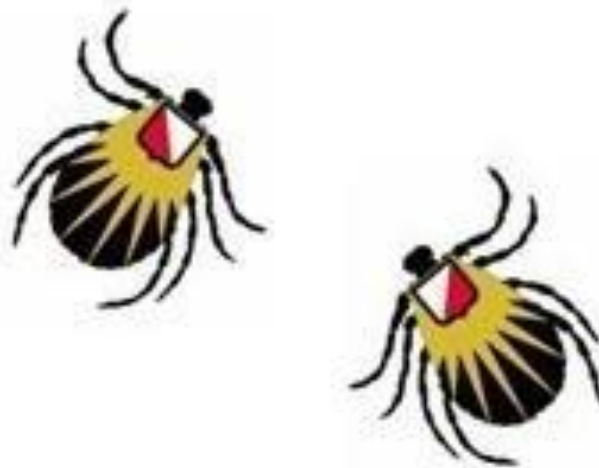


Universiteit Utrecht



IN VITRO SUSCEPTIBILITY ASSESSMENT OF VARIOUS TICK SPECIES AGAINST FIPRONIL



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ABSTRACT

The number of infections transmitted by ticks has significantly increased over the last decades. It is of importance that ticks are controlled in order to prevent the transmission of tick-borne infections. Humans are also susceptible to some of the tick-borne infections that occur in dogs and cats; therefore controlling ticks on cats and dogs is also of importance for public health.

It is desirable for an acaricide to exhibit specificity to insects over mammals and birds. Fipronil shows specificity to insects over mammals by binding to GABA and glutamate receptors in the arthropod cell. Binding to these receptors causes inhibition of chloride channels leading to hyper excitation of the nerve cell of the tick and death of the tick.

To determine the susceptibility of known and unknown acaricides, in vitro testing of acaricides is the best possible way because there is no influence from a host. In this in vitro study the efficacy of fipronil in killing adult ticks was tested. Fipronil produced 100% mortality in all tick species tested. The LD₅₀ value of fipronil in adult *Amblyomma americanum*, *Rhipicephalus turanicus* and *Rhipicephalus sanguineus* at 24 hours was 3,558, 0,094 and 0,283 respectively. The LD₅₀ value of fipronil in *Amblyomma americanum*, *Rhipicephalus turanicus* and *Rhipicephalus sanguineus* at 48 hours was 0,022, 0,006 and 0,005 respectively. Fipronil was shown to be least effective against *Amblyomma americanum*, compared to the other tick species tested. From the tick species tested fipronil is most effective against adult *Rhipicephalus* ticks. The efficacy of fipronil against adult *Rhipicephalus turanicus* and adult *Rhipicephalus sanguineus* was highly comparable.

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1. INTRODUCTION

TICKS AND TICK-BORNE DISEASES

Over the last couple of decades, the tick-borne diseases such as Lyme disease, babesiosis and ehrlichiosis have significantly increased. For instance, the Department of Health in Vermont, USA, reported an increase in the number of positive screenings of Lyme disease in dogs from 9.51% in 2003 to 15.76% in 2010 [Health Department Vermont, 2011]. Factors that could have contributed to the increase of tick-borne infections are public awareness and increase of traveling and recreation. It is of importance that ticks are controlled to prevent the transmission of tick-borne infections. Humans are also susceptible to some of the tick-borne infections that occur in dogs and cats, i.e. Lyme disease, which can lead to severe complications in humans. In the United States the number of Lyme disease infections in humans is 20,000 to 30,000 cases a year. For ehrlichiosis the reported cases in the United States increased from 200 cases in 2000 to 961 cases in 2008. Anaplasmosis infections also increased from 348 cases in 2000 to 1761 cases in 2010 [Shah & Sood, 2013]. Therefore controlling ticks on cats and dogs is also of importance for the public health [Stanneck, 2012].

IN VITRO TEST

In vitro testing of acaricides is ideal to determine the susceptibility of known and unknown acaricides because there is no influence from a host. In vitro studies can provide useful information on the spectrum of a potential acaricide, synergistic effects or possible tick resistance. The ideal acaricide would be broad-spectrum, easy to administer, have a long residual action and would contain an active ingredient (or a combination of ingredients) that are non-toxic to animals, humans and the environment. However, in vitro studies often do not give more than a broad indication of a dose for a systemic or a topical administration to an animal [Stanneck, 2012]. The downside of frequent use of acaricides on pets is that this increases the possibility of tick resistance. Ticks that were susceptible to the used concentration of an acaricide died. Ticks that survived were not susceptible to the used concentration of an acaricide and are still able to reproduce. As a result of the frequent use of acaricides where a large number of the susceptible ticks die, the surviving ticks can reproduce because there are less competitors. Due to this it is possible that a population of ticks is created that is resistant to the used acaricide [FAO, 2004].

LD₅₀

LD stands for lethal dose. LD₅₀, median lethal dose, is the amount of a chemical that is given all at once, which is expected to cause the death of 50% of the subjects tested. The LD₅₀ is a way to measure the short-term poisoning potential (acute toxicity) of a chemical. In general it is stated that the smaller the LD₅₀ value, the more toxic the chemical is. The opposite is also true, the larger the LD₅₀ value, the lower the toxicity of the chemical [Canadian Centre for Occupational Health and Safety, 2013]. The LD₅₀ value is currently the basis for toxicology classifications of chemicals [Walum, 1998].

FIPRONIL

In the late 1970s after it was discovered that insects died on plants that had been treated with herbicides, research was performed regarding the working mechanism of fipronil. This led to the

recognition of fipronil to control ticks and fleas on pets. The veterinary product, Frontline[®], was created by Rhône Mérieux, and launched in 1994 [Merial Limited, 2014]. Fipronil was first registered for use by the United States Environmental protection Agency (U.S. EPA) [Smith, 2009]. However, Mérieux' patent expired and thus many generics became available.

Characteristics of fipronil

The International Union of Pure and Applied Chemistry (IUPAC) name for fipronil is (±)-5-amino-1-(2,6-dichloro- α,α,α -trifluoro-*p*-tolyl)-4-trifluoromethylsulfinylpyrazole-3-carbonitrile. Fipronil is part of the phenylpyrazole group and is a broad-spectrum insecticide. Structurally fipronil is a white powder and has a moldy odor [Jackson *et al*, 2009]. Fipronil is lipophilic and photostable [Narahashi *et al*, 2010].

TABLE 1. Fipronil characteristics. Jackson *et al*, 2009.

Molecular formula	C ₁₂ H ₄ Cl ₂ F ₆ N ₄ OS (figure 1)
Molecular weight	437.2 g/mol
Vapor pressure	2.8 x 10 ⁻⁹ mmHg at 25 °C
Solubility (water)	0.0019 g/L (pH 5); 0.0024 g/L (pH 9) at 20 °C
Henry's constant	3.7 x 10 ⁻⁵ atm·m ³ /mol
Octanol-Water Partition Coefficient (K _{ow})	1.00 x 10 ⁴

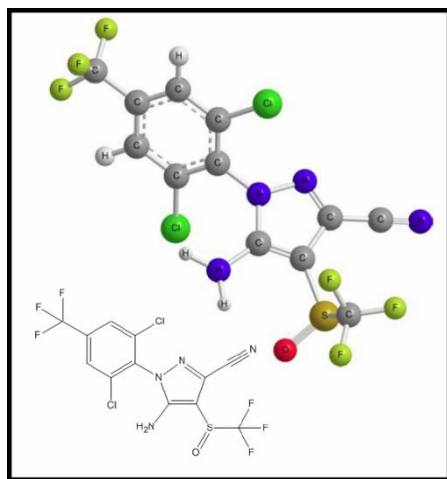


Figure 1. Molecular structure of fipronil. Jackson *et al*, 2009.

Mode of action of fipronil

In the arthropods cell fipronil binds to GABA and glutamate receptors. GABA and glutamate receptors are neurotransmitters which inhibit the muscle activity in insects and acaridae. Binding to these receptors causes the inhibition of chloride channels. Therefore hyper excitation of the nerve cell of the tick is maintained which leads to the death of the tick (figure 2) [Beugnet and Franc, 2012].

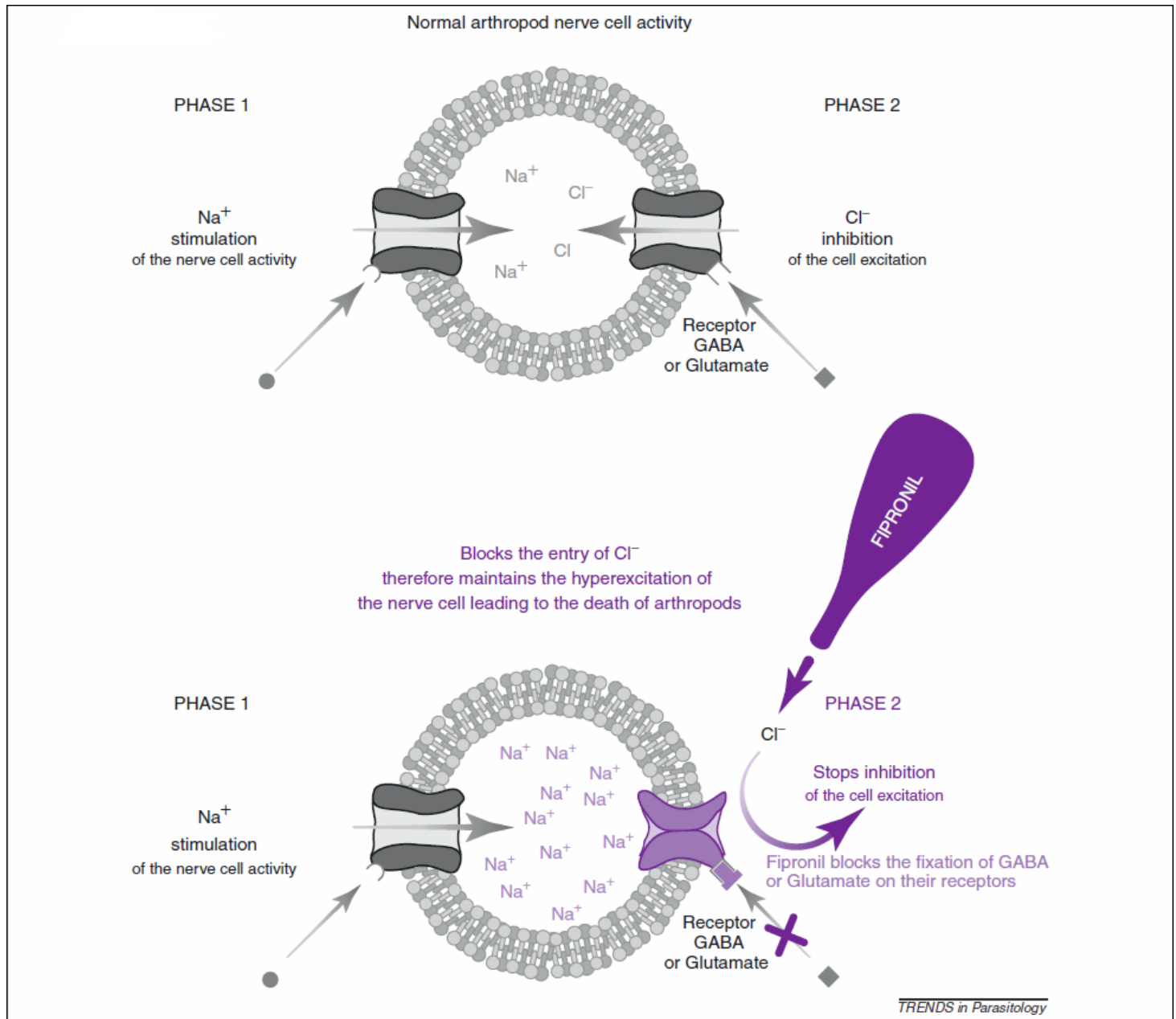


Figure 2: Mode of action of fipronil in insects and acarida. Beugnet and Franc, 2012.

It is desirable for an acaricide to exhibit selectivity to insects over mammals and birds. It has recently become more clear that the sensitivity of target sites for acaricides in many cases is higher in insects than in mammals and birds (non-target organisms). Fipronil shows specificity to insects over mammals. The LD₅₀ value for fipronil in houseflies was 0,13 mg/kg and for rats the LD₅₀ value was 41 mg/kg, a 315-fold difference. These values show that fipronil possesses a high selectivity to insects over mammals. The half maximum inhibitory concentration, IC₅₀, of fipronil on GABA receptors has also been measured. The IC₅₀ value in cockroaches has been estimated to be 30 nM and for rats it has been estimated to be 1600 nM, a 53-fold difference. GABA receptors are not the only target sites of fipronil. Fipronil also

inhibits glutamate-activated chloride channels (GluCl_s). GluCl_s are present in invertebrates, such as acaridae, but are not present in mammals. Glutamate-activated chloride channels and GABA-gated chloride channels are closely related ligand-gated chloride channels. Between these two channels there is a high degree in similarity in the pharmacological properties [Narahashi *et al*, 2010].

Fipronil is also used in combination with amitraz which gives a desired synergic effect. This combination of fipronil and amitraz causes a rapid lethal effect in ticks [Prullage *et al*, 2011]. This results in a significant risk decrease of ticks transmitting pathogens.

Metabolic pathways of fipronil

The predominant pathway of the fipronil metabolism is S-oxidation of fipronil into fipronil-sulfone. This chemical reaction (figure 3) can be considered as a bioactivation of fipronil as the sulfone metabolite was found to have a 6-fold greater binding affinity for GABA receptors in the brain compared to unchanged fipronil. Also, fipronil-sulfone has a 20-fold greater potency to block GABA-activated chloride channels in rats than fipronil itself. The sulfone metabolite is thought to be more toxic than unchanged fipronil in different species. Also, fipronil-sulfone was shown to be present in blood much longer than fipronil itself [Cravedi *et al*, 2013]. Fipronil is widely distributed in mammalian tissues and is mainly found in fatty tissues. Fipronil has been injected in mice and researchers detected the sulfone metabolite in the brain, liver, kidney, fat and feces. Fipronil and the oxidation metabolite, fipronil-sulfone, were excreted in the feces (45-75%) and in the urine (5-25%) by rats after they were given an oral dose of fipronil. Another metabolite of fipronil, fipronil-desulfinyl (figure 3), the primary photodegrade of fipronil, has been detected in the brain, liver, kidney, fat, skin and feces of mice, rats and lactating goats after injection or oral exposure [Jackson *et al*, 2009].

The half-life is a term used for the time that is needed for half of the chemical to break down in the environment. In aerobic soils fipronil has a half-life of 122-128 days, where fipronil is broken down by naturally present soil organisms to form fipronil-sulfone. Under these conditions fipronil can also be hydrolyzed to form fipronil-amide. On soil surfaces fipronil degrades to form fipronil-desulfinyl by ultraviolet radiation (i.e. sunlight). The half-life of fipronil in loamy soil is 34 days. The mobility of fipronil is low and is therefore not expected to leach into groundwater [Jackson *et al*, 2009]. In water fipronil rapidly degrades to form fipronil-desulfinyl by ultraviolet radiation. The half-life of fipronil under these conditions is 4-12 hours. At pH 5 and pH 7 fipronil is resistant to hydrolysis. In alkaline conditions however, fipronil degrades in direct ratio to increasing pH values. The main residue formed by hydrolysis of fipronil is fipronil-amide. The half-lives of fipronil-desulfinyl in aerated water and static water are 120 (± 18) hours and 149 (± 39) hours, respectively. Fipronil and fipronil-desulfinyl are less volatile than water and under field conditions fipronil and fipronil-desulfinyl can concentrate [Jackson *et al*, 2009]. Plants treated with soil only absorb about 5% of fipronil present in the soil. In plants fipronil partially degrades to form fipronil-sulfone and fipronil-amide. In foliage fipronil partially photodegrades to form fipronil-desulfinyl [Jackson *et al*, 2009]. Fipronil-sulfone and fipronil-desulfinyl are more persistent and less selective between mammals and arthropods [Bigelow Dyk *et al*, 2012].

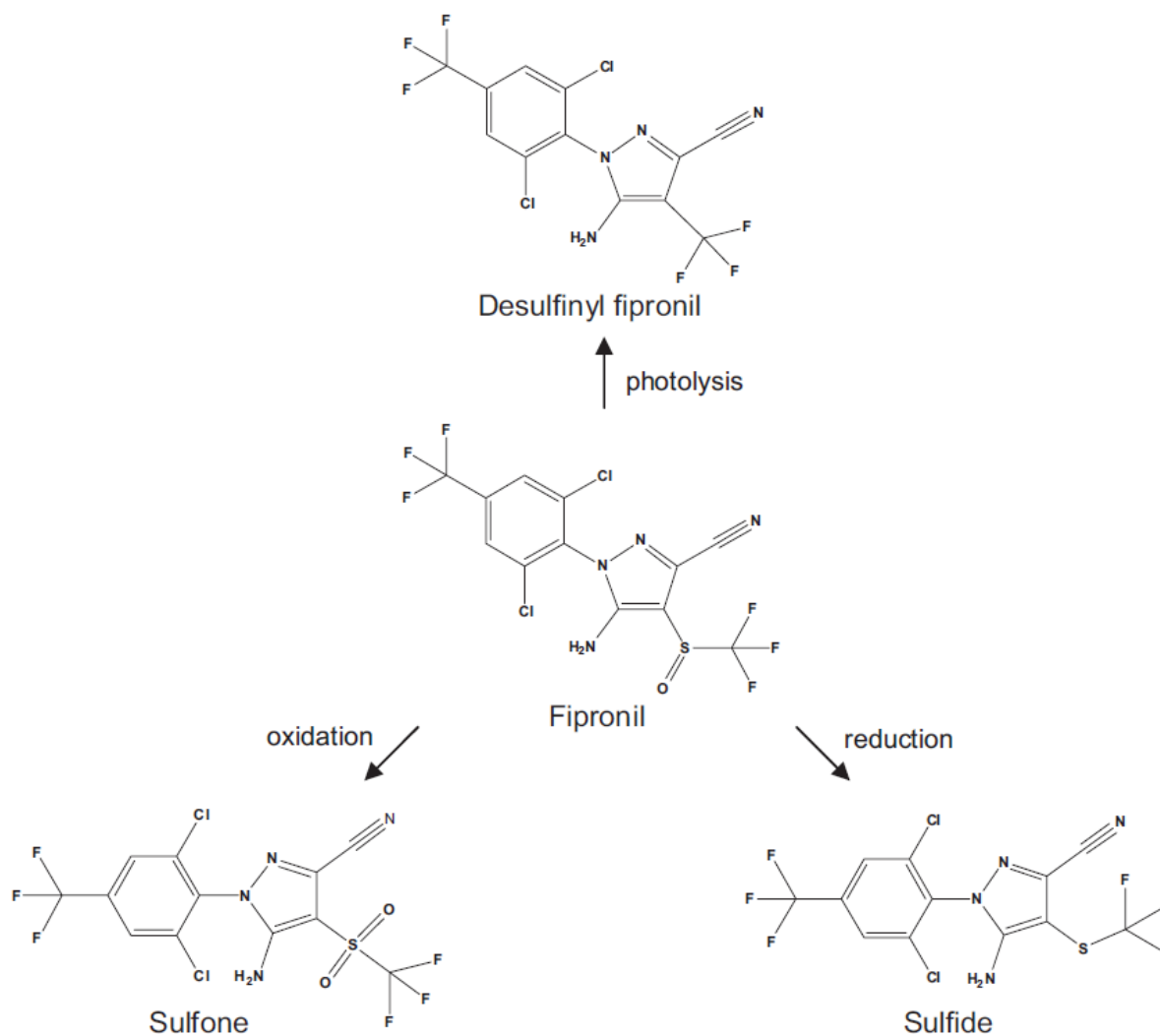


Figure 3. Metabolites of fipronil. Bigelow Dyk *et al*, 2012.

Ecotoxicity

Fipronil can be highly toxic to several birds, such as the bobwhite quail and pheasants. The mallard ducks are not susceptible to fipronil with no reported acute, sub-acute or chronic effects. The oxidation metabolite of fipronil, fipronil-sulfone, is particularly toxic to upland game birds and medium toxic to the waterfowl by ingestion [Jackson *et al*, 2009].

Marine and freshwater fish are very susceptible to fipronil. The sulfone metabolite is more toxic than fipronil itself. In fish fipronil accumulates. 14 days after being transferred to clean water fipronil is completely eliminated by fish. In fish the primary metabolites are fipronil-sulfone and fipronil-sulfide. Fipronil is also highly toxic to freshwater invertebrates (i.e. daphnids). The sulfone metabolite and the desulfinyl metabolite are more toxic to freshwater invertebrates than fipronil itself. Furthermore, fipronil is highly toxic to oysters and particularly toxic to mysid shrimp. Toxic effects caused by fipronil

affected the mysid shrimps growth, reproduction and survival. When fipronil is applied to water, it varies much in its toxicity and its potential to bioaccumulate in aquatic arthropods, which depends on the species affected [Jackson *et al*, 2009].

Further, honeybees are highly susceptible to fipronil and fipronil can cause toxic effects by contact and ingestion when applied to plant foliage. Worms are not susceptible to fipronil treated soil, including earthworms or the *Pheretima* group [Jackson *et al*, 2009].

Humans exposed to fipronil

Fipronil may persist on the coat of dogs and cats that are treated with a product containing fipronil and can transfer to humans and their residences by direct contact. Clinical signs and symptoms reported after being exposed to fipronil by humans can be headache, nausea, sweating, vomiting, abdominal pain, agitation, weakness, dizziness and seizures. Clinical signs and symptoms of exposure to fipronil are generally reversible and in most cases resolve spontaneously [Bigelow Dyk *et al*, 2012; Jackson *et al*, 2009; Smith, 2009].

Other uses

Besides the use of fipronil in commercial pet care products to control ticks and fleas, fipronil is also used to control cockroaches, ants, termites, beetles, weevils, thrips, mole crickets, rootworms and other insects. Fipronil is used in agriculture, granular turf products, seed treatments, gel baits and liquid termiticides [Bigelow Dyk *et al*, 2012; Jackson *et al*, 2009].

2. AIM OF THE STUDY

The aim of this study is (1) to determine the LD₅₀ concerning the susceptibility of *Rhipcephalus turanicus*, *Rhipcephalus sanguineus*, and *Amblyomma americanum* against fipronil; (2) to determine the reproducibility of the test; (3) to possibly adjust the dilution series to improve the test; and (4) to use this study to set up a standard operating procedure (SOP) for similar tests.

3. MATERIALS & METHODS

MATERIALS

Ticks. Adult ticks of the species *Rhipicephalus turanicus*, *Rhipicephalus sanguineus* and *Amblyomma americanum* obtained from the acaridarium from the Utrecht Centre for Tick-Borne Diseases (UCTD). Later on in the study two different strains of *Rhipicephalus sanguineus* have been used to compare the susceptibility within *R. sanguineus*, Greece and France.

Acaricide. Fipronil will be used as acaricide, batch SZBA033XV. Acetone will be used to make the 10-fold and the 5-fold dilutions.

Other materials. Each dilution will be pipetted with disposable pipettes into each of three 25ml glass vials with a diameter of 2.5 cm and a height of 5.2 cm. The vial area is 50cm².

METHODS

To cover the whole range between 0% mortality in ticks exposed to the lowest concentration of fipronil and 100% mortality in ticks exposed to the highest concentration of fipronil, tenfold serial dilutions are made. Ticks exposed to acetone alone will serve as negative controls. All test dilutions are carried out in triplicate.

A solution of 12 mg/ml fipronil in acetone is prepared as a stock solution. From this stock solution a tenfold dilution is made to acquire 1.2 mg/ml fipronil in acetone. This dilution is diluted again tenfold to acquire 0.12 mg/ml fipronil in acetone. 0,5 ml of this dilution is taken to treat the vials 1a, 1b and 1c (table 2A); these vials contain 1,2 µg fipronil/cm². The 0.12 mg fipronil/ml concentration is tenfold serially diluted and 0.5 ml of each dilution is used to treat the corresponding vials in triplicate (as listed in table 2, starting with vials 2a, 2b and 2c).

Later on in the study a higher concentration of fipronil was used. A stock solution of 40 mg/ml fipronil in acetone was prepared. Tenfold dilutions series were made to acquire the concentrations as listed in table 2B.

After the vials are closed with a cap, they are rotated for at least 2 hours at room temperature on a test-tube rotator at 10 rpm to ensure that all internal surfaces are coated. Afterwards, the vials are opened in a safety cabinet to allow the excess acetone to evaporate completely, whereby the bottom of the vials will also be coated. Thereafter, ten adult ticks are placed into each vial, starting with the control vials en working towards the vials with the highest concentration of fipronil. The ticks will be exposed to seven serial dilutions of fipronil concentrations. Each vial is loosely closed with a lid to allow air exchange. All vials are incubated, standing up at 20 °C and 80% relative humidity.

Mortality will be evaluated at 24 hours and at 48 hours post exposure. The vials are opened and the ticks are breathed on to activate them with carbon dioxide and the ticks that remain immobile are gently probed with a pair of forceps. Ticks that are able to walk are considered alive. After counting the dead ticks at 24 hours, all ticks are returned into the same vials and re-evaluated at 48 hours.

The efficacy is expressed as a percentage of mortality: the number of dead ticks in each vial divided by the total amount of ticks in the vial multiplied by 100%. The mortality is expressed as an average of the three triplicates.

These tests are carried out on various tick species in order to compare the susceptibility of the ticks against fipronil. Therefore, it can be determined which tick species is more susceptible to fipronil and which tick species is less susceptible to fipronil.

TABLE 2A AND 2B. Fipronil concentrations used in the study

Vial numbers	Concentrations of fipronil
(1a, 1b en 1c)	1,2 µg/cm ²
(2a, 2b en 2c)	0,12 µg/cm ²
(3a, 3b en 3c)	0,012 µg/cm ²
(4a, 4b en 4c)	0,0012 µg/cm ²
(5a, 5b en 5c)	0,00012 µg/cm ²
(6a, 6b en 6c)	0,000012 µg/cm ²
(7a, 7b en 7c)	0,0000012 µg/cm ²
(C1, C2 en C3)	0,0 µg/cm ² (negative control)

TABLE 2A

Vial numbers	Concentrations of fipronil
(1a, 1b en 1c)	4,0 µg/cm ²
(2a, 2b en 2c)	0,4 µg/cm ²
(3a, 3b en 3c)	0,04 µg/cm ²
(4a, 4b en 4c)	0,004 µg/cm ²
(5a, 5b en 5c)	0,0004 µg/cm ²
(6a, 6b en 6c)	0,0004 µg/cm ²
(7a, 7b en 7c)	0,00004 µg/cm ²
(C1, C2 en C3)	0,0 µg/cm ² (negative control)

TABLE 2B

Statistical Analysis

The number of dead ticks, live ticks, total ticks in the vials, and the mortality rate for each tick species, each dilution and each time point (24h and 48h) are tabulated. A logistic regression (Probit analysis) with a logit transformation is performed on the data, with the mortality rate as the dependent variable and the concentrations as the independent variable.

The function is:

$$\text{Logit}(M) = \log_e(M/(1-M)) = b_0 + b_1x_1$$

where M = mortality rate (between 0 and 1); x_1 = log concentrations; b_0 and b_1 = regression coefficients

LD₅₀, LD₉₀ and LD₉₉ are obtained by back-transformation from the logarithmic scale to the original scale.

4. RESULTS

The efficacy of fipronil in killing *Rhipicephalus turanicus* is given in Table 3. Fipronil did not produce 100% mortality with the used dilutions within 48 hours. Statistical analysis was performed and the LD₅₀ was estimated to be 4,178 at 24 hours and 0,426 at 48 hours (Table 7).

TABLE 3. Efficacy of fipronil in killing *Rhipicephalus turanicus* (12 mg/ml stock solution).

Tick species	Percentage of ticks killed after 24 h/48 h of exposure to different dilutions of fipronil; dilutions in mg/ml.							
	1,2	0,12	1,2 · 10 ⁻²	1,2 · 10 ⁻³	1,2 · 10 ⁻⁴	1,2 · 10 ⁻⁵	1,2 · 10 ⁻⁶	Aceton
<i>Rhipicephalus turanicus</i>	48 /88	41/72	20/39	22/35	16/20	12/18	9/15	5/16

The comparative efficacy of fipronil in killing *Amblyomma americanum*, *Rhipicephalus turanicus* and *Rhipicephalus sanguineus* is given in Table 4. Fipronil produced 100% mortality in *A. americanum*, *R. turanicus* and *R. sanguineus* within 48 hours at dilutions as high as 4,0 mg/ml. Fipronil did not produce 100% mortality of any of these tick species within 24 hours. The mortality rate of these tick species against fipronil are also given in figure 4.

TABLE 4. Comparative efficacy of fipronil in killing different tick species.

Tick species	Percentage of ticks killed after 24 h/48 h of exposure to different dilutions of fipronil; dilutions in mg/ml.					
	4,0	0,4	4,0 · 10 ⁻²	4,0 · 10 ⁻³	4,0 · 10 ⁻⁴	Aceton
<i>Amblyomma americanum</i>	50/100	30/77	10/63	ND ^a	ND	0/13
<i>Rhipicephalus turanicus</i>	83/100	73/87	30/60	17/47	13/27	3/17
<i>Rhipicephalus sanguineus</i>	69/100	50/93	39/66	19/37	22/35	16/22

^aND; not done

Statistical analysis showed that adults of *R. turanicus* and *R. sanguineus* are significantly more susceptible to fipronil than *A. americanum* is to fipronil, based on the LD₅₀, LD₉₀ and LD₉₉ values (Tables 5-6). Comparing *R. turanicus* and *R. sanguineus*, *R. turanicus* has a greater susceptibility to fipronil within 24 hours, based on the LD₅₀, LD₉₀ and LD₉₉ (Table 5). In contrast, *R. sanguineus* has a greater susceptibility to fipronil within 48 hours, based on the LD₉₀ and LD₉₉. The difference in value of the LD₅₀ for both *R. turanicus* and *R. sanguineus* is marginal, being 0,006 and 0,005 respectively (Table 6).

TABLE 5. Susceptibility of adult ticks to fipronil evaluated in laboratory bioassays 24h after treatment.

Tick species	No. of ticks	Concentration of fipronil as % active ingredient		
		LD ₅₀ (95% confidence limits)	LD ₉₀ (95% confidence limits)	LD ₉₉ (95% confidence limits)
<i>Amblyomma americanum</i>	120	3,558 (1,162 to 70,268)	391,725 (30,945 to 4495564,442)	18096,557 (365,054 to 45849512301,325)
<i>Rhipicephalus</i>	180	0,094 (0,039 to 0,254)	13,644 (3,093 to	787,883 (77,377 to

<i>turanicus</i>			173,626)	50460,244)
<i>Rhipicephalus sanguineus</i>	510	0,283 (0,125 to 0,788)	973,340 (116,148 to 34995,685)	743928,136 (23059,100 to 284795898,643)

TABLE 6. Susceptibility of adult ticks to fipronil evaluated in laboratory bioassays 48h after treatment.

Tick species	No. of ticks	Concentration of fipronil as % active ingredient		
		LD ₅₀ (95% confidence limits)	LD ₉₀ (95% confidence limits)	LD ₉₉ (95% confidence limits)
<i>Amblyomma americanum</i>	120	0,022	0,811	15,621
<i>Rhipicephalus turanicus</i>	180	0,006 (0,002 to 0,014)	0,655 (,202 to 4,696)	29,537 (4,236 to 1019,044)
<i>Rhipicephalus sanguineus</i>	510	0,005 (0,000 to 0,023)	0,351 (,058 to 92,913)	12,042 (,704 to 646575,100)

The efficacy of fipronil in killing *Rhipicephalus sanguineus* is given in Table 8. Fipronil did produce 100% mortality of de strains Greece and France within 48 hours at dilutions as high as 0,4 and 4,0 respectively. Statistical analysis showed that the France strain is more susceptible to fipronil than the Greece strain, based on the LD₅₀, LD₉₀ and LD₉₉ (Table 9-10). The LD₅₀ for the Greece and the France strain at 24 hours are 0,208 and 0,016 respectively. The LD₅₀ for the Greece and the France strain at 48 hours are 0,007 and 0,000 respectively.

TABLE 7. Susceptibility of adult *Rhipicephalus turanicus* (12 mg/ml stock solution) to fipronil evaluated in laboratory bioassays 24h and 48h after treatment.

Hours after treatment	No. of ticks	Concentration of fipronil as % active ingredient		
		LD ₅₀ (95% confidence limits)	LD ₉₀ (95% confidence limits)	LD ₉₉ (95% confidence limits)
24	962	4,178 (0,851 to 44,068)	2350084,646 (49511,006 to 1012733865,468)	114520029553,815 (339447259,663 to 1135685161970254,800)
48	962	0,426 (0,016 to 96805,819)	128651,060 (65,710 to 13685560980255057000000,000)	3772938929,800 (33581,374 to 2,290E+036)

TABLE 8. Comparative efficacy of fipronil in killing adult *Rhipicephalus sanguineus*.

Tick strain	Percentage of ticks killed after 24 h/48 h of exposure to different dilutions of fipronil; dilutions in mg/ml.					
	4,0	0,4	4,0 · 10 ⁻²	4,0 · 10 ⁻³	4,0 · 10 ⁻⁴	Aceton
GREECE	73/100	53/90	40/77	17/30	13/23	17/20
FRANCE	87/100	63/100	60/87	37/80	30/47	20/23

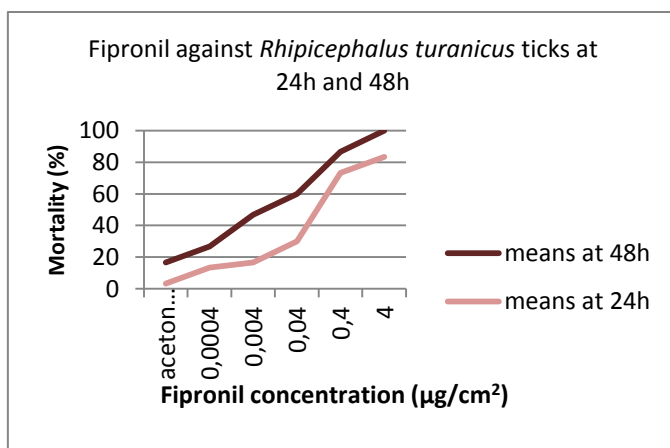
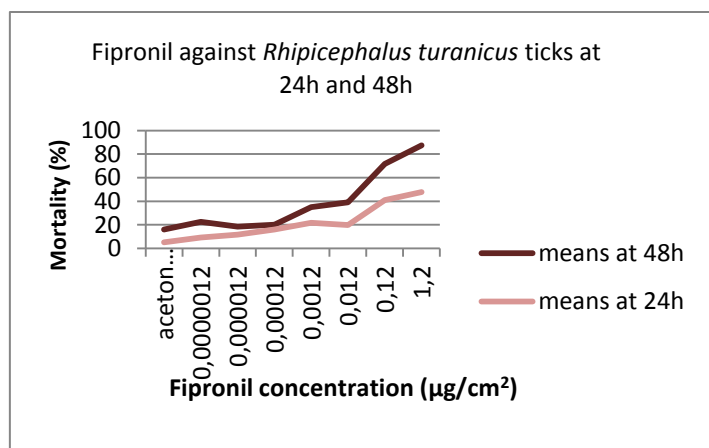
TABLE 9. Susceptibility of adult *Rhipicephalus sanguineus* to fipronil evaluated in laboratory bioassays 24h after treatment.

Tick strain	No. of ticks	Concentration of fipronil as % active ingredient		
		LD ₅₀ (95% confidence limits)	LD ₉₀ (95% confidence limits)	LD ₉₉ (95% confidence limits)
GREECE	180	0,208 (0,069 to 0,901)	134,115 (14,198 to 12501,827)	26158,263 (745,342 to 43716880,556)
FRANCE	180	0,016 (0,003 to 0,057)	33,101 (3,395 to 5017,205)	16959,198 (325,000 to 162440542,656)

TABLE 10. Susceptibility of adult *Rhipicephalus sanguineus* to fipronil evaluated in laboratory bioassays 48h after treatment.

Tick strain	No. of ticks	Concentration of fipronil as % active ingredient		
		LD ₅₀ (95% confidence limits)	LD ₉₀ (95% confidence limits)	LD ₉₉ (95% confidence limits)
GREECE	180	0,007 (0,003 to 0,015)	0,300 (,113 to 1,386)	6,434 (1,391 to 88,492)
FRANCE	180	0,000 (0,000 to 0,001)	0,026 (,010 to ,147)	0,676 (,126 to 26,744)

From the tick species tested fipronil is most effective against adult *Rhipicephalus* ticks. The efficacy of fipronil against adult *Rhipicephalus turanicus* and adult *Rhipicephalus sanguineus* is comparable. Fipronil has proven to be least effective against *Amblyomma americanum*, compared to the other tick species tested.



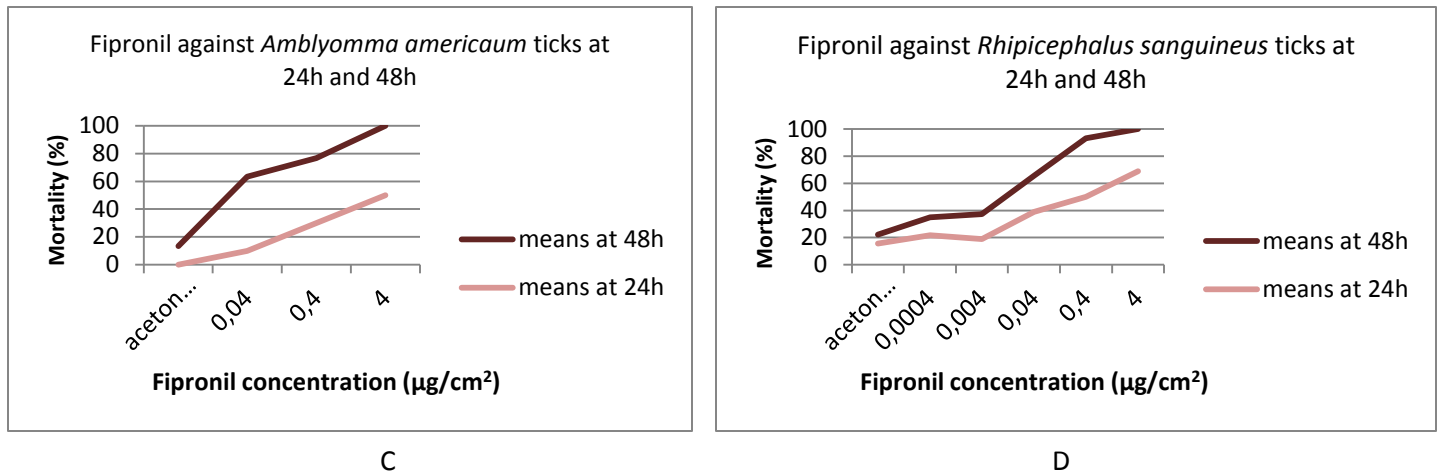


Figure 4. The mortality rate of *Rhipicephalus turanicus* (A-B), *Amblyomma americanum* (C) and *Rhipicephalus sanguineus* (D) against fipronil at 24 and 48 hours.

5. DISCUSSION

EVALUATION OF TICKS

Mortality was evaluated at 24 hours and at 48 hours post exposure. Ticks that were able to walk were considered alive. All ticks were returned into the vial and re-evaluated at 48 hours. When processing the data obtained from the tests, it became apparent that in one test the mortality rate was higher at 24 hours than at 48 hours. This is not possible and was caused by ticks that were considered dead at 24 hours but were in fact not dead. Furthermore, ticks that were unjustly considered dead at 24 hours may have died before the evaluation at 48 hours which prevents the researcher to detect the error that has been made. Thus, for the reliability of the study it is of great importance that the researcher has the proper ability to determine the mortality of the ticks.

DILUTION SERIES

In the first part of the study a tenfold dilution series was made from a 12 mg fipronil/ml stock solution. However, 100% mortality within 48 hours was not produced in any of the tests performed. Therefore, the dilution series has been adjusted to 40 mg fipronil/ml as a stock solution. This resulted in a 100% mortality rate for *A. americanum*, *R. turanicus* and *R. sanguineus* within 48 hours.

TICK QUALITY

Ticks exposed to acetone alone served as negative controls. In both dilution series used there was mortality in the negative control group. Also, there was mortality in the highest dilutions which was not expected. This questions the quality of the ticks that were used in the study. The ticks were held under the following conditions; each vial was loosely closed with a lid to allow air exchange and then incubated, standing up at 20 °C and 80% relative humidity. Unfortunately the age of the ticks was not registered and therefore it is not possible to determine whether old age may have played a part in the loss of quality. Due to the ticks dying without being exposed to fipronil, a small control test was set up during the holidays (two weeks). 25 ticks of the *R. sanguineus* species, strain Greece, were placed in a vial with a ventilation gap and accommodated with a filter paper. They were placed under the same conditions as the ticks used in the study, standing up at 20 °C and 80% relative humidity. After two weeks the ticks were evaluated where a mortality of 100% was found. Although these ticks do not represent all the ticks used in the study, it does give an indication of the general quality of the ticks, which in this study was not optimal. For more reliable results a follow-up study should be performed where the conditions in which the ticks are held under also should be tested.

AVAILABILITY OF TICKS

With the set up of the study it was anticipated that a certain amount of ticks would become available for the study after they had transformed into their adult stage. However, many ticks died shortly after they transformed into their adult stage and could therefore not be used for the study. This resulted in a shortage of ticks that were needed for the study. For that reason in the test with *Amblyomma americanum* the four highest dilutions ($4,0 \cdot 10^{-3}$ to $4,0 \cdot 10^{-6}$) were not carried out. In the tests with *Rhipicephalus turanicus* and *Rhipicephalus sanguineus* the two highest dilutions ($4,0 \cdot 10^{-5}$ and $4,0 \cdot 10^{-6}$) were not carried out. These dilutions were chosen not to be done because of the lowest active

ingredient of fipronil present and therefore little mortality was expected.

FIPRONIL

In this study the effect of fipronil is tested on various tick species. Within 48 hours fipronil causes 100% mortality in all tick species tested. Fipronil is therefore rapid and effective in killing ticks. Furthermore, fipronil is selective between mammals and acaridae, thus fipronil could be used as a safe acaricide for ticks on dogs and cats. However, little research has been done about the effects the metabolites of fipronil have on mammals; therefore follow-up research should be done.

LD₅₀ VALUE

The significance of the LD₅₀ value has been extensively discussed and criticized. First, the LD₅₀ value is not an absolute value but is a variable biological parameter and can therefore not be compared to constants such as molecular weight or the melting point. Therefore the median lethal dose cannot be described in terms of accuracy, only in terms of precision. Furthermore, the precision is only relevant for the test from which the median lethal dose is obtained and does not increase the probability that in following tests the LD₅₀ value will be identical or even similar. Secondly, the LD₅₀ value only refers to mortality and is therefore not illustrative of any other clinical expression of toxicity [Walum, 1998].

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ATTACHMENTS

ATTACHMENT 1: Overview of fipronil-based products on the market for usage on dogs and cats. Ringenier, 2013.

Brand name	Application	Active ingredient(s)	Acaricide/repellent	Further details
1. Frontline Combo (Merial)	Spot-on	Fipronil (adulticide) + (S)Methopreen (ovi- en larvicide)	Acaricide	-
2. Certifect (Merial)	Spot-on	Fipronil + (S)Methopreen + Amitraz	Acaricide, repellent	Not used on cats.
3. Flevox (Vetoquinol)	Spot-on	Fipronil	Acaricide	-
4. Bob Martin Clear (Bob Martin)	Spot-on	Fipronil	Acaricide	Not used on cats.
5. Exil fiproline (Alfamed)	Spot-on	Fipronil	Acaricide	-
6. Fibrospot (IDT Biologika)	Spot-on	Fipronil	Acaricide	Does not prevent tick infestations.
7. Pestigon (Norbrook laboratories)	Spot-on	Fipronil	Acaricide	Not used on cats.
8. Ectofend (Krka)	Spot-on	Fipronil	Acaricide	Does not prevent attachment of ticks.
9. Fleanil (Norbrook laboratories)	Spot-on	Fipronil	Acaricide	-
10. Effipro (Virbac)	Spot-on; skin spray	Fipronil	Acaricide	-
11. Eliminal (Pfizer)	Spot-on	Fipronil	Acaricide	Does not prevent attachment of ticks.
12. Eilminall (Krka)	Skin spray; spot-on	Fipronil	Acaricide	-
13. Frontline (Merial)	Skin spray	Fipronil	Acaricide	-
14. Amflee (Krka)	Skin spray	Fipronil	Acaricide	-

ATTACHMENT 2: *In vitro* susceptibility assay on ticks procedure

UTRECHT CENTRE FOR TICK-BORNE DISEASES (UCTD)
 FAO REFERENCE CENTRE FOR TICKS AND TICK-BORNE DISEASES



UCTD/13/008/5
 26 Nov 2013

In vitro susceptibility assay on ticks

IN VITRO SUSCEPTIBILITY ASSAY ON TICKS PROCEDURE

Room	
-------------	--

Wear gloves and use non-filter pipet tips
--

Tick species	
Tick strain	
Fipronil batch	
Shaker ID	
Desiccator ID	

Strictly follow the one-way route: Acaridarium → Dirty lab

Preparation fipronil stock		Done
1	Clean workspace with 70% ethanol.	
2	If needed, prepare a fipronil stock of 40mg/ml.	
3	Make a 10-fold or 5-fold dilution series in 2.0ml tubes. (See pipetting scheme,) Pipet each dilution up and down and homogenize before continuing with the next.	
4	Add 500µl of each dilution in triplicate to 25ml glass vials and close the caps well. Use acetone as the negative control. Label vials and caps accordingly.	
5	Place the vials on their sides on a shaker at room temperature at 10rpm for 2 hours.	
6	Place the vials in the fume hood without the caps and leave them until all excess acetone is evaporated.	
7	Turn off all equipment and clean workspace.	

Preparation ticks		Done
1	Take the required ticks from the acaridarium to the dirty lab.	
2	Clean workspace with 70% ethanol.	
3	Place 10 ticks in each vial and loosely close the caps. Start with the control and continue towards the highest concentration.	
4	Incubate the vials in standing position at 20°C and 80% relative humidity for 24 hours.	
5	Turn off all equipment and clean workspace.	

24 hour check		Done
10	Open the vials one by one and breath into them to activate the ticks. Immobile ticks are gently probed with forceps. Start with the control and continue towards the highest concentration.	
11	Count the dead ticks; ticks are considered dead when they are not able to walk.	
12	Return all 10 ticks to the vial and close the cap loosely and continue with the next vial.	
13	Incubate the vials in standing position at 20°C and 80% relative humidity for 24 hours.	
14	Clean workspace.	

48 hour check		Done
15	Open the vials one by one and breath into them to activate the ticks. Immobile ticks are gently probed with forceps. Start with the control and continue towards the highest concentration.	
16	Count the dead ticks; ticks are considered dead when they are not able to walk.	
17	Return all 10 ticks to the vial, add 70% ethanol, close the cap tightly and leave it for 1 day. Continue with the next vial.	
18	Clean workspace.	
19	Process all acquired data.	
20	Dispose all ticks in a vial with 70% ethanol in a seal bag and into the bio-waste bin.	
21	Dispose all used vials in a seal bag and into the bio-waste bin.	

Susceptibility assay fipronil preparation done:

by _____ on _____
Signature

Susceptibility assay tick preparation done:

by _____ on _____
Signature

Susceptibility assay 24 hour check done:

by _____ on _____
Signature

Susceptibility assay 48 hour check done:

by _____ on _____
Signature

Comments:

IN VITRO SUSCEPTIBILITY ASSAY ON TICKS - PIPETTING SCHEME

Fipronil batch	
-----------------------	--

Pipet each dilution up and down and homogenize before making the next

Wear gloves and use non-filter pipet tips
--

Strictly follow the one-way route: Acaridarium → Dirty lab

10-fold dilution series					
Dilution #	Conc. (mg/ml)	Aceton (ml)	Fipronil	End volume (ml)	Done

1	4.0	1.8	0.2ml from stock	1.8	
2	0.4	1.8	0.2ml from #1	1.8	
3	$4.0 \cdot 10^{-2}$	1.8	0.2ml from #2	1.8	
4	$4.0 \cdot 10^{-3}$	1.8	0.2ml from #3	1.8	
5	$4.0 \cdot 10^{-4}$	1.8	0.2ml from #4	1.8	
6	$4.0 \cdot 10^{-5}$	1.8	0.2ml from #5	1.8	
7	$4 \cdot 10^{-6}$	1.8	0.2ml from #6	2.0	

10-fold dilutions series done:

by _____ on _____

Signature

Comments:

--

IN VITRO SUSCEPTIBILITY ASSAY ON TICKS - PIPETTING SCHEME

Fipronil batch	
----------------	--

Pipet each dilution up and down and homogenize before making the next

Wear gloves and use non-filter pipet tips

Strictly follow the one-way route: Acaridarium → Dirty lab

5-fold dilution series

Dilution #	Conc. (mg/ml)	Aceton (ml)	Fipronil	End volume (ml)	Done
------------	---------------	-------------	----------	-----------------	------

1	4.0	1.6	0.4ml from stock	1.6	
2	0.4	1.6	0.4ml from #1	1.6	
3	$4.0 \cdot 10^{-2}$	1.6	0.4ml from #2	1.6	
4	$4.0 \cdot 10^{-3}$	1.6	0.4ml from #3	1.6	
5	$4.0 \cdot 10^{-4}$	1.6	0.4ml from #4	1.6	
6	$4.0 \cdot 10^{-5}$	1.6	0.4ml from #5	1.6	
7	$4.0 \cdot 10^{-6}$	1.6	0.4ml from #6	1.6	
8	$4.0 \cdot 10^{-7}$	1.6	0.4ml from #7	1.6	
9	$4.0 \cdot 10^{-8}$	1.6	0.4ml from #8	1.6	
10	$4.0 \cdot 10^{-9}$	1.6	0.4ml from #9	2.0	

5-fold dilutions series done:
 by _____ on _____
 Signature

Comments:

IN VITRO SUSCEPTIBILITY ASSAY ON TICKS - TICK SEEDING RECORD

Tick species	
Tick strain	

Fipronil batch	
----------------	--

Tick tube ID #	Dose ($\mu\text{g}/\text{cm}^2$)	# of ticks/vial	Time	Comments
C1	0.00	10		
7a	$4.0 \cdot 10^{-6}$			
6a	$4.0 \cdot 10^{-5}$			
5a	$4.0 \cdot 10^{-4}$			
4a	$4.0 \cdot 10^{-3}$			
3a	$4.0 \cdot 10^{-2}$			
2a	0.4			
1a	4.0			
C2	0.00	10		
7b	$4.0 \cdot 10^{-6}$			
6b	$4.0 \cdot 10^{-5}$			
5b	$4.0 \cdot 10^{-4}$			
4b	$4.0 \cdot 10^{-3}$			
3b	$4.0 \cdot 10^{-2}$			
2b	0.4			
1b	4.0			
C3	0.00	10		
7c	$4.0 \cdot 10^{-6}$			
6c	$4.0 \cdot 10^{-5}$			
5c	$4.0 \cdot 10^{-4}$			
4c	$4.0 \cdot 10^{-3}$			
3c	$4.0 \cdot 10^{-2}$			
2c	0.4			
1c	4.0			

Tick seeding done:

By _____ on _____

Signature

IN VITRO SUSCEPTIBILITY ASSAY ON TICKS - 24 HOUR TICK ASSESSMENT RECORD

Tick species	
Tick strain	

Fipronil batch	
----------------	--

Tick tube ID #	Dose ($\mu\text{g}/\text{cm}^2$)	Dead ticks	Live ticks	Time	Comments
C1	0.00				
7a	$4.0 \cdot 10^{-6}$				
6a	$4.0 \cdot 10^{-5}$				
5a	$4.0 \cdot 10^{-4}$				
4a	$4.0 \cdot 10^{-3}$				
3a	$4.0 \cdot 10^{-2}$				
2a	0.4				
1a	4.0				
<hr/>					
C2	0.00				
7b	$4.0 \cdot 10^{-6}$				
6b	$4.0 \cdot 10^{-5}$				
5b	$4.0 \cdot 10^{-4}$				
4b	$4.0 \cdot 10^{-3}$				
3b	$4.0 \cdot 10^{-2}$				
2b	0.4				
1b	4.0				
<hr/>					
C3	0.00				
7c	$4.0 \cdot 10^{-6}$				
6c	$4.0 \cdot 10^{-5}$				
5c	$4.0 \cdot 10^{-4}$				
4c	$4.0 \cdot 10^{-3}$				
3c	$4.0 \cdot 10^{-2}$				
2c	0.4				
1c	4.0				

24 hour assessment done:

By _____ on _____

Signature

IN VITRO SUSCEPTIBILITY ASSAY ON TICKS - 48 HOUR TICK ASSESSMENT RECORD

Tick species	
Tick strain	

Fipronil batch	
----------------	--

Tick tube ID #	Dose ($\mu\text{g}/\text{cm}^2$)	Dead ticks	Live ticks	Time	Comments
C1	0.00				
7a	$4.0 \cdot 10^{-8}$				
6a	$4.0 \cdot 10^{-5}$				
5a	$4.0 \cdot 10^{-4}$				
4a	$4.0 \cdot 10^{-3}$				
3a	$4.0 \cdot 10^{-2}$				
2a	0.4				
1a	4.0				
C2	0.00				
7b	$4.0 \cdot 10^{-8}$				
6b	$4.0 \cdot 10^{-5}$				
5b	$4.0 \cdot 10^{-4}$				
4b	$4.0 \cdot 10^{-3}$				
3b	$4.0 \cdot 10^{-2}$				
2b	0.4				
1b	4.0				
C3	0.00				
7c	$4.0 \cdot 10^{-8}$				
6c	$4.0 \cdot 10^{-5}$				
5c	$4.0 \cdot 10^{-4}$				
4c	$4.0 \cdot 10^{-3}$				
3c	$4.0 \cdot 10^{-2}$				
2c	0.4				
1c	4.0				

48 hour assessment done:

By _____ on _____

Signature

