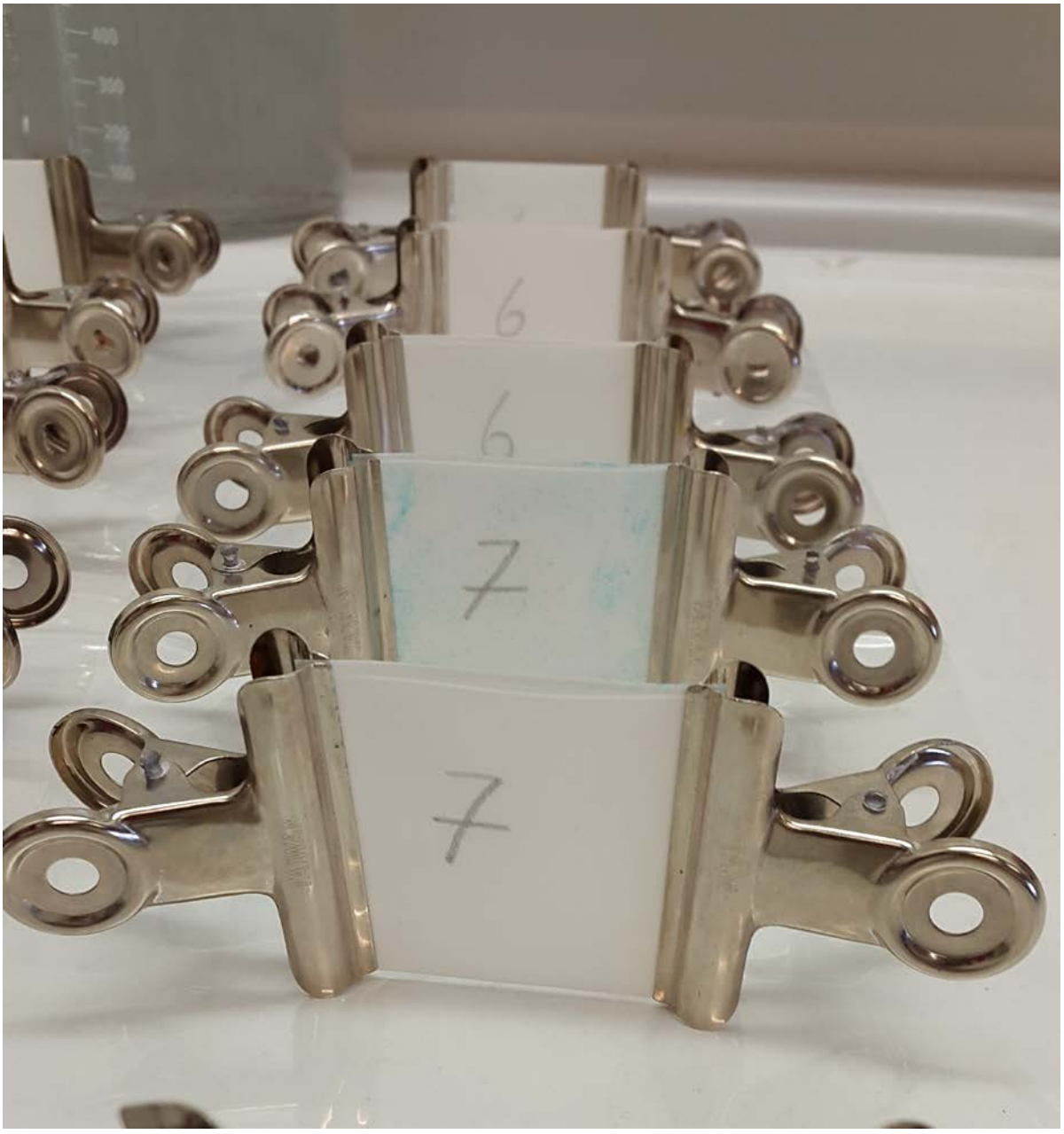
Comparative efficacy of a novel combination of permethrin and fipronil against *Rhipicephalus sanguineus*, *Ixodes hexagonus* and *Dermacentor reticulatus* ticks



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Preambule

This paper is the final report of the research project carried out by Oïfa de Jong at the Utrecht Centre for Tick-borne Diseases (UCTD) at Utrecht University.

Research was executed to make baseline data of the efficacy of a novel combination of Fipronil and Permethrin (Tri-Act®/Frontect®) against *Rhipicephalus sanguineus, Ixodes hexagonus* and *Dermacentor reticulatus*. Also synergism between fipronil and permethrin was evaluated.

Contents

Abstract

Background:

The purpose of this study was to determine the efficacy of a novel combination of fipronil (Tri-Act®/Frontect®) and permethrin against *Rhipicephalus sanguineus, Ixodes hexagonus* and *Dermacentor reticulatus*. Also synergism between fipronil and permethrin was evaluated.

Methods

The Larval Packet Test was used to determine the LC_{50} for fipronil, permethrin as well as for the combination of fipronil and permethrin. The values for LC_{50} were used to calculate synergistic ratios by dividing the LC_{50} of fipronil or permethrin alone by the LC_{50} of the same acaricide when combined with the other.

Results

For *R. sanguineus* the LC_{50} for fipronil was $1.2169*10^{-2}$ mg/ml in Tri-Act®/Frontect® and $3.5654*10^{-2}$ mg/ml for fipronil alone. The LC_{50} for permethrin was $9.0249*10^{-2}$ mg/ml in Tri-Act®/Frontect® and $9.6677*10^{-2}$ mg/ml for permethrin alone. Synergy ratios were 2.95 for fipronil and 1.07 for permethrin.

For *I. hexagonus* the LC_{50} for fipronil was $1.0096*10^{-2}$ mg/ml and $7.8443*10^{-2}$ mg/ml for permethrin in Tri-Act®/Frontect®.

No reliable results were obtained for *D. reticulatus* due to too high control mortality.

Conclusions

The LC_{50} for fipronil, permethrin and the combination in Tri-Act®/Frontect® for *R.sanguineus* and *I. hexagonus* were determined. Synergy ratios for *R. sanguineus* of 2.95 for fipronil and 1.07 for permethrin were found indicating some synergism between fipronil and permethrin.

Introduction

Resistance was been reported to occur in several tick species. Resistance to both permethrin and fipronil has been confirmed in *R. sanguineus* (Eiden et al., 2015 and Miller, George, Guerrero, Carpenter, & Welch, 2001). Consultation of the Arthropod Pesticide Resistance Database revealed no (reported) resistance for *Dermacentor reticulatus* or *Ixodes hexagonus* ("Arthropod Pesticide Resistance Database," n.d.).
However it is important to monitor resistance and because of this it is important to establish baselines for acaricides. A possible synergy can contribute to the reduced use of acaricides, which can contribute to the delay in development of resistance against acaricides.

Background information

Ticks belong to the phylum Arthropoda. They are blood-feeding parasites which can cause direct damage to their host due to their feeding behaviour and they can also be vectors for several different diseases (Dantas-Torres, 2008). The features of the ticks used in this research can be found in table A. The most important tick-transmitted diseases in dogs that can cause serious illness are babesiosis and ehrlichiosis. Several infections that affect dogs, like borreliosis and ehrlichiosis, can also cause illness in humans (Shaw, Day, Birtles, & Breitschwerdt, 2001). Because of this it is important to use tick control, especially in endemic areas. In most situations it is impossible to eradicate tick because of maintenance of the tick life cycle on reservoir hosts (Shaw et al., 2001). For tick control both chemical and non-chemical strategies can be used. Chemical control may consist of spot-on formulations, impregnated collars, shampoos, sprays, dips and powders and more recently also oral compounds. The most frequently used acaricides in tick control are fipronil, amitraz, carbaryl and pyrethroids (deltamethrin, permethrin and cypermethrin) (Dantas-Torres, 2008). In this paper we will discuss permethrin and fipronil as these are the acaricides used in Frontline Tri-Act®/Frontect®. It contains 6.76% fipronil and 50.48 % permethrin w/v. (Jongejan et al, 2015).
Permethrin is used for tick control on domestic animals and for the environment. It is a pyrethroid; this pesticide causes a disruption in the function of the sodium channel of the parasite. At first it has a repellent effect due to irritation by contact with the tick. This is followed by a killing effect (Jongejan et al , 2015).
Fipronil is only used for treatment directly on the animal. Fipronil belongs to the phenylpyrazole class and acts on the GABA-gated chloride channels. It blocks the flow of chlorine ions which leads to neuroexcitation (Eiden et al., 2015). It induces a progressive onset of tick mortality (Jongejan et al., 2015). There are no products with fipronil that can be used for (indoor) area applications. Therefore it appears that there has been less tick exposure to fipronil than to permethrin (Eiden et al., 2015). Both fipronil and permethrin have a different mode of action and therefore combining both can be an advantage (Jongejan et al., 2015).

Because ticks can be vectors it is not only important that the ticks are killed but also that tick control prevents the transmission of diseases. This can be achieved by repelling ticks or disrupting the feeding process once they have already attached themselves to the dog. Frontline Tri-Act®/Frontect® has been shown to reduce the risk of *Babesia canis* with 87.5% over a period of 28 days (Jongejan et al., 2015). In another research by Dumont et al the same combination was used to investigate the repellency, prevention of attachment and acaricidal efficacy against *D. reticulatus*. Here a repellency

ranging between 56.5-73.5% at 4 hours after infestation and between 83.9-96.5% 24 hours after infestation was found. The effect lasted up to 4 weeks post-treatment. Prevention of attachment was 84.2-99.6% 24 hours after infestation up to 4 weeks post-treatment. The acaricidal efficacy was 99.5% for 4 weeks post-treatment (Dumont et al ., 2015).

Tick species	*Dermacentor reticulatus*	*Ixodes hexagonus*	*Rhipicephalus sanguineus*
Common name	Ornate dog tick (Estrada-Pena et al., 2013)	Hedgehog tick.	Brown dog tick or kennel tick (Taylor, Coop, & Wall, 2007).
Host(s)	Adults: sheep, cattle, dogs, pigs and humans. Nymphs & larvae: smaller mammals and occasionally birds (Taylor et al., 2007).	Dogs and medium-sized wild carnivores like hedgehogs (Estrada-peña, 2004).	Adults: dogs, larger mammals and humans. Nymphs & larvae: primarily dogs, but are sometimes found on rodents and other small animals (Dantas-Torres, 2008).
Life cycle	Three-host tick (Taylor et al., 2007).	Three-host tick (Estrada-peña, 2004).	Three-host tick (Dantas-Torres, 2008).
Habitat and distribution	Paleartic species with a highly focal distribution pattern. Foci occur from south-western England reaching the Yenisei river basin in Siberia (Beugnet & Marié, 2009). Since 2004 autochthonous populations of *D. reticulatus* are also present in the Netherlands (Jongejan, Ringenier, et al., 2015).	Europe and northwest Africa (Taylor et al., 2007).	Worldwide (Taylor et al., 2007).
Seasonal occurrence	Early spring, persists until late autumn. There is a diapause in winter months (Buczek, Bartosik, Wiśniowski, & Tomasiewicz, 2013).	Ticks may be active from early spring to late autumn, but they are most active during April and May (Taylor et al., 2007).	In climates with a winter all stages can be found on dogs from October to May (Estrada-peña, 2004).
Possible transmitted diseases	*Babesia canis* (Taylor et al., 2007).	*Borrelia* ssp (Taylor et al., 2007).	*Babesia vogeli, Ehrlichia canis, Rickettsia conorii* (Dantas-Torres, 2008).

Table A . Features of the tree tick species used in this study.

2.2 Main research question

The purpose of this study was to determine baseline susceptibility of *Rhipicephalus sanguineus, Ixodes hexagonus* and *Dermacentor reticulatus*, for fipronil and permethrin and for a combination of fipronil and permethrin using the larval packet test. This paper also evaluated whether there is synergism between these two acaricides.

2.3 Hypothesis

H0: There is a significant synergetic effect of concurrent application of fipronil and permethrin.

H1: There is no such synergy.

2.4 Research methodology

In order to create valuable and comparable results the standard FAO-recommended research methodology, the Larval Packet Test or LPT, was used, as described further in this paper.

Purpose of the study

The purpose of this study was to test the efficacy of a new combination of fipronil and permethrin against *Rhipicephalus sanguineus, Ixodes hexagonus* and *Dermacentor reticulatus* ticks. Also a possible synergism between fipronil and permethrin was evaluated.

Material and methods

4.1 Rearing larvae

Engorged female ticks used for egg laying were kept in a climate controlled chamber of 27-28 °C with a relative humidity of 85 to 95 %. The hatched larvae were kept under the same conditions. The larvae used for testing should be between the age of 14 to 21 days (FAO, 2004) .

4.2 Acaricides

The tested acaricides in this research were fipronil and permethrin. These are the same acaricides in the commercial product Tri-Act®/Frontect®. In order to determine a possible synergy, both acaricides were tested separately and together in the same ratio as in Tri-Act®/Frontect®. (fipronil: 67.6 mg/ml & permethrin: 504.8 mg/ml).

4.3 Larval Packet Test

Each LPT was conducted over a period of 3 days.

Day 1

On the first day the packets were prepared. First a total of 21 filter papers were cut to 5*10 cm: there were 6 dilutions and 1 control and each dilution and control were done in threefold. Each paper was then moistened with 600 µl of the used acaricides (fipronil, permethrin or a combination of both) dissolved in a dilution of olive oil and trichloroethylene in a 1:2 ratio. Each series of 3 papers was moistened with a different concentration of acaricides. The control papers were moistened with the dilution of olive oil and trichloroethylene only. After complete impregnation packets were made of the papers using 3 clips for each packet. The packets were then placed in the fume hood and left overnight.

Day 2

On the second day the larvae of one of the used tick species were added to the packets. Approximately 100 larvae were added to each packet, always starting with the control group and then working from the lowest to the highest concentration. Then the packets were placed in the corresponding container, a different container for each dilution and control, with a zip bag with moistened cotton wool and left for 24 hours in the fume hood.

Day 3

On the third day the larvae were counted, 24 hours after placing them into the packets. Counting was always started with the control group and them working from the lowest to the highest concentration. Dead and live larvae are counted and results are written down in a table (see appendices).

4.4 Statistics

After each test the numbers of the larvae, dead and alive, were counted and a percentage of mortality for each dilution is calculated. This percentage is calculated by the following formula: mortality (%) = dead larvae / total larvae. If the mortality in the control packets was lower then 5% direct mortality figures were used. If the percentage of mortality of the control larvae was between the 5 and 10% the percentage in all the dilutions will be corrected with Abbotts's formula:

$$\textbf{Corrected percent mortality} = \textbf{100 x } \frac{\%\ \textbf{test mortality} - \%\ \textbf{control mortality}}{\textbf{100} - \%\ \textbf{control mortality}}$$

If mortality in the control packets was greater than 10%, the test conditions may be faulty and the test results were disregarded and the test was repeated.

After this the results were entered into an Excel spreadsheet and the LC_{50} and LC_{99} were determined by plotting the results with on the x-axis the percentage concentration and on the y-axis the probit mortality. The LC_{50} is the concentration that is lethal to 50% of the larvae, the LC_{99} is the concentration that is lethal to 99% of the larvae. This was done for Tri-Act®/Frontect® and for both acaricides separately.

Results

A test was considered valid as long as the test is replicated three times with five doses in each replication (Robertson et al, 2007). The results of the test carried out during this research will be presented according by tick species.

Rhipicephalus sanguineus

Tri-Act

For the determination of the efficacy of Tri-Act®/Frontect® against *R. sanguineus* 3 tests were carried out. The results of these tests for fipronil are summarized in figure 1 and table 1. The results of these tests for permethrin are summarized in figure 2 and table 2. The used concentrations of fipronil and permethrin can be found in table B. The determined LC_{50} and LC_{99} were $1.212*10^{-2}$ and $2.05*10^{-1}$ mg/ml respectively for fipronil and $9.02*10^{-2}$ and 1.52 mg/ml for permethrin.

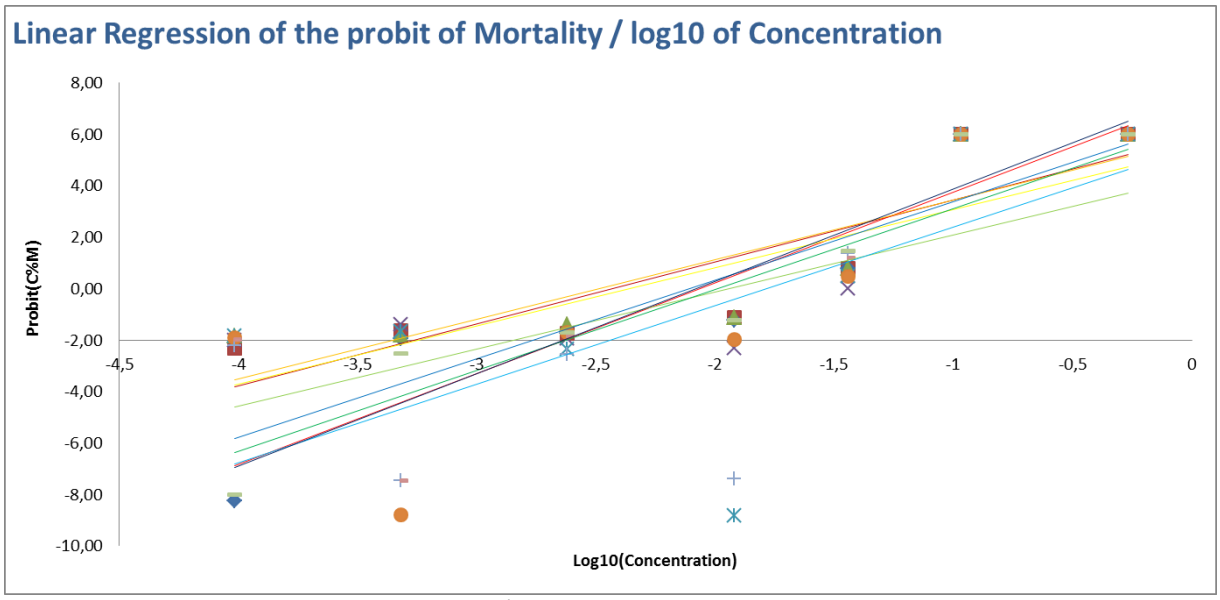

Figure 1. Summarized results for fipronil in Tri-Act®/Frontect® of 3 larval packet tests for *R. sanguineus*.

	Equation y=ax+b		Log concentration		LC	
	a	b	log50	log99	LC50	LC99
A	2,156549	5,1223602	-2,37526	-1,29652	0,004214	0,05052
B	3,638635	7,601154	-2,08901	-1,44967	0,008147	0,03551
C	2,055316	4,994231	-2,42991	-1,29804	0,003716	0,05035
D	1,862137	4,1565805	-2,23216	-0,98287	0,005859	0,10402
E	1,492559	2,1787139	-1,45972	0,098913	0,034696	1,25578
F	2,95251	5,7047028	-1,93215	-1,14423	0,011691	0,07174
G	2,624247	4,1984604	-1,59987	-0,71339	0,025126	0,19347
H	2,94429	6,1191224	-2,0783	-1,28818	0,008350	0,05150
I	3,752099	7,9265801	-2,11257	-1,49256	0,007717	0,03217
Mean					0,012169	0,20501

Table 1. Mean LC_{50} and LC_{99} of fipronil in Tri-Act®/Frontect® for *R.sanuineus.*

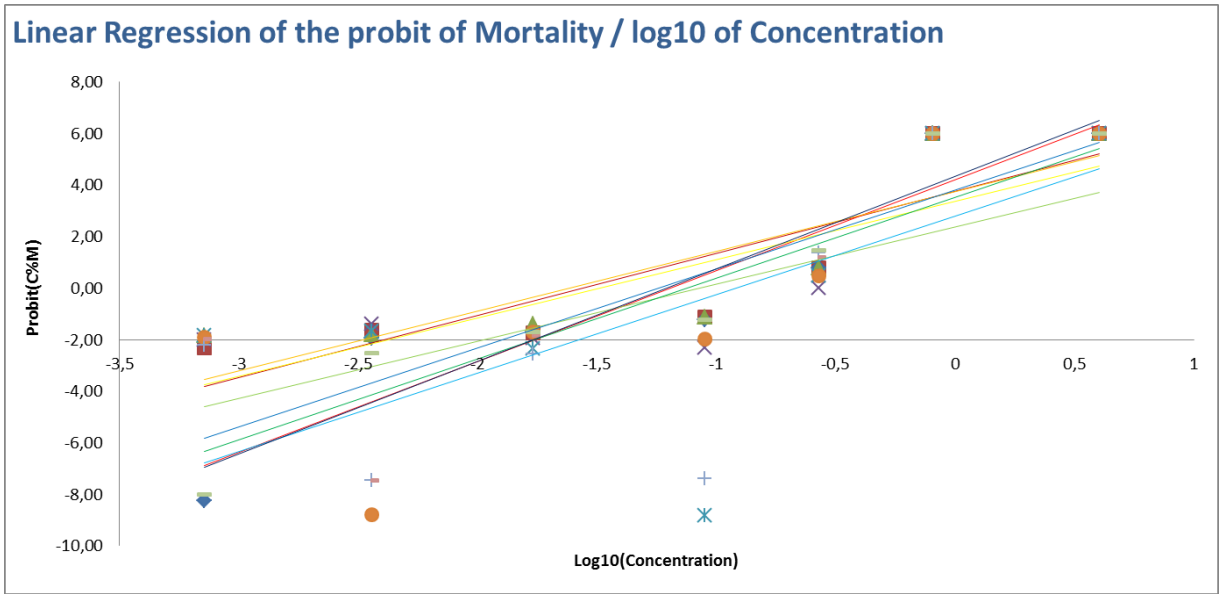

Figure 2. Summarized results for permethrin in Tri-Act®/Frontect® of 3 larval packet tests for *R. sanguineus.*

	Equation y=ax+b		Log concentration		LC	
	a	b	log50	log99	LC50	LC99
A	2,158698	3,249424	-1,50527	-0,42761	0,031241	0,37359
B	3,634426	4,4292048	-1,21868	-0,57859	0,060439	0,26388
C	2,056833	3,2084122	-1,55988	-0,42885	0,027550	0,37252
D	1,864606	2,5402643	-1,36236	-0,11472	0,043415	0,76785
E	1,493141	0,8810771	-0,59008	0,96794	0,256990	9,28838
F	2,948557	3,130065	-1,06156	-0,27258	0,086784	0,53385
G	2,621218	1,9108054	-0,72898	0,15853	0,186648	1,44056
H	2,94233	3,5546474	-1,20811	-0,41746	0,061929	0,38242
I	3,747783	4,6557544	-1,24227	-0,62154	0,057244	0,23903
Mean					0,090249	1,51801

Table2. Mean LC_{50} and LC_{99} of permethrin in Tri-Act®/Frontect® for *R.sanuineus.*

Dilution	Concentration fipronil mg/ml	Amount of fipronil per cm^2 paper (mg/cm^2)	Concentration permethrin mg/ml	Amount of permethrin per cm^2 paper (mg/cm^2)
7	0.54	0.00648	4.0384	0.048
6	0.108	0.001296	0.808	$9.68 * 10^{-3}$
5	0.036	$4.32*10^{-3}$	0.2693	$3.23*10^{-3}$
4	0.012	$1.44*10^{-4}$	0.0898	$1.08*10^{-3}$
3	0.0024	$2.88*10^{-5}$	0.017	$2.04*10^{-4}$
2	$4.8*10^{-4}$	$2.16*10^{-6}$	0.00359	$4.31*10^{-5}$
1	$9.6*10^{-5}$	$1.152*10^{-6}$	$7.18*10^{-4}$	$8.62*10^{-6}$

Table B. Used concentrations fipronil and permethrin in the dilution series for *R.sanguineus* with Frontline Tri-Act®/Frontect®.

Fipronil (Frontline)

For the determination of the efficacy of fipronil alone against *R. sanguineus* 3 tests were carried out. The results of these tests are summarized in figure 3 and table 3. The used concentrations of fipronil can be found at table C. The determined LC_{50} and LC_{99} were $3.57*10^{-2}$ and $2.98*10^{-1}$ mg/ml respectively.

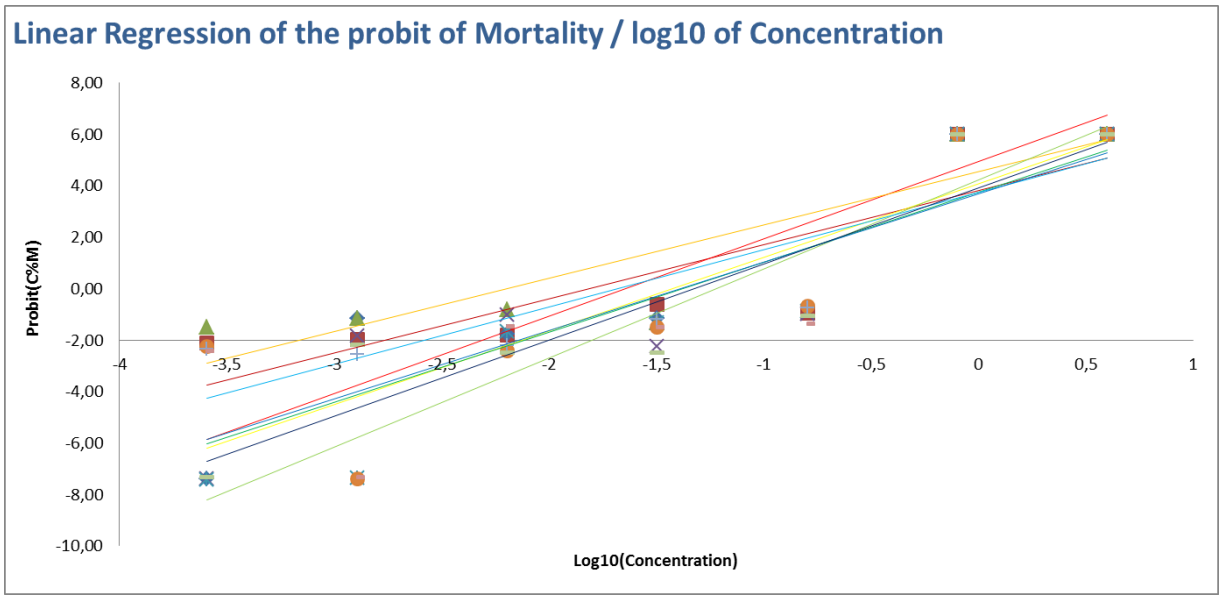

Figure 3. Summarized results for fipronil of 3 larval packet tests for *R. sanguineus*.

	Equation y=ax+b		Log concentration		LC	
	a	b	log50	log99	LC50	LC99
A	1,834817	3,137583	-1,71002	-0,44213	0,019497	0,36130
B	3,280367	5,633271	-1,71727	-1,0081	0,019175	0,09815
C	1,989062	4,328069	-2,17593	-1,00636	0,006669	0,09855
D	2,797611	3,907193	-1,39662	-0,56507	0,040122	0,27223
E	3,554452	4,434755	-1,24766	-0,59317	0,056538	0,25517
F	2,547239	3,318938	-1,30296	-0,38967	0,049779	0,40769
G	1,970781	3,11837	-1,5823	-0,40188	0,026164	0,39639
H	2,452082	3,164847	-1,29068	-0,34195	0,051206	0,45504
I	2,867036	3,687514	-1,28618	-0,47476	0,051740	0,33515
Mean					0,035654	0,29774

Table 3 Mean LC_{50} and LC_{99} of fipronil for *R.sanuineus.*

Dilution	Concentration fipronil mg/ml	Amount of fipronil per cm^2 paper (mg/cm^2)
7	4.0	0.048
6	0.8	$9.6*10^{-3}$
5	0.16	$1.92*10^{-3}$
4	$3.2*10^{-2}$	$3.84*10^{-4}$
3	$6.4*10^{-3}$	$7.68*10^{-5}$
2	$1.28*10^{-3}$	$1.536*10^{-5}$
1	$2.56*10^{-4}$	$3.07*10^{-6}$

Table C. Used concentrations fipronil in the dilution series for *R.sanguineus*

Permethrin

For the determination of the efficacy of permethrin alone against *R. sanguineus* 4 tests were carried out. The results of these tests are summarized in figure 4 and table 4. The used concentrations of fipronil can be found at table D. The determined LC_{50} and LC_{99} were $9.6677*10^{-2}$ and $1.8632*10^{-1}$ mg/ml respectively.

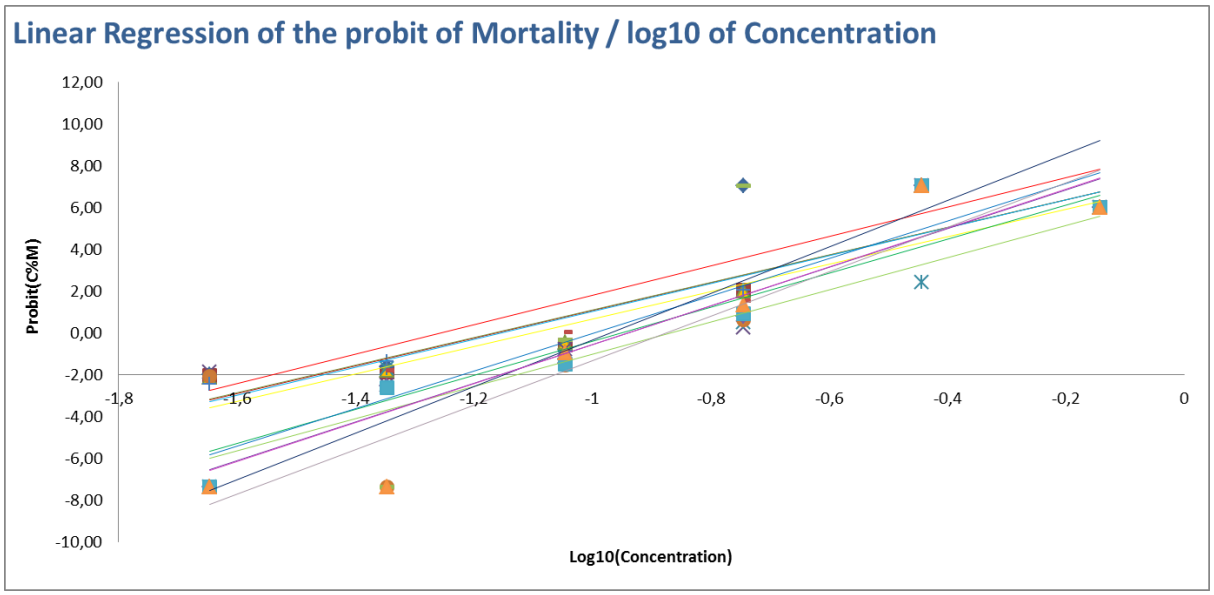

Figure 4. Summarized results for permethrin of 4 larval packet tests for *R. sanguineus*.

	Equation y=ax+b		Log concentration		LC	
	a	b	log50	log99	LC50	LC99
A	6,601149	7,680598	-1,16352	-0,81111	0,068624	0,15449
B	7,040958	8,837304	-1,25513	-0,92473	0,055574	0,11893
C	6,601024	7,695353	-1,16578	-0,81336	0,068268	0,15369
D	6,552351	7,21971	-1,10185	-0,74681	0,079095	0,17914
E	7,682927	6,666136	-0,86766	-0,56486	0,135626	0,27236
F	8,147911	7,74454	-0,95049	-0,66498	0,112074	0,21628
G	6,689127	7,717427	-1,15373	-0,80595	0,070190	0,15633
H	8,995896	8,968591	-0,99696	-0,73836	0,100701	0,18266
I	11,13847	10,80815	-0,97034	-0,76149	0,107067	0,17319
J	9,277728	8,721558	-0,94005	-0,68931	0,114801	0,20450
K	9,318347	8,760266	-0,94011	-0,69046	0,114786	0,20396
L	10,66024	9,329077	-0,87513	-0,6569	0,133313	0,22034
Mean					0,096677	0,18632

Table 4. Mean LC_{50} and LC_{99} of permethrin for *R.sanuineus*.

Dilution	Concentration permethrin mg/ml	Amount of permethrin per cm^2 paper (mg/cm^2)
6	0.72	$8.64*10^{-3}$
5	0.26	$3.12*10^{-3}$
4	0.18	$2.16*10^{-3}$
3	0.09	$1.08*10^{-3}$
2	$4.5*10^{-2}$	$5.4*10^{-4}$
1	$2.26*10^{-2}$	$2.712*10^{-4}$

Table D. Used concentrations permethrin in the dilution series for *R.sanguineus*.

Synergistic ratios

R. sanguineus was tested with Tri-Act®/Frontect® and fipronil and permethrin alone. Therefore synergistic ratios could be calculated. These ratios were calculated by dividing the LC_{50} or LC_{99} of fipronil or permethrin alone by the LC_{50} or LC_{99} of the same acaricide when combined with the other. This is the same method as used by Prullage et al (Prullage, Cawthorne, & Timmons, 2011). The synergistic ratios can be found in table E .

	LC_{50} mg/ml	LC_{99} mg/ml	Synergystic ratio LC_{50}	Synergistic ratio LC_{99}
Fipronil in Tri-Act®/Frontect®	$1.2169*10^{-2}$	0.20501	-	-
Permethrin in Tri-Act®/Frontect®	$9.0249*10^{-2}$	1.51801	-	-
Fipronil alone	$3.5654*10^{-2}$	0.29774	2.95	1.45
Permethrin alone	$9.6677*10^{-2}$	0.18632	1.07	0.12

Table E. Found values for LC_{50} and LC_{99} for fipronil and permethrin and calculated synergistic rations for fipronil and permethrin.

Ixodes hexagonus

For the determination of the efficacy of Tri-Act®/Frontect® against *I. hexagonus* 3 tests were carried out. The results of these tests are summarized in figure 5 and table 5 for fipronil and figure 6 and table 6 for permethrin. The used concentrations of fipronil and permethrin can be found at table F. The determined LC_{50} and LC_{99} were $1.01*10^{-2}$ and $5.85*10^{-2}$ mg/ml respectively for fipronil and $7.84*10^{-2}$ and $4.46*10^{-1}$ mg/ml for permethrin.

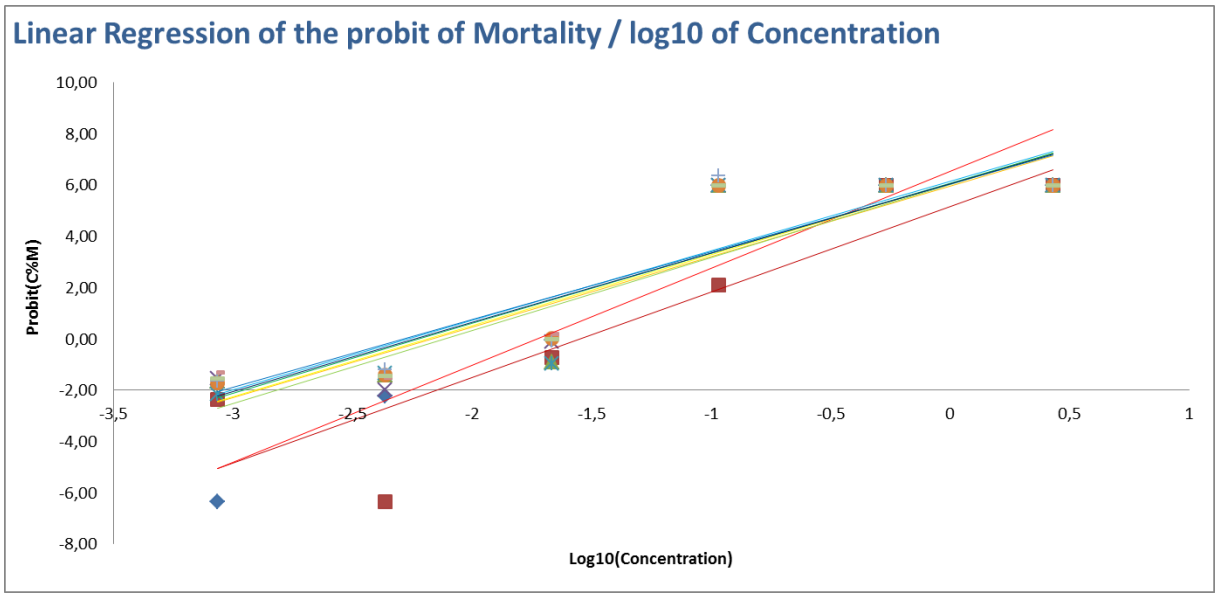

Figure 5. Summarized results for fipronil in Tri-Act®/Frontect® of 3 larval packet tests for *I. hexagonus*.

	Equation y=ax+b		Log concentration		LC	
	a	b	log50	log99	LC50	LC99
A	3,341546	5,16625589	-1,54607	-0,84988	0,028440	0,14129
B	3,782558	6,5357369	-1,72786	-1,11284	0,018713	0,07712
C	2,755793	5,96373535	-2,16407	-1,31991	0,006854	0,04787
D	2,769468	6,03558197	-2,17933	-1,33933	0,006617	0,04578
E	2,849784	6,00550125	-2,10735	-1,29103	0,007810	0,05116
F	2,731691	6,07368687	-2,22342	-1,3718	0,005978	0,04248
G	2,710861	6,13483959	-2,26306	-1,4049	0,005457	0,03936
H	2,652118	6,04807735	-2,28047	-1,4033	0,005242	0,03951
I	2,701168	6,05024462	-2,23986	-1,37862	0,005756	0,04182
Mean					0,010096	0,05849

Table 5. Mean LC_{50} and LC_{99} of fipronil in Tri-Act®/Frontect® for *I. hexagonus.*

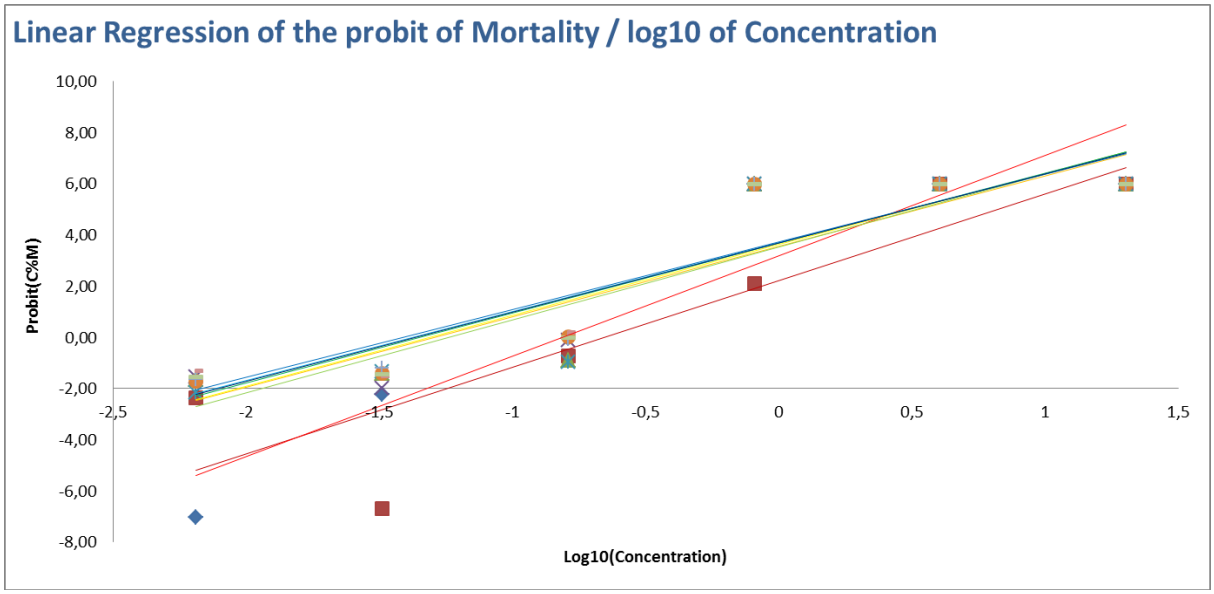

Figure 6. Summarized results for permethrin in Tri-Act®/Frontect® of 3 larval packet tests for *I. hexagonus*.

	Equation y=ax+b		Log concentration		LC	
	a	b	log50	log99	LC50	LC99
A	3,383982	2,20729294	-0,65228	0,035182	0,222702	1,08438
B	3,920394	3,17879648	-0,81084	-0,21744	0,154584	0,60612
C	2,755956	3,55543645	-1,29009	-0,44598	0,051275	0,35812
D	2,769635	3,61533329	-1,30535	-0,4654	0,049506	0,34245
E	2,849954	3,51506427	-1,23338	-0,4171	0,058428	0,38274
F	2,731857	3,68645237	-1,34943	-0,49787	0,044727	0,31778
G	2,696164	3,69864925	-1,37182	-0,50898	0,042480	0,30975
H	2,652278	3,73038149	-1,40648	-0,52937	0,039221	0,29555
I	2,701331	3,68968441	-1,36588	-0,50469	0,043065	0,31283
Mean					0,078443	0,44553

Table 6. Mean LC_{50} and LC_{99} of permethrin in Tri-Act®/Frontect® for *I. hexagonus.*

Dilution	Concentration of Fipronil mg/ml	Amount of fipronil per cm^2 paper (mg/cm^2)	Concentration Permethrin mg/ml	Amount of permethrin per cm^2 paper (mg/cm^2)
7	13.5	0.162	100.96	1.21
6	2.7	0.0324	20.192	0.242
5	0.54	0.00648	4.0384	0.048
4	0.108	0.001296	0.808	$9.68 * 10^{-3}$
3	$2.16 * 10^{-2}$	$2.59 * 10^{-4}$	0.162	$1.94 * 10^{-3}$
2	$4.32 * 10^{-3}$	$5.18 * 10^{-5}$	$3.32 * 10^{-2}$	$3.872 * 10^{-4}$
1	$8.64 * 10^{-4}$	$1.04 * 10^{-5}$	$6.46 * 10^{-3}$	$7.75 * 10^{-5}$

Table F. Used concentrations fipronil and permethrin in Tri-Act®/Frontect® in the dilution series for *I. hexagonus*.

Dermacentor reticulatus

For the determination of the efficacy of Tri-Act®/Frontect® against *D. reticulatus* 4 tests were carried out. 3 tests had a control mortality rate above 10% and were disregarded. The results of the remaining test are summarized in figure 7 and table 7 for fipronil and figure 8 and table 8 for permethrin. The used concentrations of fipronil and permethrin can be found in table G. The values for LC_{50} found in the remaining test were $3.527*10^{-3}$ mg/ml for fipronil and $2.657*10^{-2}$ mg/ml for permethrin.

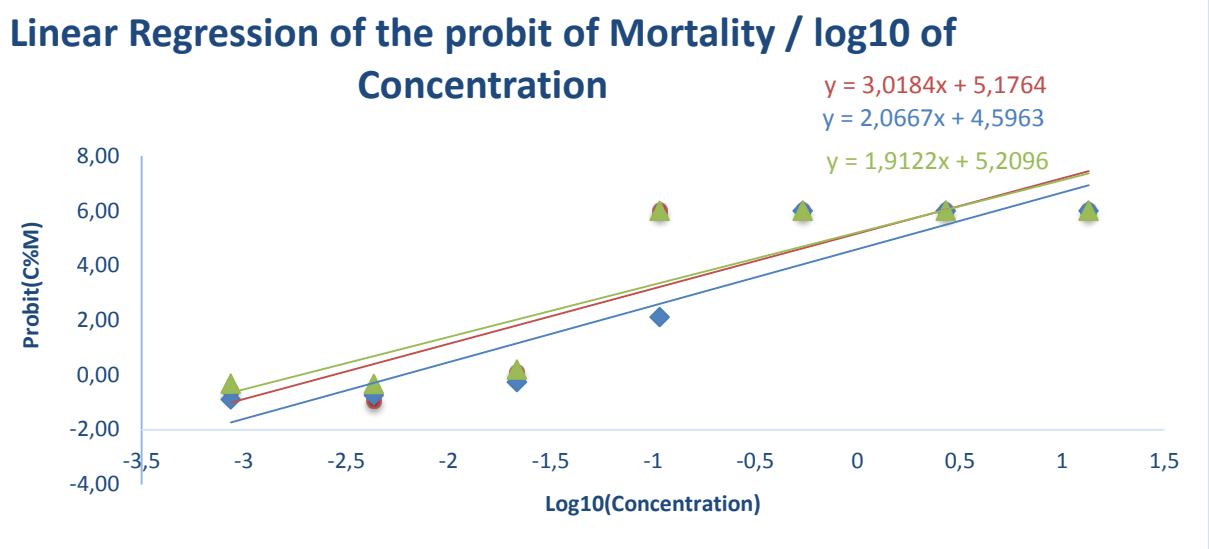

Figure 7. Summarized results for fipronil in Tri-Act®/Frontect® of one larval packet test for *D. reticulatus.*

	Equation y=ax+b		Log concentration			LC	
	a	b	log50	log99		LC50	LC99
A	2,018352426	5,176346	-2,56463934	-1,41204		0,002725	0,03872
B	2,066682112	4,596321	-2,2240098	-1,09837		0,005970	0,07973
C	1,912248183	5,209552	-2,72430723	-1,50776		0,001887	0,03106
MEAN						0,003527	0,04984

Table 7. Mean LC_{50} and LC_{99} of fipronil in Tri-Act®/Frontect® for *D. reticulatus.*

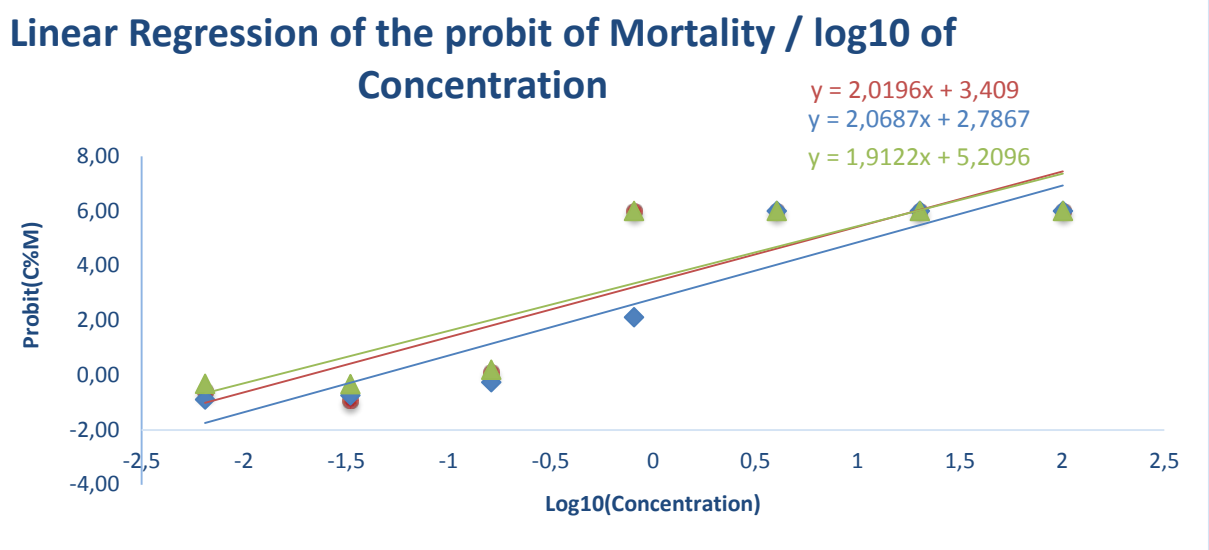

Figure 8. Summarized results for permethrin in Tri-Act®/Frontect® of one larval packet test for *D. reticulatus.*

	Equation y=ax+b		Log concentration			LC	
	a	b	log50	log99		LC50	LC99
A	2,019606259	3,408998	-1,68795169	-0,53607		0,020514	0,29102
B	2,068733961	2,786723	-1,3470671	-0,22254		0,044971	0,59905
C	1,913618183	3,535129	-1,84735331	-0,63167		0,014212	0,23352
MEAN						0,026566	0,37453

Table 8. Mean LC_{50} and LC_{99} of permethrin in Tri-Act®/Frontect® for *D. reticulatus.*

Verdunning	Concentratie Fipronil mg/ml	Fipronil per cm^2 papier (mg/cm^2)	Concentratie Permethrin mg/ml	Permethrin per cm^2 papier (mg/cm^2)
7	13.5	0.162	100.96	1.21
6	2.7	0.0324	20.192	0.242
5	0.54	0.00648	4.0384	0.048
4	0.108	0.001296	0.808	$9.68 * 10^{-3}$
3	$2.16 * 10^{-2}$	$2.59 * 10^{-4}$	0.162	$1.94 * 10^{-3}$
2	$4.32 * 10^{-3}$	$5.18 * 10^{-5}$	$3.32 * 10^{-2}$	$3.872 * 10^{-4}$
1	$8.64 * 10^{-4}$	$1.04 * 10^{-5}$	$6.46 * 10^{-3}$	$7.75 * 10^{-5}$

Table G. Used concentrations fipronil and permethrin in Tri-Act®/Frontect® in the dilution series for *D.reticulatus.*

Discussion

LC_{50} and LC_{99}

For the establishment of LC_{50} a basic bioassay can be used. In such an assay a total sample size of at least 500 larvae is needed with eight doses. This means each dose contains approximately 63 larvae. The bioassay will be valid as long as the test is replicated three times with five doses in every replication (Robertson J. L., Russel R.M., Preisler H.K., 2007). In this study 6 or 7 doses were used with approximately 300 larvae per dose and was repeated 3 or 4 times, so it can be concluded that the found value for LC_{50} is reliable.

For the establishment of LC_{99} (meaning the concentration that kills 99% of the larvae) a larger sample size and more doses are required. For the determination of LC_{99} a minimum of 7 doses is required with a sample size of 100.000 larvae. This large sample sizes are mostly impossible to reach in practice (Robertson J. L., Russel R.M., Preisler H.K., 2007). Also in this study the required sample sizes to establish LC_{99} are not reached. Therefore the found values for LC_{99} in this study are unreliable and must be disregarded.

Dilution series

Before a Larval Packet Test is performed the dilution series must be determined. Normally dilution series are based on serial dilutions, which consist of seven randomly chosen doses. These are used to determine a narrower range of effective concentrations between the lowest concentration of the active ingredient, where all the ticks are killed, and the highest concentration where all the ticks survive. This is called the dose-fixing phase in which only about ten larvae per dose are used for the determination of the mortality (Robertson J. L., Russel R.M., Preisler H.K., 2007). In this study the dilution series are based on previous studies from the UCTD and the dose-fixing phase was therefore not used.

For the determination of the efficacy of fipronil and permethrin alone against *R. sanguineus* dilution series were used from a previous study at the UCTD about the susceptibility of *R. sanguineus* to fipronil and permethrin. No adjustment was made at these dilution series. The used series can be found at table C and D. Also the dilution series for Tri-Act®/Frontect® are based on this study but adjusments were made to cover a better 0-100% dilution series. The used dilution series can be found at table B.

For *I. hexagonus* with Tri-Act®/Frontect® the same dilution series as *D. reticulatus* was used. Used concentration can be found at table F.

The dilution series for *D. reticulatus* with Tri-Act®/Frontect® were based on several previous studies with other tick species. The used concentrations can be found at table G.

Dermacentor reticulatus

For *D. reticulatus* 4 tests were carried out. Because of the high mortality rate in the control groups 2 control tests were carried out. Results of the control mortality can be found in table H.

Test no.	Average control mortality	Serial dilution
1	28.33%	1:10
2	28.6%	1:10
Control test 1	10.46%	Olive oil and trichloroethylene only
Control test 2	14.3%	Olive oil and trichloroethylene only
3	4.8%	1:10
4	52%	1:10

Table H. Control mortality of 6 tests with *Dermacentor reticulatus*

Only one of four carried out tests had a control mortality below 10%. Therefore only this test could be used. However, a test is only valid when it is replicated three times with five doses in each replication. Because in this research only one test was conducted the results are not reliable and must be disregarded.

After ruling out contamination and the possible influence of the age of the larvae it must be concluded that *D. reticulatus* larvae are possible too sensitive to be used in the Larval Packet Test. More research regarding the use of this tick species in the LPT is required.

Ixodes hexagonus

For *I. hexagonus* 3 test with Tri-Act®/Frontect® were carried out. Because the acaricides were not tested separately the found LC_{50} values for fipronil and permethrin cannot be used as baseline data for fipronil and permethrin alone.

Synergy

The definition of synergy is 'the interactions of two or more agents so that their combined effect is greater than the sum of their individual effects'. It is a term used not only for the study of acaricides but also in human and veterinary medicine. However it is unclear when synergy is proven. Ting-Chao Chou et al stated in his article about cancer research that there is no standardized definition for synergism and that 'the meaning of synergism has become an individual's preference' (Chou, 2010).

Several papers are published about synergy in acaricides and they all have their own way to approach synergy. Prullage et al (Prullage et al., 2011) did research on the synergistic effect between fipronil and amitraz. They calculated the synergistic ratios by dividing the EC_{50} or EC_{90} for fipronil alone by the EC_{50} or EC_{90} of fipronil combined with amitraz. The found synergistic ratios for EC_{50} is significant according to the research paper but the conditions for the 'cut off point' are not mentioned.

Hernández et al (Hernández MM1, Martínez-Villar E, Peace C, Pérez-Moreno I, 2012) studied the compatibility of flufenoxuron and azadirachtin against the two-spotted spider mite. They used a χ^2

test to analyse their data. Because a different method was used to obtain their lethal concentrations it is difficult to apply the same technique in this research.

As there are different ways to calculate synergy and none of the methods are scientifically proven it is difficult to state if and when there is synergy. If we calculate the synergistic ratios as is done by Prullage et al (only for LC_{50} because, as mentioned above, the LC_{99} are not reliable) we find the values 2.95 for fipronil and 1.07 for permethrin, as mentioned before in table E .

With the values found during this research it is unlikely that there is high level of synergy between fipronil and permethrin. However, as previously discussed the value of this statement is debatable depending on the applied definition and statistics. A result of 1.07 for permethrin and 2.95 for fipronil does imply that the effect of the combination of fipronil and permethrin has a higher kill rate when compared to both substances alone. However these low values are in contrast to Prullage who stated a synergistic ratio up to 137 (Prullage et al., 2011).

We can conclude from this outcome that a slight decrease in the need of fipronil appears to be required, however the needed concentration for permethrin stays nearly the same. As the ratio fipronil:permethrin was fixed in this research, results may differ when other ratios are tested.

Further research is required which is beyond the scope of this article.

Conclusion

For *R. sanguineus* a LC_{50} for permethrin and fipronil alone and in Tri-Act®/Frontect® were found. These can be found in table I. The LC_{50} for fipronil and permethrin alone can be used in the future as baseline data. The LC_{99} were not reliable due to the number of larvae needed to obtain reliable results. Therefore the found values for LC_{99} must be disregarded. The found values for the synergistic ratios where >1, indicating synergism between fipronil and permethrin. Therefore the H_0 hypothesis is confirmed and the H_1 hypothesis is rejected.

	LC_{50} mg/ml	Synergystic ratio LC_{50}
Fipronil in Tri-Act®/Frontect®	$1.2169*10^{-2}$	-
Permethrin in Tri-Act®/Frontect®	$9.0249*10^{-2}$	-
Fipronil alone	$3.5654*10^{-2}$	2.95
Permethrin alone	$9.6677*10^{-2}$	1.07

Table I. Found values for LC_{50} and LC_{99} for fipronil and permethrin and calculated synergistic rations for fipronil and permethrin.

For *I. hexagonus* a LC_{50} of $1.0096*10^{-2}$ mg/ml for fipronil and $7.8443*10^{-2}$ mg/ml for permethrin in Tri-Act®/Frontect® were found. The LC_{99} were not reliable due to the number of larvae needed to obtain reliable results. Therefore the found values for LC_{99} must be disregarded. Because fipronil and permethrin weren't test separately no synergistic ratios could be calculated.

For *D. reticulatus* no reliable result regarding the LC_{50} or LC_{99} for fipronil or permethrin were found due to too small number of performed tests.

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