™ 50 mg/kg BW per treatment) was fed to nursing mother mice to determine if acaricidal activity might be passed to their offspring. One nursing female (M1) received three weekly treatments of lotilaner throughout her 23-day nursing period. A second nursing female (M2) received only one lotilaner treatment on postpartum day 10, slightly less than half-way through her 25-day nursing period. A third female was given untreated bait as a control. After weaning, pups were exposed to serial larval tick infestations. Pups from M1 exhibited excellent acaricidal activity for 15 days after weaning. Acaricidal activity was not present at 31 days. No acaricidal activity was evident in a second litter born to M1 44 days after her last lotilaner treatment. Pups from M2 exhibited strong acaricidal activity for 8 days after weaning but not at 40 days. The transfer of acaricidal drugs from mother to offspring could increase the abundance of tick-killing rodents in a treated white-footed mouse population and increase the efficiency of reservoir-targeted approaches to control ticks.

Introduction

Lyme disease, caused by the bacterium *Borrelia burgdorferi* (Nguyen et al. 2019, Johnson et al. 1984) is currently the most prevalent vector-borne illness in North America, with the CDC estimating that around 476,000 individuals may contract the disease every year in the United States (Kugeler et al. 2021). The vector of the disease, the black-legged tick (*Ixodes scapularis*) (Say, 1821) has been increasing its range over the years. This increase in range puts more people at risk, making the need for control methods necessary.

A possible control method would be to treat reservoir hosts with acaricides to aim to control tick population and also control the transfer of *B. burgdorferi* between vector and reservoir host. A main target for treatment would be white footed mice (*Peromyscus leucopus* Rafinesque, 1818), the main reservoir host (Nguyen et al. 2019). Lotilaner (Credelio™) shows promise as a reservoir targeted control approach effective against the black legged tick due to its long lasting acaricidal protection in dogs (Murphy et al 2017, Baker et al 2018, Cavalleri et al 2017). Lotilaner belongs to a veterinary class of drugs called isoxazolines, which are lipophilic compounds that work by selectively blocking γ-aminobutyric acid (GABA) gated chloride channels in arthropods, which leads to failure of inhibitory regulation of the central nervous system and fatal excito-neurotoxicity (Gonçalves 2021, Gassel et al. 2014, Weber & Selzer 2016).

Veterinary drugs that are lipophilic are not approved for the use in lactating animals due to the possibility of drug secretion to offspring via nursing. Studies show that ivermectin, another lipophilic acaricide/insecticide, was detected in lambs after treatment of ewes (Cerkvenik et al. 2002). Although this property of lipophilic drugs is often viewed as a negative quality, it could be beneficial for tick control. Theoretically, the transfer of acaricidal drugs from mother to

offspring could increase the abundance of tick-killing rodents in a reservoir population treated with lotilaner. This could increase the efficiency of reservoir targeted approaches to control ticks.

Currently no research exists that explores the transfer of isoxazoline drugs to offspring through lactation. In our study I explored the transfer of lotilaner acaricidal activity against larval blacklegged ticks and American dog ticks from mother to nursing pups in. I hypothesized that lotilaner would pass through the milk to offspring and provide a short amount of protection after weaning.

Materials & Methods

2.1 Animal Models and Safety

All collections and handling were done in accordance with University of North Dakota Institutional Animal Care and Use Committee policies (IACUC # 2103-7, 2102-2, 2209-0043) Colony establishment and screening was done by methods referred to in the previous chapter. Pathogen free larval *Ixodes scapularis* and *Dermacentor variabilis* were obtained from BEI Resources (Manassas, VA).

2.2 Lotilaner Preparation

The lotilaner-based product, Credelio®, was diluted appropriately to achieve an oral dose of 50mg/kg BW which is twice the recommended dose for dogs. To do this, mice were weighed prior to being provided a single lotilaner-laced ‘bait’. To make the treats, a Credelio® tablet was crushed into powder, solubilized in hot water to an appropriate concentration, and mixed with a basic paste composed of peanut butter and powdered rat chow. The appropriate weight of lotilaner-laced bait for the intended recipient was placed on a small carrot slice and introduced

into the cage housing the nursing female and her pups. A control female was left untreated during her nursing period.

2.3 Mouse Selection and Treatment

Three pregnant females were randomly selected from the breeding colony. Two mice received baits that contained 50 mg/kg body weight lotilaner (Credelio™) per bait. Female one (M1) gave birth to 3 pups and was treated 3 times during her lactation period on day 3, 10, and 22 postpartum. Female 2 (M2) birth to 5 pups and was treated one time on day 10 postpartum, about half way through her lactation period. Female 3 (control) gave birth to 6 pups and received an untreated placebo bait. Pups were removed from the mother 23-25 days after birth, when they had fully weaned. Mice are able to become pregnant immediately on giving birth, then will go through diapause until the first litter is weaned. Females M1 and M2 gave birth again within days after weaning their first litter. The second litters were not treated again but were weaned on 23-25. The second litter from M1 was tested for acaricidal activity post weaning, but the second litter from M2 was not tested.

2.4 Tick Infestation and Collection

Pups from female M1 were tested for acaricidal activity by serial tick infestations on days 4, 15, and 31 post weaning. Pups from female M2 were tested on days 8 and 40 post weaning. Mice were anesthetized with pentobarbital (Sigma St. Louis, MO) by intraperitoneal injection to achieve better tick attachment rates (Levin & Fish, 1998). Pentobarbital doses were determined by weight class, with each mouse receiving approximately 60 mg/kg body weight. Forty (20 of each species) larval ticks were applied with a paint brush to the head, shoulders, and ears. Mice were rolled in a paper towel sheet to keep the mouse warm, for use of bedding, and to keep ticks

from falling off prematurely. Mice were placed in a wire bottom cage over a tray of water. Water trays and bedding were checked daily for unattached flat and engorged ticks. All ticks were assessed as alive, dead, or moribund. Each infestation was concluded on day 5 at which point mice were anesthetized with a Pentobarbital injection. Any remaining attached ticks were removed and counted. Mice were then returned to normal cages. The tick species was determined by characteristics identifiable in flat and engorged ticks.

2.5 Data Analysis

For each tick species and serial infestation, two-sample t-tests were used to compare the mean number of engorged ticks produced by pups nursed by treated mothers with that of pups nursed by the untreated mother (R Team, 2022, using the *t.test()* package).

Drug efficacy was defined as the percent reduction in counts of fully engorged ticks dropping off lotilaner-exposed mice compared with counts of fully engorged ticks dropping off control mice. Efficacies were calculated according to the formula as follows;

$$\text{Efficacy (\%)} = 100 \times (MC - MT) / MC,$$

where MC is the mean number of engorged ticks from control mice, and MT is the mean number of engorged ticks from mice from treated offspring.

The relationship between the number of engorged ticks produced and the number of infestations a mouse had experienced was examined with linear regression (control group only) using linear regression using the *lm()* function in R and graphed with 95% confidence intervals using *ggplot2* (Wickham, 2016). Residuals were plotted against fitted values to confirm that the assumption of normality was reasonable.

Results

One female *P. leucopus* was given three oral doses of lotilaner during nursing (*i.e.*, M1) and all three of her pups remained strongly acaricidal against larval *I. scapularis* and *D. variabilis* ticks for up to 15 days after weaning (Table 5). Similarly, all five pups from another female *P. leucopus* given a single dose during nursing (M2) were also strongly acaricidal against larval ticks for 8 days after weaning (Table 6). However, the acaricidal activity in both sets of pups subsided by 30 to 36 days after weaning, and none of five pups born in a second litter to M1 at 51 days after she had ingested lotilaner, displayed acaricidal activity.

Efficiency of tick engorgement on untreated control mice decreased with increased number of tick exposures (linear regression, $F_{1,10} = 14.63$, $p = 0.003$, $R^2 = 0.59$) according to the function; Number of engorged ticks = 25 + -4.93* Number of Infestations (*Figure 2*).

Discussion

The acaricidal activity of lotilaner-treated baits consumed by two different lactation *P. leucopus* mice was transferred to their pups during nursing and acaricidal protection conferred to the pups lasted up to 2 weeks after weaning. However, the maternal transfer of acaricidal activity from mother to pup only occurred during the first reproduction cycle and no acaricidal protection was conferred to the pups born in a second litters from a treated mother that had consumed lotilaner 51 days earlier.

Successful grooming is a major factor in larval tick mortality (Shaw et al. 2003). Tick attachment was less successful on control mice after serial exposures to a high tick load. We believe that this is due to learned grooming behavior over exposures leading mice to be more successful at grooming off ticks rather than tick fitness. Only active ticks that were actively crawling or questing were selected to be applied to the mice.

Lipophilic drugs are not advised for the use in pregnant or lactating animals due to potential negative effects on the offspring. During our study, pups and mothers were visually monitored often. All offspring appeared to be unaffected by the drug treatment and were robust in size and energy. Since mice were only monitored visually, it remains unknown if treatment affected pups in ways not visual, such as fertility. One study has suggested that ivermectin may have adverse effects on male rat fertility (El-Nahas & El-Ashmawy 2008). To-date, no published studies have investigated the potential adverse health effects of isoxazolines to suckling animals from lactation. Credelio™ was found to be safe for the use in puppies with little to no adverse effects on dog health (Kuntz & Kammanadiminti 2017). Further testing in white-footed mice may be necessary to assess any negative effects to the health of the animal. In addition, the efficacy and duration of tick protection in a treated mother mouse could be affected by pregnancy and lactation. Further testing could determine if there is any loss of drug effectiveness in mating female mice.

Our study suggests that lotilaner will be transferred to offspring via mothers milk and offspring will gain protection for up to two weeks after weaning. Theoretically, more than one mouse will receive protection from a single dose, amplifying the effectiveness of lotilaner in the field. Lotilaner continues to look like an excellent candidate for further field trials. Further testing of lotilaner out in the environment is necessary to truly understand its effectiveness going forward.

Table 5. Production of engorged larvae successfully engorging and detaching (='*repletes*') from untreated control *Peromyscus leucopus* pups versus *Peromyscus leucopus* pups after mother (M1) received three oral doses of lotilaner (50 mg/kg BW) during her 25-day nursing period. Efficacy of pups after weaning in preventing the successful engorgement of infesting larval ticks; *Ixodes scapularis* and *Dermacentor variabilis*. Numbers in parentheses indicate numbers of mice.

Litter	Infestation No. (Days Post Weaning)	Tick Species	Control		Lotilaner M1 (High Dose)			
			% Mice yielding repletes (N)	Mean SD ± repletes	% Mice yielding repletes (N)	Mean SD ± repletes	T-test comparison of means	% Efficacy
1	1 (4)	Ixodes	100% (3)	9 ± 2.65	0% (3)	0	P=0.0041 T=5.892	100%
		Dermacentor	100% (3)	12.34 ± 3.51	0% (3)	0	P=0.0037 T=6.083	100%
	2 (15)	Ixodes	66.7% (3)	4.67 ± 4.51	0% (3)	0	P=0.148 T=1.793	100%
		Dermacentor	100% (3)	6 ± 5.57	0% (3)	0	P=0.1354 T=1.867	100%
	3 (31)	Ixodes	100% (3)	8.67 ± 5.03	100% (3)	11.67 ± 2.89	P=0.4211 T=0.42	0%
		Dermacentor	100% (3)	5 ± 1.73	100% (3)	5 ± 4.58	P=1 T=0	0%
2	1 (7)	Ixodes	100% (3)	4.67 ± 3.79	100% (5)	11 ± 1.87	P=0.1552 T=-1.63	0%
		Dermacentor	100% (3)	6 ± 1.73	100% (5)	12.4 ± 4.04	P=0.0316 T=-2.789	0%

Table 6. Production of engorged larvae successfully engorging and detaching (='*repletes*') from untreated control *Peromyscus leucopus* pups versus *Peromyscus leucopus* pups after mother (M2) received a single oral dose of lotilaner (50 mg/kg BW) during her 25-day nursing period. Efficacy of pups after weaning in preventing the successful engorgement of infesting larval ticks; *Ixodes scapularis* and *Dermacentor variabilis*. Numbers in parentheses indicate numbers of mice.

Litter	Infestation No. (Days Post Weaning)	Tick Species	Control		Lotilaner M2 (Low Dose)			
			% Mice yielding repletes (N)	Mean SD ± repletes	% Mice yielding repletes (N)	Mean SD ± repletes	T-test comparison of means	% Efficacy
1	1 (8)	Ixodes	100% (3)	8.67 5.03	0% (5)	0	P=0.0153 T=8	100%
		Dermacentor	100% (3)	5 1.73	0% (5)	0	P=0.1835 T=2	100%
	2 (37)	Ixodes	100% (3)	2.67 0.58	100% (5)	6.6 1.82	P=0.315 T=5.5	0%
		Dermacentor	67% (3)	0.67 0.58	80% (5)	1.8 1.924	P=0.299 T=1.387	0%

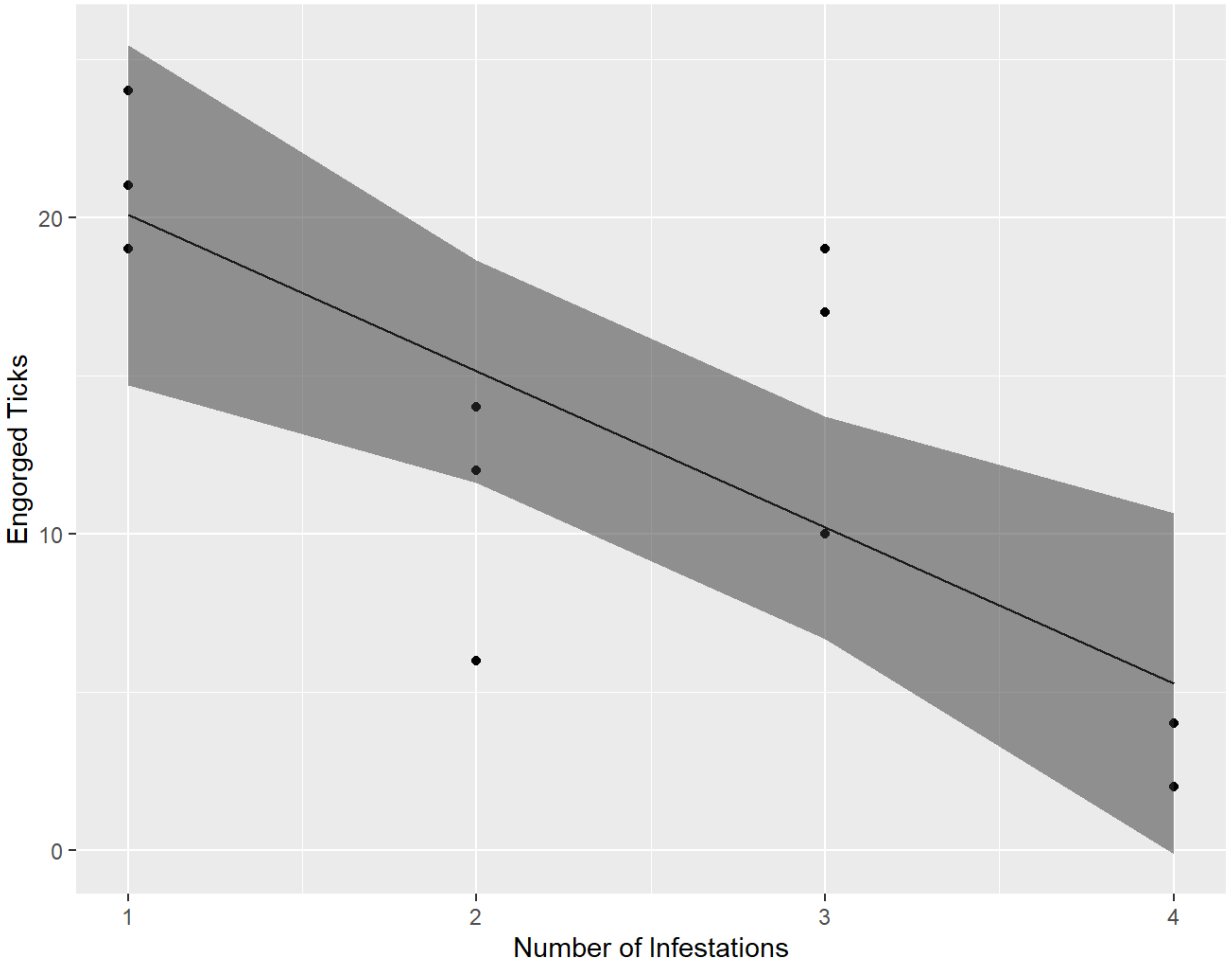

Figure 2. The number of successfully engorged ticks decreased in control mice as the number of serial infestations a mouse had experienced increased. Linear regression, F (1,10) = 14.63, p = 0.003, R2 = 0.59 according to the function; Number of engorged ticks = 25 + -4.93* Number of Infestations.

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