SUSCEPTIBILITY OF AMBLYOMMA HEBRAEUM AND DERMACENTOR RETICULATUS TICKS TO FIPRONIL

12/3/2015

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PREAMBULE

This paper is the final report of the research project carried out by Alice Abma at the Utrecht Centre for Tick-borne Diseases (UCTD) at Utrecht University.
Research was executed to compare and improve the FAO recommended Larval Packet Test and the Larval Immersion Test. Now that the Larval Packet Test is perfected and standardized, more research can be done by new research students for future purposes.

CONTENTS

1. ABSTRACT

The purpose of the study was to test the susceptibility of *A. hebraeum* and *D. reticulatus* to fipronil. Both the Larval Packet Test and Larval Immersion Test were carried out, for comparison and to determine which test has the best sensitivity. For *A. hebraeum* a LC_{50} of $2{,}15 \cdot 10^{-2}$ mg/mL for fipronil was found. In future studies this LC_{50} can be used as a baseline. No reliable results were obtained for *D. reticulatus* because larvae were too old. Therefore, no conclusion can be drawn regarding this tick species, the second hypothesis remains unproven. This study proves that larval age is important and that the test should be performed when the larvae are 14 to 28 days old. When comparing more tick species at once and larvae of these species are all the same age, it is important that these tests are carried out in parallel for reliable results. If not, larvae of one tick species may be too old of age and results will be unreliable.

In the future, more research needs to be done. Not only to determine baseline data for other tick species, as well as to detect changes in susceptibility to acaricides at an early stage. Now that the Larval Packet Test is standardized and more reliable, a more accurate LC_{50} can be determined.

No conclusion can be drawn regarding difference in sensitivity of the Larval Immersion Test and Larval Packet Test. This hypothesis remains unproven. It is in the future, however, recommended to perform the Larval Packet Test rather than the Larval Immersion Test. The Larval Packet Test is significantly easier to perform and less time consuming and it takes less working space and less materials. The Larval Packet Test also has a higher chance of succeeding. Furthermore, the Larval Immersion Test is not recommended or standardized by the FAO, as opposed to the Larval Packet Test [15].

2. INTRODUCTION

Long-term use of acaricides to control and eradicate tick populations has caused acaricide resistance in multiple tick species. Regularly monitoring of ticks regarding resistance against common acaricides is therefore important and frequently practiced [2]. Acaricide resistance in cattle ticks, especially *Rhipicephalus microplus*, has been reported, whereas resistance in ticks infesting dogs and cats is relatively uncommon [3].

Some tick species do not show acaricide resistance yet, but may develop this in the future [4]. The frequency of resistant genes initially increases slowly in a certain species, however, this frequency is usually high at the time decreased efficiency of treatment is noticed [2]. Therefore, early detection of loss of susceptibility is important and depends on the availability of accurate baseline data [5]. Thus, it is important to determine baseline data for these tick species (i.e. acaricide LC_{50} and LC_{99} values for susceptible strains), to enable detection of changes in susceptibility to acaricides.

2.1 Background information

Consultation of the Arthropod Pesticide Resistance Database [4] at http://www.pesticideresistance.com, regarding the tick species used in this study, revealed that there were six reports of acaricide resistance in *Amblyomma hebraeum*. Based on the number of papers on *R. microplus* (102 reports for eight different acaricides) in the Arthropod Pesticide Resistance Database, this again shows that resistance in *R. microplus* is widespread. The database does not include any reports of acaricide resistance in *Dermacentor reticulatus*, *Ixodes hexagonus* and *Ixodes ricinus*, although the database does show slightly more cases of acaricide resistance for *Dermacentor* spp. in general than for *Ixodes* spp. This indicates that acaricide resistance does not (yet) exist in these tick species. [4]

A. hebraeum resistance was found for BHC/cyclodienes, carbamates, DDT, organophosphates, sodium arsenite and toxaphene in South Africa [4]. There are no reports of acaricide resistance in ticks in Europe. This variation in resistance in different countries can possibly be explained because of variations in their use of frequency of acaricides [2].

The remarkable differences in resistance in different tick species might be explained by their refugia (i.e. the portion of the species population that is not exposed to the chemical). On this part of the population there is no selection pressure, thus the refugia contribute to the reservoir of pesticide-susceptible genes. *I. ricinus* is not host specific and hosts of *I. ricinus* are usually not treated with acaricides. Therefore *I. ricinus* has more refugia than *R. microplus*, which is a one-host tick that remains on a host usually treated with acaricides. This may explain why *I. ricinus* has not developed acaricide resistance as opposed to *R. microplus*. The same applies to other ticks that are not host specific and whereby hosts for the immature stages are usually not treated with acaricides. [3]

A question that arises regarding resistance is the duration of the period before reintroducing an insecticide after resistance has caused problems. Abbas et al. states that when a drug, for which parasites have developed resistance, is withdrawn from use for a period of time, the susceptibility for

this drug may return [2]. However, this appears more difficult than it seems, because it depends on both the species and the chemical itself. Dieldrin, for example, has not been used for over 30 years, what led to a decreased selection pressure. It is to be expected that this will cause a decrease in the prevalence of resistant genes, but studies have showed that this is not the case. In contrast to Dieldrin, the withdrawal of use of DDT and organophosphates and the associated decreased selection pressure showed a rapid recurrence of susceptibility for these chemicals. [3]

2.2 Main research question

Testing the susceptibility of two Ixodid tick species to fipronil using the Larval Packet Test and the Larval Immersion Test for determining baseline data for these tick species.

2.3 Hypothesis

$H_{1.1}$ = the Larval Immersion Test has a better sensitivity than the Larval Packet Test.
$H_{1.0}$ = the Larval Immersion Test is less sensitive than the Larval Packet Test.
$H_{2.1}$ = *D. reticulatus* has the highest susceptibility for fipronil.
$H_{2.0}$ = *D. reticulatus* does not have the highest susceptibility for fipronil.

2.4 Research methodology

In order to create valuable and comparable results, the standard FAO-recommended research methodology, the Larval Packet Test, was applied. The second research methodology that was used is the Larval Immersion Test/Shaw Larval Test, as carried out in Dr. R.J. Taylor's Laboratory, Pietermaritzburg, South Africa.

3. PURPOSE OF STUDY

The purpose of the study was to test the susceptibility of two tick species to fipronil, in order to determine baseline data for these tick species, for detection of changes in susceptibility to acaricides. To achieve this, larvae of *A. hebraeum* and *D. reticulatus* were used (see *table 1*). Both of these species are three-host ticks, which means that each stage (larvae, nymph and adult) must find a new host [3]. The acaricide used in the tests was fipronil, making use of the formulated product (i.e. veterinary medicine that contains the pharmacological active ingredient)(see *table 2*).
Two different tests were carried out, for comparison and to determine which test has the highest sensitivity. The two tests used in this study were the Larval Packet Test and the Larval Immersion Test. In this study LC_{50} and LC_{99} values for both tick species were determined (the LC is the lowest concentration needed to provide a particular mortality rate). Thus, when working with a potential resistant strain of studied tick species in the future, a factor of resistance can be calculated based on these LC_{50} and LC_{99} values. The factor of resistance can be obtained by comparing the LC_{50} and LC_{99} values obtained from the potential resistant strain with the LC_{50} and LC_{99} values for susceptible strains, as determined in this study (see *figure 2*).
In the future this test will be carried out more often at the UCTD for other tick species and to enable detection of changes in susceptibility to acaricides at an early stage. Therefore, it is important to perfect and standardize the Larval Packet Test for future purposes. While executing the Larval Packet Test some elements were modified and improved. Also, a protocol was created to make sure the test is standardized for reliable results in the future.
For a detailed protocol description, see *Appendix A*.

Tick species	*Amblyomma hebraeum*	*Dermacentor reticulatus*
Common name	The South African bont tick	The ornate dog tick
Hosts	**Adults:** - Large hosts such as cattle and large wild ruminants, particularly giraffes, buffaloes, elands and rhinoceroses. - Sheep, goats **Immature stages:** - Same hosts as the adults - Small antelopes, scrub hares, helmeted guineafowls and tortoises - Very rarely rodents	Dogs and other medium-sized (wild) carnivores, sheep, cattle, horses, ungulates
Life cycle	Three-host tick	Three-host tick
Habitat and distribution	South eastern Africa *N.B.: in South Africa it is found along the coastal belt.* *It is also most commonly found in eastern Swaziland, southern Mozambique, eastern Botswana and in southern and eastern Zimbabwe as well as parts of the Zimbabwean highveld.*	The Palearctic region *N.B.: Southern Europe represents the southern limits of its distribution, while it is common in northern Europe and British Isles.*
Seasonal occurrence	**Larvae:** - Late summer, autumn **Nymphs:** - Winter, early spring **Adults:** - Summer *N.B.: in the north-eastern lowveld regions of the KwaZuluNatal, Northern and Mpumalanga provinces of South Africa and in southern Zimbabwe, the life cycle continues throughout the year*	**Adults:** - In humid climates in the Mediterranean region: from October to March - In the northern limit of its distribution: in summer
Transmitted diseases	*Ehrlichia ruminantium* (heartwater), benign theileriosis	
Transmitted pathogens	*Rickettsia africae*, *Rickettsia conorii*, *Theileria mutans*	*Babesia canis*

Table 1. Features of the six tick species used in this study. **[6, 7]**

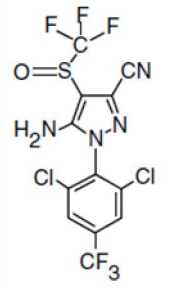
Acaricide	Frontline
Scientific name	Fipronil
Chemical group	Phenylpyrazoles
Molecule	
Veterinary medicine	**Frontline:** - Pump spray - Spot-on - Dog and cat **Frontline Combo/Plus:** - Spot-on (combined with S-methopren) - Dog and cat **Certifect:** - Spot-on (combined with amitraz and S-methopren) - Dog only **Effitix:** - Spot-on (combined with permethrin) - Dog only
Spectrum	Insects, ticks, mites
Mode of action	Gaba-gated chloride channel antagonist
Targeted tick species	*Ixodes* spp. *Rhipicephalus sanguineus* (spray only) *Dermacentor* spp.
Duration of action (after first treatment)	4 weeks
Warnings	Not for rabbits. Not for puppies <8 weeks and/or <2kg (spot-on only) Not for kittens <8 weeks and/or <1kg (spot-on only)

Table 2. Features of acaricides used in this study. **[8, 9]**

4. MATERIALS AND METHODS

4.1 Conditions during testing

Engorged females were placed in a climate-controlled chamber with a temperature of 27-28 °C and 85-95% relative humidity (RH), for egg laying. The eggs were collected and kept under the same circumstances regarding temperature and humidity conditions as the engorged females. [10]

4.2 Acaricide resistance testing

Larvae of two tick species (*A. hebraeum* and *D. reticulatus*) were used for this research.
The acaricide for which susceptibility was tested is fipronil. The dilutions were based on the concentrations between the lowest concentration that kills all ticks and the highest concentration in which all ticks are still alive (i.e. 0-100% mortality series; see *table 3*).

Larval Packet Test

For the Larval Packet Test, about 100 larvae were placed in a sheet of filter paper (10 x 5 cm). Since all filter papers are equal, it is possible to determine the amount of product per square centimeter (see *table 3*).
These sheets were folded in the center and the edges were secured with clips. Both sides of the packet were homogeneously moistened with 600 µL of the acaricide to be tested, at different dilutions in olive oil and trichloroethylene (1:2) (see *table 3*), with three repetitions for each dilution per test.
Three control packets were also formed, using only olive oil and trichloroethylene (1:2).
The packets were placed in eight separate climate-controlled containers under the same circumstances as mentioned before for a period of 24 hours. At first, a vacuum desiccator was used for this purpose. However, after 24 hours all packets, including the control, showed 100% mortality. Thus, to prevent contamination of control packets and lower concentration packets with higher concentrations of acaricide, all packets were stored separately. Therefore, eight different containers were used, one for each of the seven different concentrations and one for the control, with each container containing three duplicate packets. After 24 hours the packets were opened and the larvae, both dead and alive, were counted.
For a more detailed protocol description, see *Appendix A*.

Larval Immersion Test

For the Larval Immersion Test, two sheets of filter paper were soaked with 10 mL of the acaricide to be tested, at different dilutions (see *table 3*), with two repetitions for each dilution per test. About 100 larvae were placed between the two sheets. After exactly ten minutes, the larvae were transferred to a clean and dry folded packet of filter paper which were then secured with clips. [11]
As opposed to the Larval Packet Test, for the Larval Immersion Test it is important to use different brushes per concentration (i.e. one brush for each concentration). Larvae usually get stuck in the brush

and if you use this same brush for the next concentration, larvae might end up in the wrong packet (thus creating false results).
Three control packets were also formed, using water.
The packets were placed in eight separate climate-controlled containers for a period of 24 hours. After 24 hours the packets were opened and the larvae, both dead and alive, were counted. [11]
For a more detailed protocol description, see *Appendix B*.

4.3 Statistics

Data acquired with the Larval Packet Test and Larval Immersion Test were analyzed and compared with each other.
After counting the larvae, the numbers of alive and dead ticks were entered in to an EXCEL spreadsheet and a percentage of mortality for each concentration was calculated. If, for the control packets, mortality revealed to be below 5%, then the direct mortality figures were used. If they were found to be between 5% and 10% in the control, then the percentage mortality in all of the concentrations were corrected using Abbott's formula (see *figure 1*).
If mortality in the control was higher than 10% the results were disregarded and the test was repeated.
Results were plotted with percent concentration (x-axis) by Probit mortality (y-axis) for each acaricide and all tick species, hereby determining the LC_{50} and LC_{99}. These populations are homogeneously susceptible, so a straight line was obtained for all tests.

Tick species	*A. hebraeum*	*D. reticulatus*
Used concentrations of fipronil	4.0, 0.8, 0.16, $3.2 \cdot 10^{-2}$, $6.4 \cdot 10^{-3}$, $1.28 \cdot 10^{-3}$ and $2.56 \cdot 10^{-4}$ mg/mL	4.0, 0.8, 0.16, $3.2 \cdot 10^{-2}$, $6.4 \cdot 10^{-3}$, $1.28 \cdot 10^{-3}$ and $2.56 \cdot 10^{-4}$ mg/mL
Amount of Frontline per cm² filter paper	$4.8 \cdot 10^{-1}$, $9.6 \cdot 10^{-2}$, $1.92 \cdot 10^{-2}$, $3.84 \cdot 10^{-3}$, $7.68 \cdot 10^{-4}$, $1.536 \cdot 10^{-4}$ and $3.072 \cdot 10^{-5}$ µL/cm²	$4.8 \cdot 10^{-1}$, $9.6 \cdot 10^{-2}$, $1.92 \cdot 10^{-2}$, $3.84 \cdot 10^{-3}$, $7.68 \cdot 10^{-4}$, $1.536 \cdot 10^{-4}$ and $3.072 \cdot 10^{-5}$ µL/cm²
Amount of fipronil per cm² filter paper	$4.8 \cdot 10^{-2}$, $9.6 \cdot 10^{-3}$, $1.92 \cdot 10^{-3}$, $3.84 \cdot 10^{-4}$, $7.68 \cdot 10^{-5}$, $1.536 \cdot 10^{-5}$ and $3.072 \cdot 10^{-6}$ mg/cm²	$4.8 \cdot 10^{-2}$, $9.6 \cdot 10^{-3}$, $1.92 \cdot 10^{-3}$, $3.84 \cdot 10^{-4}$, $7.68 \cdot 10^{-5}$, $1.536 \cdot 10^{-5}$ and $3.072 \cdot 10^{-6}$ mg/cm²

Table 3. Amount of product used for the LPT per tick species.

$$\text{Corrected percent mortality} = \frac{\%\text{ test mortality} - \%\text{ control mortality}}{100 - \%\text{ control mortality}} \times 100$$

Fig. 1. Abbott's formula.

$$\text{Factor of Resistance} = \frac{LC_{50}\text{ of acaricide read from graph}}{LC_{50}\text{ for susceptible strain}}$$

Fig. 2. Formula to determine Factor of resistance.

5. RESULTS

As long as the experiment is replicated three times with five doses in each replication, the test will be valid [12]. For *A. hebraeum* a total of seven tests were carried out, with seven doses in each replication, to determine a reliable LC_{50} (dose-fixing phase, consisting of three tests, not included). With three repetitions for each dilution per test there will be a total of 21 results per dilution. Eventually five tests were used for the results, because two tests had to be disregarded because of high larval mortality in the controls (see *page 15: Larval age*).

All results are summarized and shown in *figure 3*. In *Table 4* the average mortality for each concentration is given and in *Table 5* the LC_{50} and LC_{99} for *A. hebraeum* regarding fipronil are shown. One may assume that this LC_{50} is pretty accurate, thus can be used for baseline data. However, the LC_{99} is not accurate, due to sample size, and must be disregarded (see *page 16: Sample size, dose number, LC_{50} and LC_{99}*).

For *D. reticulatus* only one test was carried out. The control mortality in this test was 36,23 % and thus this test was disregarded. Unfortunately, at this stage the larvae were already too old for reliable results (see *page 15: Larval age*), so no useful results were obtained regarding *D. reticulatus*.

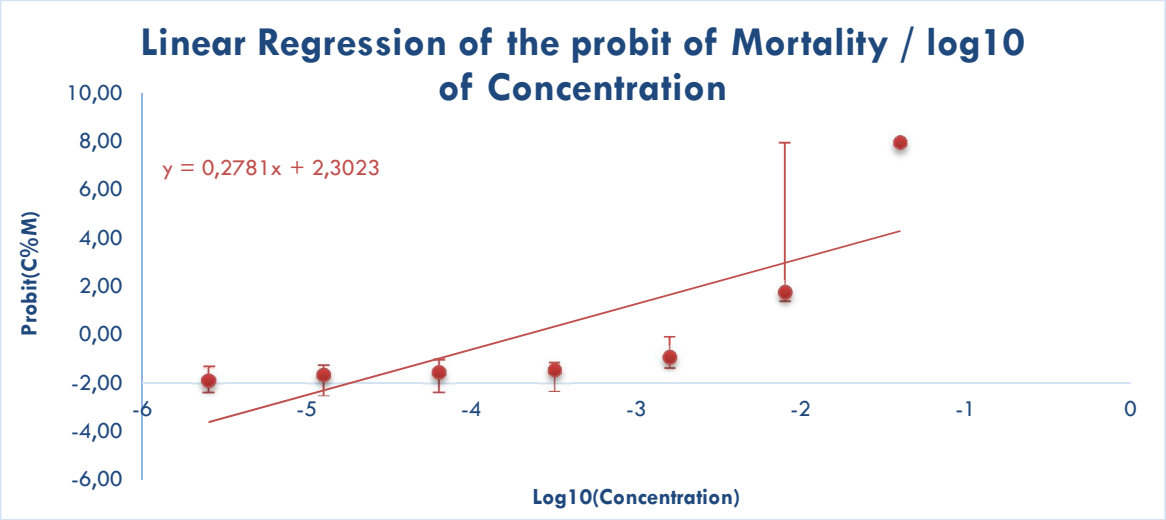


Fig. 3. Summarized results of a total of five Larval Packet Tests for *A. hebraeum*.

Concentration of fipronil	Average mortality rate
$2.56 \cdot 10^{-4}$ mg/mL	3,05 %
$1.28 \cdot 10^{-3}$ mg/mL	5,00 %
$6.4 \cdot 10^{-3}$ mg/mL	6,18 %
$3.2 \cdot 10^{-2}$ mg/mL	7,32 %
0.16 mg/mL	17,96 %
0.8 mg/mL	96,14 %
4.0 mg/mL	100,0 %

Table 4. Average mortality for each concentration.

Lethal concentrations	Frontline	fipronil
LC_{50}	$2,15 \cdot 10^{-4}$ mL/mL	$2,15 \cdot 10^{-2}$ mg/mL
LC_{99}	$3,69 \cdot 10^{-3}$ mL/mL	0,369 mg/mL

Table 5. Lethal concentrations of Frontline and fipronil to kill 50% and 99% of all ticks, extracted from *figure 1*.

6. DISCUSSION

6.1 Duration Larval Immersion Test

The Shaw Larval test, as carried out in Dr. R.J. Taylor's Laboratory, Pietermaritzburg, South Africa, indicates to leave the packets in the incubator for 72 hours. This seems a bit long, since the incubation period for the Larval Packet Test is 24 hours. Is it really necessary to wait 72 hours before counting? Research shows that most articles indicate a waiting period of 24 hours and only a few mention 72 hours. But it is also explained why they use 72 hours and why it is aberrant.
Each acaricide and each tick species has its own end point, meaning the point where additional time does not result in further mortality. For example, the end point for dichlorvos is reached at 17 hours, but for toxaphene, this point is not reached until 48 hours, and possibly even after 72 hours. In this case, a waiting period of 72 hours is only indicated for toxaphene and not for other acaricides. [13]
Rodriguez-Vivas *et al*. also mention an incubation period of 72 hours, but the reason for this deviation is that their test is modified. They did not use multiple dilutions as such, but only used the discriminating dose to check for resistance. Instead of exposing the larvae to the acaricide for ten minutes, they exposed the larvae for 72 hours. This to prevent false results regarding resistance. They did not want to create a baseline but merely wanted to test strains for resistance (if one or more larvae were found alive after 72 hours, the strain was considered resistant). For the original Larval Immersion Test this argument is invalid, because larvae are only exposed to the acaricide for ten minutes. [14]
So in the case of fipronil and in the case of creating a baseline, it is unnecessary to wait 72 hours before counting. Therefore a waiting period of 24 hours was maintained during this test.

6.2 Comparing the Larval Packet Test and Larval Immersion Test

To compare the Larval Packet Test and Larval Immersion Test and to determine which test has the best sensitivity, both tests were carried out multiple times using the same tick species and acaricides.
The Larval Packet Test was carried out twelve times: one time using *D. reticulatus*, seven times for *A. hebraeum*, three times during the dose-fixing phase and one time using controls only.
The test with *D. reticulatus* and the two final tests for *A. hebraeum* were disregarded because of larval age. The control mortality for the five remaining tests was always below 10%, with the highest control mortality at 6,91% and four out of five tests remaining below 5%, with two tests even at 0% control mortality.
During the dose-fixing phase, the control mortality rates were also all below 10%.
One test was performed with controls only, to test the quality of the eight separate plastic containers. One control packet went in each container, using a total of eight packets. Only one of these packets showed mortality above 10%. However, this was the first time this test was carried out and the test was not perfected yet at this point. Also, for this test, very limited numbers of larvae were used (for this particular packet 28 larvae were used and four of them died during the test), so high percentages are easily gained. Furthermore, five packets showed 0% mortality and the two remaining packets showed mortality below 5%. See *table 6* for detailed mortality rates.

The Larval Immersion Test was carried out five times, using *A. hebraeum* for all tests. One test was disregarded because of contamination of the immersion stock. The control mortality for the four remaining tests was always above 10%, with the highest control mortality at 31,82% and the lowest at 27,07%. Multiple attempts were performed to find out the cause of this extremely high mortality rate. First, four tubes with larvae were filled with pure water for exactly ten minutes. The larvae were then transferred into four packets and counted after 24 hours. The mortality of these packets was respectively 8,89%, 17,02%, 42,19% and 14,58%.

It seemed that, for some reason, the larvae were not able to survive being under water for ten minutes. Therefore, the entire Larval Immersion Test was performed dry, meaning all steps of the test were performed, the ten minutes of waiting time included, but without being immersed in water. Again, four packets were filled with larvae and counted after 24 hours. Mortality in this test ranged from 10 to 35%. This may proof that the larvae are unable to survive this test for some other reason than being immersed for ten minutes. There are unexplained circumstances to why this test does not seem to work. A research student performed this exact same test in South-Africa and it is remarkable that, in this test, mortality sometimes did get below 10%, but not always, and never below 5%. There might be a factor in South-Africa that creates better circumstances for this test. Further research will be necessary to prove this and find the cause.

Since the Larval Packet Test and Larval Immersion Test were not performed simultaneously, the mortality rate in the Larval Immersion Test might be caused by larval age. However, it seems that larval age has a greater influence on the Larval Immersion Test, because later on in this study another Larval Packet Test was performed and the mortality, although higher than in previous tests, was still significantly low.

Since not all larvae die in the Larval Immersion Test, one of these factors might be the quality of the larvae. Therefore, another test was carried out, using only “good” larvae. For this, larvae were chosen individually, based on speed and activity. The only way you can select larvae, is either using a forceps or a suction device. Mortality in this test was 63%. So it seems that, even though these larvae appeared to be strong, they are not strong enough to withstand being handled with forceps or the suction device. Presumption arises that there are too many factors that influence the mortality in this test, making this test very unreliable, as opposed to the Larval Packet Test, were mortality is often between 0 and 5%.

Test	Control mortality
Controls only	Respectively 3,45%, 0%, 0%, 0%, 0%, 2,49%, 14,29% and 0%
Dose-fixing phase test 1	1,69%
Dose-fixing phase test 2	0,65%
Dose-fixing phase test 3	9,89%
***A. hebraeum* test 1**	6,91%
***A. hebraeum* test 2**	0%
***A. hebraeum* test 3**	0%
***A. hebraeum* test 4**	1,71%
***A. hebraeum* test 5**	2,03%

Table 6. Control mortality for the Larval Packet Tests.

6.3 Larval age

While performing the Larval Packet Tests, it showed that mortality went up as the larvae were aging. The first five tests with *A. hebraeum* to determine the LC_{50} were performed at the age of 19 to 28 days. Test six and seven were carried out at the age of 50 to 56 days. During this period, average control mortality went up from respectively 2,13% to 29,94%. This suggests that larval age can influence the test.

Multiple studies state that the ideal larval age is 14 to 28 days after hatching for both the Larval Packet Test and the Larval Immersion Test [5, 10, 11, 13].

R.D. Shaw was the first to describe the Larval Immersion Test and to determine the ideal circumstances for this test he thoroughly tested several aspects of the test, larval age being one of them. To test the changes in the susceptibility of larvae to insecticides and thus to determine the ideal larval age, he observed the sequential susceptibility of larvae to an insecticide during a period from the 7th to the 35th day after hatching. The lowest susceptibility was observed during the third week (14 to 21 days after hatching). In addition, there was less variation in mortality during the third week than during the preceding or subsequent weeks. The variation in mortality was especially remarkable before the age of 14 days, so larvae can be too old for the tests as well as too young. [13]

The FAO standardized Larval Packet Test recommends a larval age of 14 to 21 days for all Ixodid tick larvae, stating that, regardless of species or developmental stage, age greatly affects the susceptibility to acaricides. For reliable results, standard ages for the testing of each species and stage should be established. [10]

6.4 Sample size, dose number, LC_{50} and LC_{99}

Robertson *et al*. determined the required sample size for bioassays to obtain a reliable LC_{50} and LC_{99}. A comparison of designs for estimation of a LC_{50} and LC_{90} was made. They found that precision increases

as the number of doses increases and estimates become more precise as total sample size increases and each lethal concentration (a.o. LC_{50}, LC_{90}, LC_{95} and LC_{99}) requires a different sample size (see *table 7*). The final conclusion was that, if possible, a total sample size of 500 test subjects with eight doses (63 insects/dose) should be tested for optimal results. However, it is noted that, when seven to twelve doses are tested, most estimates of LC_{50} are precise, regardless of dose placement or sample size, but the LC_{90} is less precise, unless large sample sizes are used. [12]

In this study a LC_{99} was determined for *A. hebraeum* regarding fipronil. Although, Robertson *et al.* states that estimation of extreme levels such as the LC_{95} and LC_{99} require more doses and larger sample sizes than the numbers mentioned in *table 7*. For precise estimation of the LC_{99}, none of the designs with four to six doses is acceptable and use of at least seven doses is necessary. Furthermore, the required sample sizes for this estimation are 3.000-3.600 larvae per test, which is nearly impossible in practice. [12]

No. of doses	Sample size	
	LC_{50}	LC_{90}
4-5	100 per dose	200 per dose
6-10	50 per dose	100 per dose
Estimation	300-500	600-1.000

Table 7. Required sample size related to number of doses for LC_{50} and LC_{90}.

6.5 Dose-fixing phase

Before performing the actual Larval Packet Test or Larval Immersion Test, dose selection must be determined. In this study, therefore, a serial dilution of seven doses was randomly chosen to determine a narrower range of effective concentrations between the lowest concentration that kills all ticks and the highest concentration in which all ticks are still alive (i.e. 0-100% mortality series). This is called the dose-fixing phase, which consists of using a broad series of dilutions to test about ten insects per dose, merely to determine mortality [12].

Once the effective dose ranges are identified for each population, multiple doses can be selected and these will then be tested in the basic packet or immersion tests [12].

In this study, three replications of the dose-fixing phase were performed with the following concentrations of fipronil: 10, 1.0, 0.1, $1.0 \cdot 10^{-2}$, $1.0 \cdot 10^{-3}$, $1.0 \cdot 10^{-4}$ and $1.0 \cdot 10^{-5}$ mg/mL. Results showed 100% mortality at 1,0 mg/mL and 0% mortality at $1.0 \cdot 10^{-4}$ mg/mL. The final serial dilution for the tests was based on these results (see *table 3*).

7. CONCLUSION

For *A. hebraeum* a LC_{50} of $2{,}15 \cdot 10^{-2}$ mg/mL for fipronil was found (see *table 5*). In future studies this LC_{50} can be used to determine a factor of resistance. The factor of resistance can be derived when the LC_{50} of acaricide read from graph is divided by the LC_{50} for the susceptible strain, determined in this study (see *figure 2*). [11]

No conclusion can be drawn regarding *D. reticulatus*. As mentioned before, no reliable results were obtained. In the future, it is important that the test is carried out when the larvae are 14 to 28 days old. When comparing more tick species at once and larvae of these species are all the same age, it is important that these tests are carried out in parallel for reliable results. If not, larvae of one tick species may be too old of age and results will be unreliable.

In the future, more research needs to be done. Not only to determine baseline data for other tick species, as well as to detect changes in susceptibility to acaricides at an early stage. In the future, when using a susceptible strain, more tests can be carried out to retrieve a more accurate LC_{50}. Now that the Larval Packet Test is standardized and more reliable, a more accurate LC_{50} can be determined. When using a resistant strain, the previous determined LC_{50} can be used to retrieve the factor of resistance.

For future purposes, an attempt can be made to delay or even prevent acaricide resistance through the following measures: rationale use of acaricides (i.e. regular monitoring, rotation of acaricides and using combinations of acaricides), vaccination (i.e. prevention), nutritional management, using botanicals, improving genetic resistance in cattle, environmental management (i.e. pasture burning, pasture alternation and/or rotation, house management) and improving resistance diagnostic tests (the Larval Packet Test, Larval Immersion Test and Adult Immersion Test are usually used for acaricide resistance testing; unfortunately, these tests are not sensitive enough to detect the emergence of early stages of resistance). Underdosing, which may lead to the selection of mutants initially resistant to low levels of acaricides, might play a role in the development of acaricide resistance. An important factor involved in resistance development is the frequent use of the same acaricide for a long period of time. Studies show that more than five treatments with the same acaricide per season is a risk factor for acaricide resistance. [2]

A study that compared the Larval Packet Test and Larval Immersion Test showed that the toxicity of acaricides was much higher in the Larval Immersion Test than in the Larval Packet Test and the Larval Immersion Test was approximately 400 times more sensitive than the Larval Packet Test [15]. However, based on this study no such conclusion can be drawn. No results from Larval Immersion Tests were obtained, therefore, no records can show a difference in sensitivity between the two tests. This hypothesis remains unproven.

It is in the future, however, recommended to perform the Larval Packet Test rather than the Larval Immersion Test. Even though, no records regarding sensitivity were obtained, the Larval Packet Test is significantly easier to perform. Besides that, it is less time consuming, it takes less working space and less materials. The Larval Packet Test also has a higher chance of succeeding. Furthermore, the Larval

Immersion Test is not recommended or standardized by the FAO, as opposed to the Larval Packet Test [15].

It might be assumed that *D. reticulatus* has a higher susceptibility for fipronil than *A. hebraeum*, based on the records from the Arthropod Pesticide Resistance Database (a.o.) regarding resistance in these tick species. However, in this study, it is not proven that *D. reticulatus* has a higher susceptibility for fipronil than *A. hebraeum*. No results for *D. reticulatus* were obtained due to larval age, therefore, no records can show a difference in susceptibility between the two tick species. Further research is necessary. The second hypothesis remains unproven.

Normally the chances of *D. reticulatus* being susceptible to acaricides would be higher than for *A. hebraeum*. However, in case of fipronil this might not be the case. Fipronil is not used as an acaricide in the region were *A. hebraeum* has his natural habitat. Thus, *A. hebraeum* is usually not exposed to fipronil and therefore will not develop resistance to this acaricide. This in contrary to *D. reticulatus*, which is often being exposed to fipronil and might lose its susceptibility for this acaricide. Therefore, in the future, it might be more plausible for *D. reticulatus* to develop resistance to fipronil than for *A. hebraeum*.

8. ACKNOWLEDGEMENTS

I would like to thank prof. dr. F. Jongejan for the opportunity to perform my research project at the UCTD and for his guidance during the process. It has been a very interesting and educational experience.
Furthermore, I want to thank ing. L. Berger and Gabriel Goderski for their guidance and assistance with the practical part of the project, being mainly lab work. When working with exotic species, it is very important that there are no escapees and an extra pair of hands can come in extremely handy sometimes.
Finally, I want to thank AnneKim Geerdes for teaching me how to perform the Larval Packet Test and Nikky Kok for teaching me how to perform the Larval Immersion Test. These test are complicated and it makes it a lot easier if you can see how it is been done rather than merely reading the protocol.

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LARVAL PACKET TEST

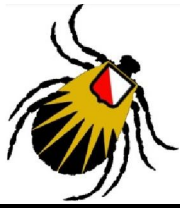
Acaricide			
Active ingredient		**Batch number**	
Dilution range			
Tick species			
T = 0		**T = 24H**	

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

Wear gloves and work in the fume hood

All escaped larvae can be caught with tape

	Preparation	Done
1	Calculate serial dilutions (in triplicate) and write it down in a labjournal.	
2	Tape the “acaricide testing in process” sign on the door and lock the door.	
3	Cut filter papers to10x5cm. The control and each dilution are done in triplicate.	
4	Fold the filter papers in length in half and write the corresponding concentrations on each with pencil.	
5	Put the filter papers, unfolded, in a zip bag per dilution/control.	
6	Prepare the diluent of olive oil and trichloroethylene in a ration of 1:2.	
7	Prepare the serial dilution in 15ml Falcon tubes.	
8	Impregnate the filter papers with 1800µl of corresponding dilution/control. Ensure all filter papers are completely impregnated. **Start with the control and work from the lowest to the highest concentration.**	
9	Rip 2 large zip bags open and cover the workspace of the fume hood with them.	
10	Make packets of all impregnated filter papers by taking them with forceps from the zip bag, folding them in half with a large bulldog clip and placing a large bulldog clip on both sides. **Start with the control and work from the lowest to the highest concentration.**	
11	Place the packets upright on the covered workspace with sufficient space between them.	
12	Leave the packets overnight in the fume hood.	
13	Discard all waste in a closed zip bag in the acaricide bio-waste container.	
14	Close the fume hood and turn off the lights.	



	Setup	Done
1	Put the needed ticks from the acaridarium in the “acaricide” dessicator.	
2	Fill the enthanol squeeze bottle if necessary.	
3	Perforate 1 zip bag for each dilution and the control.	
4	Fill each perforated zip bag with moisturized cotton wool and place one in each container.	
5	Fill the testing bin and the acaricide waste bin with 1% delladet.	
6	Fill each control packet with approximately100 larvae using the “control” brush and seal the packet with a small bulldog clip.	
7	Place the control packets in the corresponding container and close the container well.	
8	Fill each sample packet with approximately100 larvae using the “acaricide” brush and seal the packet with a small bulldog clip. **Work from the lowest to the highest concentration.**	
9	Place the sample packets in the corresponding containers and close the containers well. **Work from the lowest to the highest concentration.**	
10	Write down the time for T=0 and leave the containers for 24 hours in the fumehood.	
11	Fill the used tick tube with ethanol, place it in a zip bag with ethanol and discard in the acaricide bio-waste container.	
12	Clean the testing island with 1% delladet. Ensure no larvae are left behind.	
13	Discard the delladet from the testing bin with a funnel.	
14	Rinse the funnel and the testing bin with 1% delladet to ensure no larvae are left behind.	
15	Discard waste in a zip bag with ethanol, close it and discard in the acaricide bio-waste container.	
16	Discard the delladet from the acaricide waste bin with a funnel.	
17	Rinse the funnel and waste bin with 1% delladet to ensure no larvae are left behind.	
18	Discard waste in a zip bag with ethanol, close it and discard in the acaricide bio-waste container.	
19	Discard the liquid waste in a type IV halogen-rich organic waste (in solution) jerry can.	
20	Rinse funnel with 1% delladet to ensure no larvae are left behind.	
21	Check the entire fume hood for escaped larvae; catch them with tape.	
22	Clean work space with 70% ethanol.	
23	Discard waste (including escaped larvae) in a zip bag with 70% ethanol in the acaricide bio-waste container.	
24	Close the fume hood and turn off the lights.	

	Reading	Done
1	Prepare a table in a labjournal for easy filling of the data and place the labjournal in the fume hood.	
2	Fill the enthanol squeeze bottle if necessary.	
3	Fill the testing bin and the acaricide waste bin with 1% delladet.	
4	Fill a beaker with water and put the “tick count” brush in it.	
5	Place 4 skewers and a counter in the fume hood.	
6	Write down the time for T=24H.	
7	Count the number of live and dead larvae in the packets with a skewer, brush and counter. Check the filter papers and clips well for any larvae. **Start with the control and work from the lowest to the highest concentration.**	
8	Counted packets can be discarded; clean and dry the island after counting each packet.	
9	Write down the data in the labjounal.	
10	Clean the testing island with 1% delladet. Ensure no larvae are left behind.	
11	Discard the delladet from the testing bin with a funnel.	
12	Rinse the funnel and the testing bin with 1% delladet to ensure no larvae are left behind.	
13	Discard waste in a zip bag with ethanol, close it and discard in the acaricide bio-waste container.	
14	Discard the delladet from the acaricide waste bin with a funnel.	
15	Rinse the funnel and waste bin with 1% delladet to ensure no larvae are left behind.	
16	Discard waste in a zip bag with ethanol, close it and discard in the acaricide bio-waste container.	
17	Discard the liquid waste in a type IV halogen-rich organic waste (in solution) jerry can.	
18	Rinse funnel with 1% delladet to ensure no larvae are left behind.	
19	Clean all used bulldog clips with water and store them.	
20	Check the entire fume hood for escaped larvae; catch them with tape.	
21	Clean work space with 70% ethanol.	
22	Discard waste (including escaped larvae) in a zip bag with 70% ethanol in the acaricide bio-waste container.	
23	Close the fume hood and turn off the lights.	
24	Remove the “acaricide testing in progress” sign from the door and unlock the door.	

25	Process the data in Excel; calculate the mortality, LD50 and make a graph.	
26	Write down the results and conclusion(s) in the labjournal.	

Larval packet test preparation done:

by _____ on _____ _____

Signature

Larval packet test setup done:

by _____ on _____ _____

Signature

Larval packet test reading done:

by _____ on _____ _____

Signature

Comments:

Appendix B: Larval Immersion Test Guideline

THE SHAW LARVAL TEST

As carried out in Dr R.J. Taylor's Laboratory, Pietermaritzburg, South Africa

B. Dipping the larvae

OVERVIEW:

Approximately 100 larvae will be transferred with a soft brush to a filter paper placed upon a disposable aluminium plate. 5 mL of clean water or diluted acaricide (starting with the lowest concentration) is poured onto the ticks on the filter paper; and then a second filter paper is placed on top of the first filter paper containing the ticks; and the remaining 5 mL of water or acaricide is poured onto the filter paper sandwich; which is then set aside for exactly ten minutes.

PROCEDURE:

1. Place a few sheets of paper towel on a tray. This is to soak up any drops of water or liquid which may fall.
2. On this, place an aluminium foil plate and in the plate place 1 sheet of 12,5 cam filter paper.
3. Place the weakest dip concentration on the stirrer and switch it on.
4. Draw up 10 mL of the dipwash with the syringe and lay it on the side of the tray. Place the second concentration on the stirrer.
5. Pick up the flask of ticks from the petri dish, dry the bottom on the paper towel, and, using the forceps, remove the cotton wool plug from the flask and place it on the filter paper in the plate.
6. Take a no. 5 brush and push some ticks from the neck of the flask onto the bristles (when picking up ticks with the brush, **always push** the brush forwards into the ticks. When brushing ticks off the bristles, stroke the brush backwards on the filter paper).
7. Place the brushful of ticks on the filter paper and push the plug back into the neck of the flask with the forceps and place the flask back in the petri dish.
8. Rinse the forceps in acetone tube A and lay them back between the bundles of brushes.
9. Stroke about 100 of the larvae onto the filter paper and plunge the head of the brush into acetone tube B. Leave it there for the moment.
10. Pick up the syringe with 10 mL of water in the right hand, and at the same time as you start the stopwatch, start to squirt 5 mL of water in a zig-zag pattern over the ticks on the filter paper.
11. Place another sheet of 12,5 cm paper over the ticks, and squirt another 5 mL on the top of the "sandwich".
12. Lift the aluminium foil plate and place it on the counter to your right.
13. Repeat movements 2, 5, 6, 7, 8 and 9.
14. Pick up the syringe and when the stopwatch reaches exactly 60 seconds, squirt 5 mL of the dipwash from the pipette onto the ticks.
15. Repeat movement 11.
16. Lift the aluminium foil plate and place it next to the one on your right.

17. Fill the pipette with dipwash from the bowl on the stirrer and place the pipette on the side of the tray once more.
18. Take the aluminium foil dish off the stirrer and place the next one there.
19. Rinse the used brush from tube B in tube A then set it aside.
20. Movements 2, 5, 6, 7, 8, 9, 11-19 may be repeated until all the concentrations have been used.
21. Stop the stirrer.

C. Packeting of larvae

OVERVIEW

Following the dipping of the larvae, the filter paper "sandwich" is opened and the papers placed on dry paper towel to absorb excess moisture. The larvae are then transferred, with a clean brush, into each of two replicates of a dry, pre-folded, conical filter paper envelope which are then sealed off with a paper crimper. The dilution is marked onto each of the two envelopes as well as details: date of test, isolate number and acaricide concentration.

PROCEDURE:

1. After all the concentrations have been used, wash the pipette with water, acetone and water again and place it out of the way, ready to use in the next test.
2. Wash all the used no. 5 brushes in acetone and dry them. Make sure that all the larvae have been removed by the washing. The easiest way to clean them is to place them in a glass beaker, heads down. Squirt acetone over the bristles and shake the brushes in the acetone. Fold a sheet of paper towel in half, and holding the brushes at right angles to the paper, rub the bristles over the towel until no more larvae fall out.
3. Place the brushes back in place ready for use again.
4. Using the paper towel, wipe down the tray, squashing any stray larvae and mopping up drops of liquid. Throw the paper away.
5. Take the ticks out of the petri dish and wipe any stray larvae from the flask and plug. They can now be taken back to the incubator. Remove the petri dish and paper towel.
6. Place the filter paper envelopes on top of the pile of 12,5 cm filter papers.
7. Place two fresh sheets of paper towel on the tray and bring the first aluminium plate i.e. the one which was dipped first, onto the tray next to the paper.
8. Pick up the forceps.
9. After exactly 10 minutes, as the second hand reaches 60 seconds, pick up the filter paper "sandwich" with the forceps and place it on one section of the paper towel.
10. Throw the aluminium foil plate away.
11. Open the "sandwich" with the forceps and place each half, tick side up, on a dry portion of paper.
12. Press the papers down gently with the tip of the forceps to dry them.
13. Rinse the forceps in acetone tube A.
14. Pick up the first filter paper envelope and open it. Holding it open with your left hand, pick up a no. 5 paintbrush (N.B. this is the water control, so use an uncontaminated brush) and push the brush through the larvae.

15.Stroke the larvae as close to the center of the open envelope as possible.
16.Put down the envelope and do the same with the replicate envelope.
17.Place the paintbrush in the acetone tube A (contaminated brushes are placed in tube C).
18.Fold up the envelope you are holding, place it with the open edges next to the cog of the crimper.
19.Turn the crimper handle and allow the open edges to run between the cogs, sealing them.
20.Label each packet with the date, species, strain number and active ingredient.
21.Sealed packets containing the larvae are placed in a vertical position on a metal rack.
22.Fold in all the edges of the paper towels, use the bundle to mop the tray, then throw the papers away.
23.Rinse the brush in tube A.
24.Place two fresh sheets of paper towel on the tray, together with the next aluminium foil plate.
25.Wait until the stopwatch reaches 60 seconds once more and repeat steps 9-22, this time using a no. 6 (contaminated) brush to pick up the ticks.
26.Once all concentrations have been done, the stopwatch is stopped.
27.Wash the brushes first in tube C, then in tube A.
28.Place the metal rack containing the sealed envelopes in the incubator.
29.Spray the waste bucket with acetone to kill the ticks.