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A new *in vitro* test to evaluate the resistance level against acaricides of the cattle tick, *Rhipicephalus* (*Boophilus*) *microplus*

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ABSTRACT

In this article we present a new bioassay to assess the resistance status of ticks to acaricides. The Larval Tarsal Test (LTT) is a sensitive, highly time-effective *in vitro* test. It allows the investigation of a large number of compounds and doses on the cattle tick *Rhipicephalus* (*Boophilus*) *microplus* in a short period of time. The ability of the LTT to assess the lethal concentration at 50% mortality (LC_{50}) and resistance ratios (RRs) of a susceptible and a resistant *R. microplus* strain was compared with the FAO-recommended Larval Packet Test (LPT). Representative compounds of the carbamate, organophosphate (OP), synthetic pyrethroid (SP), formamidine (FOR), macrocyclic lactone and pyrazole classes were used for this comparison. The resistance status against OP, SP and FOR of the resistant *R. microplus* strain was confirmed *in vivo*.

The LTT resulted in resistance ratios comparable to those obtained with the LPT. However, the lethal concentrations were up to 150-fold lower in the LTT than in the LPT. The advantage of the LTT is to simplify the methodology by avoiding the handling of larvae and using multi-well plates. The LTT is therefore a suitable test for the assessment of the level of resistance of *R. microplus* and is very promising to evaluate the resistance profile of field strains. Additionally, the LTT is also suitable to test other ixodid species.

1. Introduction

Rhipicephalus (*Boophilus*) *microplus* is an important cattle tick widely distributed in most of the countries with tropical and subtropical climate (Estrada-Pena et al., 2006; Cutulle et al., 2009). The widespread use of acaricides has led to drug and multidrug resistance and resistance has been reported against nearly all commercially available acaricides (Martins and Furlong, 2001; Li et al., 2005; Alonso-diaz et al., 2006; Castro-Janer et al., 2010). Monitoring of ticks is crucial to diagnose resistance at an early stage, to help slow down the spread of resistance and to obtain knowledge of the distribution of acaricide resistance. To do so, the Food and Agriculture Organisation (FAO) currently recommends and provides standardised protocols for two bioassays to evaluate tick resistance (2004), the Larval Packet Test (LPT), originally described by Stone and Haydock (1962), and the Adult Immersion Test (AIT), originally developed by Drummond et al. (1973). In 2004, White et al. developed an additional test, the Larval Immersion Microassay (LIM). Standardised methods are needed to assess resistance evolution and allow the comparison of resistance data between laboratories. As highlighted in the guidelines of the FAO, a suitable laboratory test for acaricide resistance needs to satisfy several requirements. Ideally, the test should be sensitive enough to identify resistance early in its emergence, cover the full range of chemical groups in use, be simple, inexpensive and provide a rapid

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Table 1
Acaricides used in the bioassays.

Class	Chemicals	[AI][a] (%)	Provider	Location (City, Country)
Technical grade compounds				
OP	Coumaphos	>90	Novartis	Basel, Switzerland
	Diazinon	>90	Novartis	Basel, Switzerland
SP	Flumethrin	97.0	Sigma–Aldrich, Riedel-de Haën	Buchs, Switzerland
	Cypermethin	>90	Novartis	Basel, Switzerland
ML	Moxidectin	>90	Novartis	Basel, Switzerland
	Ivermectin	∼95	Sigma–Aldrich	Switzerland
PYZ	Pyriprol	>90	Novartis	Basel, Switzerland
	Fipronil	>90	Novartis	Basel, Switzerland
CAR	Carbaryl	99.8	Sigma–Aldrich, Supelco	Switzerland
FOR	Amitraz	99.4	Sigma–Aldrich, Fluka	Seelze, Germany
Formulated compounds				
OP	Phoxim, Sebacil®	50	Provet SA	Lyssach, Switzerland
SP	Flumethrin, Bayticol®	1	Provet SA	Lyssach, Switzerland
FOR	Amitraz, Taktic®	12.5	Intervet, Veterinaria AG	Zürich, Switzerland

[a] Active ingredient.

and reliable result. Additionally, it should require a low number of ticks and small amounts of compounds.

Adult tests such as the AIT have the advantage to provide results within seven days after tick collection for all the compounds except growth regulators, while larval tests need 5–6 weeks to complete. However, adult tests require high numbers of engorged females, which may become a limiting factor when resistance to several compounds is evaluated or when the objective is to obtain the full dose–response mortality curve. Larval tests offer the advantage to require limited number of engorged females and are therefore very suitable for the monitoring of resistance. However, the LPT is a laborious and time-consuming test. The LIM reduces the amount of work in comparison with the LPT, enabling more samples to be handled, but an even more simplified method would be desirable. Therefore, a new Larval Tarsal Test (LTT) was developed. By avoiding the tedious handling of larvae and using multiwell plates, the LTT is a time-effective test which allows testing of a large number of compounds and doses in a single test and which could be used to evaluate resistance of field strains. In this paper the LTT capacity to provide a dose–response mortality curve and to assess the lethal concentration at 50% mortality (LC_{50}) of a resistant and a susceptible strain of *R. microplus* and of a *R. sanguineus* strain is evaluated. Representative compounds of the carbamate (CAR), organophosphate (OP), synthetic pyrethroid (SP), formamidine (FOR), macrocyclic lactone (ML) and pyrazole (PYZ) classes were used. *R. sanguineus* ticks were included to investigate whether this bioassay technique would also be suitable to test other ixodid species. Additionally, the LTT was compared with the FAO recommended LPT. The capacity to determine resistance ratios (RRs) of both tests was evaluated. The characteristics of the new test are discussed.

2. Materials and methods

2.1. Acaricides

Technical grade coumaphos, diazinon, flumethrin, cypermethrin, moxidectin, ivermectin, pyriprol, fipronil and carbaryl were used for the LTT and LPT (Table 1). Technical grade amitraz was used for the LTT while formulated amitraz was used for the LPT and the *in vivo* characterisation. Formulated flumethrin and formulated phoxim were used for the *in vivo* characterisation only (Table 1).

2.2. Ticks

The *R. microplus* Ultimo strain was originally collected in 1992 in central Queensland, Australia from ticks resistant to all SPs and to amitraz (Kunz and Kemp, 1994) and maintained at CSIRO, Australia. A colony was established in the Novartis Animal Health Research Center (CRA), St-Aubin, Switzerland in 1999 and was maintained without acaricide selection. Ticks used for the *in vitro* bioassays were from F31 and F32 generations.

The *R. microplus* Muñoz strain was collected from Zapata County in Texas, USA in 1999. It is susceptible to SP, OP and FOR. A colony was established in the Cattle Fever Tick Research Laboratory (CFTRL), Edinburg, Texas, and was reared without acaricide selection. In February 2010, some larvae from the F48 generation were transferred and established in the CRA. Ticks used for bioassays were from F49 and F50 generations.

The Corapeake strain, *R. sanguineus* was collected from Corapeake in North Carolina, USA, in 2005 and was established the same year in the CRA where it was maintained without acaricide selection. This strain is considered to be susceptible to all classes of acaricides. Ticks used for bioassays were from the F6 generation.

2.3. Larval Tarsal Test (LTT)

Stock solutions of acaricides were prepared by dissolving each test compound in dimethyl sulfoxide (DMSO; Fluka) to a concentration of 20,000 parts per million (ppm). Twofold dilutions were prepared in DMSO to test 12 concentrations ranging from 566 to 0.28 ppm. A coating solution was prepared with 100% ethanol (Sigma–Aldrich, Fluka) and olive oil (Sigma–Aldrich, Fluka) (400:1). For a standard bioassay, 20 μl of ethanol:olive oil were dispensed into each well of a flat bottom 96-well plate (NUNC,

Catalogue No. 260836, Denmark) and ethanol was allowed to evaporate for at least 6 h under a fume hood. According to our experience an evaporation period of up to 72 h did not negatively impact the outcome of the test. A volume of 5 μl of each acaricide dilution was dispensed in the bottom of the test wells, to obtain concentrations of 100–0.05 mg/m^2. In addition, 5 μl of DMSO was used as a negative control in all plates. Three replicates were prepared on separate plates. One additional plate with only DMSO was also prepared as a control plate. Plates were placed for 1 h in an N_2 sample concentrator (Techne DB-3 Dri-Block, Witec AG, Switzerland) for complete DMSO evaporation.

Plates were used for testing within three days after preparation. Fifty eggs were distributed in each well using a seed counter (elmor, Switzerland) 14–21 days after engorged females' collection. Plates were placed uncovered in an environmental chamber with ~95% relative humidity (RH) and 28 ± 1 °C. One to three days after the start of incubation, the plates were covered with a transparent sealing film (Catalogue No. 676070, VIEWseal, Greiner bio-one, Switzerland) and static electricity was removed with a discharging system (Static Line LC, HAUG Biel AG, Switzerland). Plates were incubated in an environmental chamber with 70–80% RH and 28 ± 1 °C.

Plates were removed from the environmental chamber 2 weeks after egg hatching and the larval mortality was determined by counting dead or live larvae in each well using a dissecting microscope with a magnification of 12×. Larval mortality assessment was based on the observation of the motility and general appearance. The heat of the hands was used to activate larvae.

Each test was repeated three times using ticks from different passages. Additional tests with higher or lower doses were performed to obtain mortality ranging from 0 to 100%.

2.4. FAO-Larval Packet Test (LPT)

The LPT was conducted as described previously (FAO, 2004) with a modification to facilitate the handling of tick larvae. Technical acaricide was dissolved in two parts of trichloroethylene (Sigma–Aldrich) and one part of olive oil (Sigma–Aldrich, Fluka). This formulation was subsequently diluted in trichloroethylene:olive oil performing 4-fold serial dilutions ranging from 53 to 0.05 mg/m^2. Each serial dilution had a negative control (diluent only) and each dose had three replicates. A volume of 0.7 ml of each dilution was applied to a 7.5–10 cm filter paper (Whatman No. 1, Whatman, Madstone, United Kingdom) and trichloroethylene was allowed to evaporate under a fume hood for 2 h. Treated papers were then folded in half and sides sealed with bulldog clips (Catalogue Nos. 36031and 36032, rapesco, Sevenaoks, England) forming an open-ended packet. To facilitate the introduction of tick larvae into the packets, around 120 eggs were beforehand distributed into tubes (Catalogue No. STBR96-300, REMP, Switzerland) using a seed counter. After one day incubation in an environmental chamber with ~95% RH and 28 ± 1 °C, the tubes were capped and kept at 70–80% RH and 28 ± 1 °C until larvae hatched and reached the required age (7–21 days old). The content of one tube with around one hundred larvae was then inserted with a paintbrush into each packet, which was then sealed with a third bulldog clip. Packets were incubated at 70–80% RH and 28 ± 1 °C for 24 h and then the number of dead and live larvae recorded. Larvae that moved their legs but did not walk were counted as if dead.

For amitraz, the FAO-LPT protocol modified by Miller et al. (2002) was followed. It contains two changes in comparison with the FAO standard protocol: formulated instead of technical amitraz was diluted in trichloroethylene:olive oil as in the FAO protocol, and finally was applied to a piece of nylon fabric (type 2320, Cerex Advanced Fabrics, Pensacola, FL, USA) instead of filter paper.

Additional tests with higher or lower doses were performed to obtain mortality ranging from 0 to 100%. Tests with over 10% mortality in the controls were rejected and repeated.

2.5. In vivo characterisation

Eight tick-naïve bull calves (Red Holstein × Simmental) were allocated to four groups and were housed under controlled climatic conditions. All calves were infested with about 5000 Ultimo *R. microplus* larvae in the anterior region of the back on trial days −18 and −11. On trial day 0, three groups of two animals were treated with phoxim (OP), flumethrin (SP) and amitraz (FOR), respectively according to the manufacturer guidelines, while the control group did not receive any treatment.

In all experimental groups, the engorged female ticks dropping off the hosts were collected and their number was recorded daily for each bull calf over a four-day period starting one day after treatment and over a five-day period starting seven days after treatment in order to quantify ticks from first and second infestations respectively. On each day of tick collection, a sample of 10 engorged female ticks (or less if fewer ticks dropped off) from every host animal were glued onto adhesive tape, with the ventral side facing up, and incubated at ~80% RH and 28 ± 1 °C. Oviposition was evaluated three weeks after the drop-off.

Efficacy was assessed based on the number of viable engorged ticks collected after drop-off. Reduced numbers of ticks dropping off the hosts in comparison with untreated controls was used as an indicator for efficacy. Viability of the collected ticks was based on the oviposition rate. If no oviposition was observed, the ticks were assumed to be dead. Efficacy was calculated using the following Abbott formula (Abbott, 1987):

$$\text{Efficacy}\,(\%) = \frac{\text{DropOff}\,(\text{C}) \times \text{OvipositionRate}\,(\text{C}) - \text{DropOff}(\text{T}) \times \text{OvipositionRate}(\text{T})}{\text{DropOff}\,(\text{C}) \times \text{OvipositionRate}\,(\text{C})} \times 100$$

where "DropOff" is the mean number of ticks collected from control- (C) and treatment-group (T) and "OvipositionRate" is the mean rate of ticks laying eggs in control- (C) and treatment-group (T) (value between 0 and 1).

2.6. Statistical analysis

Data were entered in Excel software (Microsoft Office 2003) and transferred to Intercooled STATA release 11.0 (StataCorp, College Station, TX, USA) for data cleaning. All mortality values were normalized for control mortality applying Abbott's formula (Abbott, 1987). Deviating values from wells located at the borders of the LTT plate were excluded from calculation. Nonlinear regression analyses of dose-mortality data was performed on the R software (version 2.9.0) using the drc package (version 1.7–2), specific for modelling dose–response curves (Ritz and Streibig, 2005). A five-parameter log-logistic function with bottom and top values locked at 0 and 100 respectively was used to model the data using the drm command. Then LC_{50} values, LC_{99} values and their 95% confidence intervals (CI) were estimated using the ED command and the delta option for the interval parameter. Difference between LC_{50} estimates was designed as significant if their 95% CI did not overlap. Resistance ratios of the *R. microplus* Ultimo strain were calculated relative to the reference susceptible *R. microplus* strain Muñoz (LC_{50} Ultimo/LC_{50} Muñoz). Potential discriminating doses (DDs) for the LTT and the LPT were computed as 2× LC_{99} of the susceptible Muñoz strain (Jonsson et al., 2007). Percentage of the population of the Ultimo strain surviving to DDs were computed (PR command, R software).

3. Results

3.1. Assessment of the dose–response curves for R. microplus

The LTT and LPT dose–response curves for the susceptible, Muñoz, and the resistant, Ultimo, *R. microplus* strains are shown in Fig. 1. The LTT produced results covering the whole range of mortality from 0 to 100% for both susceptible and resistant *R. microplus* strains for all compounds. Using the LPT, the complete dose–response range from 0 to 100% was obtained for both *R. microplus* strains for all compounds except for coumaphos tested on the resistant Ultimo strain. For coumaphos 67% mortality was obtained at 3381 mg/m^2 (36,224 ppm), the highest dose tested (Fig. 1). For the susceptible Muñoz strain, testing doses from 0.0015 to 100 mg/m^2 with the LTT provided a complete dose–response curve for all the compounds while the interval to obtain similar results with the LPT ranged from 0.05 to 845 mg/m^2. Dose–response mortality data obtained with the LTT showed very low dispersion for SP and PYZ, while the highest variability was observed with ivermectin and amitraz.

3.2. Assessment of the LC_{50} values and resistance ratios for R. microplus

The LC_{50} values of the *R. microplus* strains and their 95%CI obtained through nonlinear regression analyses of dose-mortality data are displayed in Table 2 with the RRs of the Ultimo strain in comparison with the reference susceptible Muñoz strain. When evaluated with the LTT, RRs were less than 2 for ML and PYZ, approximately 10 for OP and CAR, approximately 20 for amitraz, and greater than 100 for SP. When evaluated with the LPT, these RRs remained in the same range than the ones estimated with the LTT, except the RR of coumaphos which was 20-fold higher when estimated using the LPT than with the LTT.

Analyses revealed that the LC_{50} was reached at lower doses with the LTT than with the LPT for all compounds except carbaryl and amitraz. For SP, ML and PYZ, the concentrations (mg/m^2) required to determine the LC_{50} values of the Ultimo strain were 25–75-fold lower with the LTT. For the Muñoz strain these factors ranged between 20 and 150 (Table 2).

3.3. Use of discriminating doses

Table 3 summarises the potential DDs for the LTT and the LPT obtained by computing 2× LC_{99} of the susceptible Muñoz strain and the survival rates of the Ultimo strain at these DDs. These DDs are represented by vertical lines on the graphs of Fig. 1. The FAO-DDs for the LPT are represented by vertical lines in Fig. 2 for OP and SP. The survival rates of the Ultimo strain at these DDs are also included in Table 3.

Survival rates of the Ultimo larvae at the LTT-DDs and the LPT-DDs of OP and PYR ranged from 49 to 100% and from 78 to 100%, respectively. Survival rates were below 4% for ML and PYR, except for fipronil when measured with the LPT (11%). Eighteen percent of the Ultimo population survived at the LTT-DD of carbaryl, while it was only 2% with the LPT. Inversely, only 5% of the Ultimo population survived at the LTT-DD of amitraz, while 86% survived at the LPT-DD.

All Ultimo larvae survived at the FAO-LPT-DDs after exposure to SP and coumaphos, while 91% and 54% survived at the DDs for diazinon of 0.1 and 0.2 AI%, respectively. In contrast, no Muñoz larvae survived at the DDs of any of the compounds.

3.4. Evaluation of R. sanguineus

The LTT results of the susceptible *R. sanguineus* Corapeake strain are shown in Fig. 3 and LC_{50} values and their 95%CI are summarised in Table 4. LC_{50} values, ranging between 0.029 and 10.62 mg/m^2, were in the same range as those of the *R. microplus* susceptible Muñoz strain. Although LC_{50} values of *R. sanguineus* were significantly higher for diazinon, flumethrin, ML and PYZ, it never exceeded a factor of 7.2. The highest factors were observed for ML and pyriprol.

3.5. In vivo efficacy trials

Detailed results of the *in vivo* efficacy trials are available in Table 5, *In vivo* trials on *R. microplus* Ultimo showed an efficacy of phoxim (OP) of 25.7% against adult female ticks and 38.1% against nymphal stages. These efficacy rates were 12.3% and 70.4%, respectively, with flumethrin (SP) and 87.9% and 82.3% with amitraz (FOR).

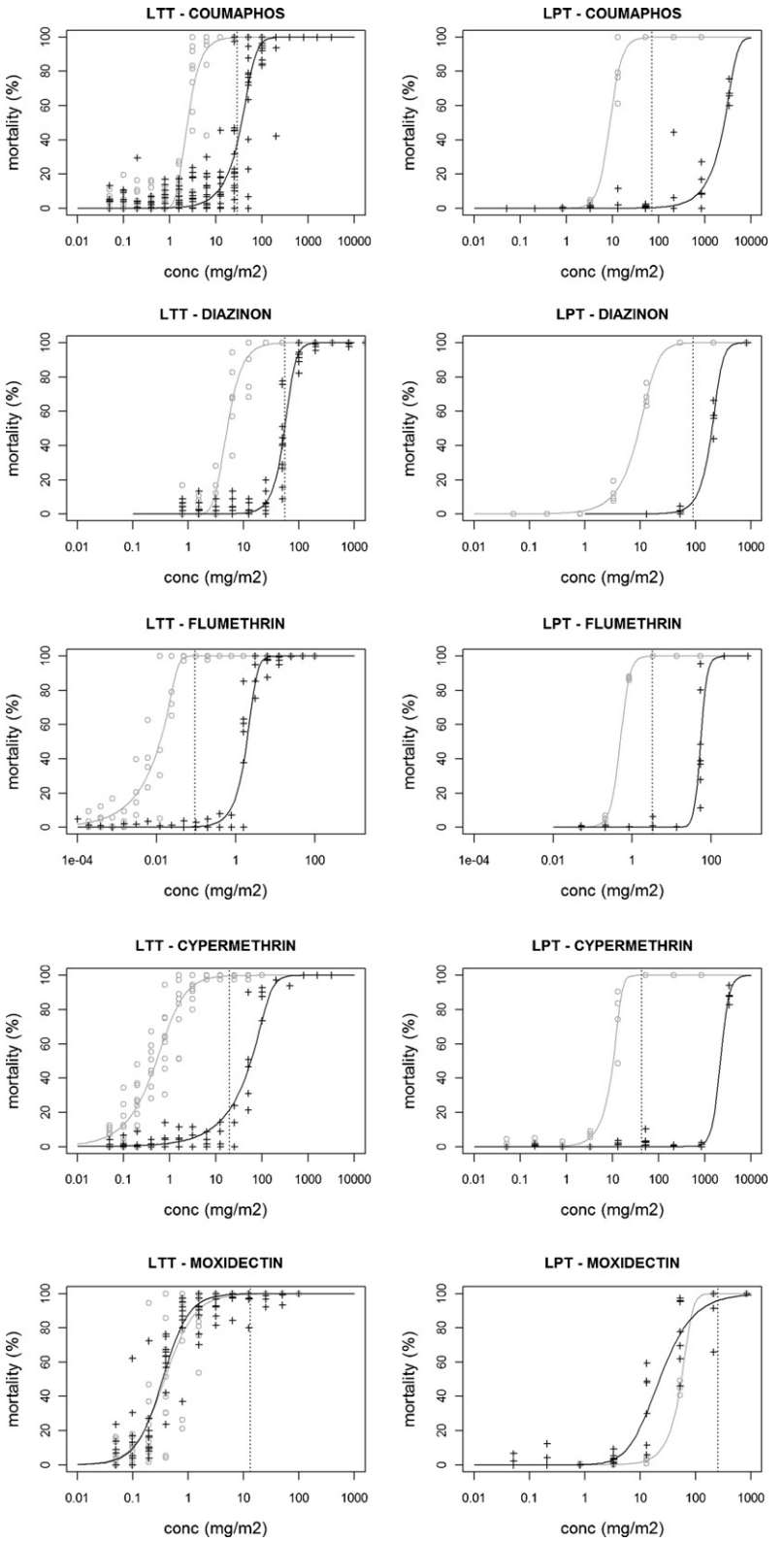

Fig. 1. Dose–response mortality for the susceptible Muñoz (○) and the resistant Ultimo (+) *R. microplus* strains obtained with the LTT (left side) and the LPT (right side). Data were analysed using a nonlinear regression model for all compounds. Dashed lines indicate the concentrations corresponding to 2× LC_{99} of the susceptible Muñoz strain when tested with the LTT and LPT respectively.

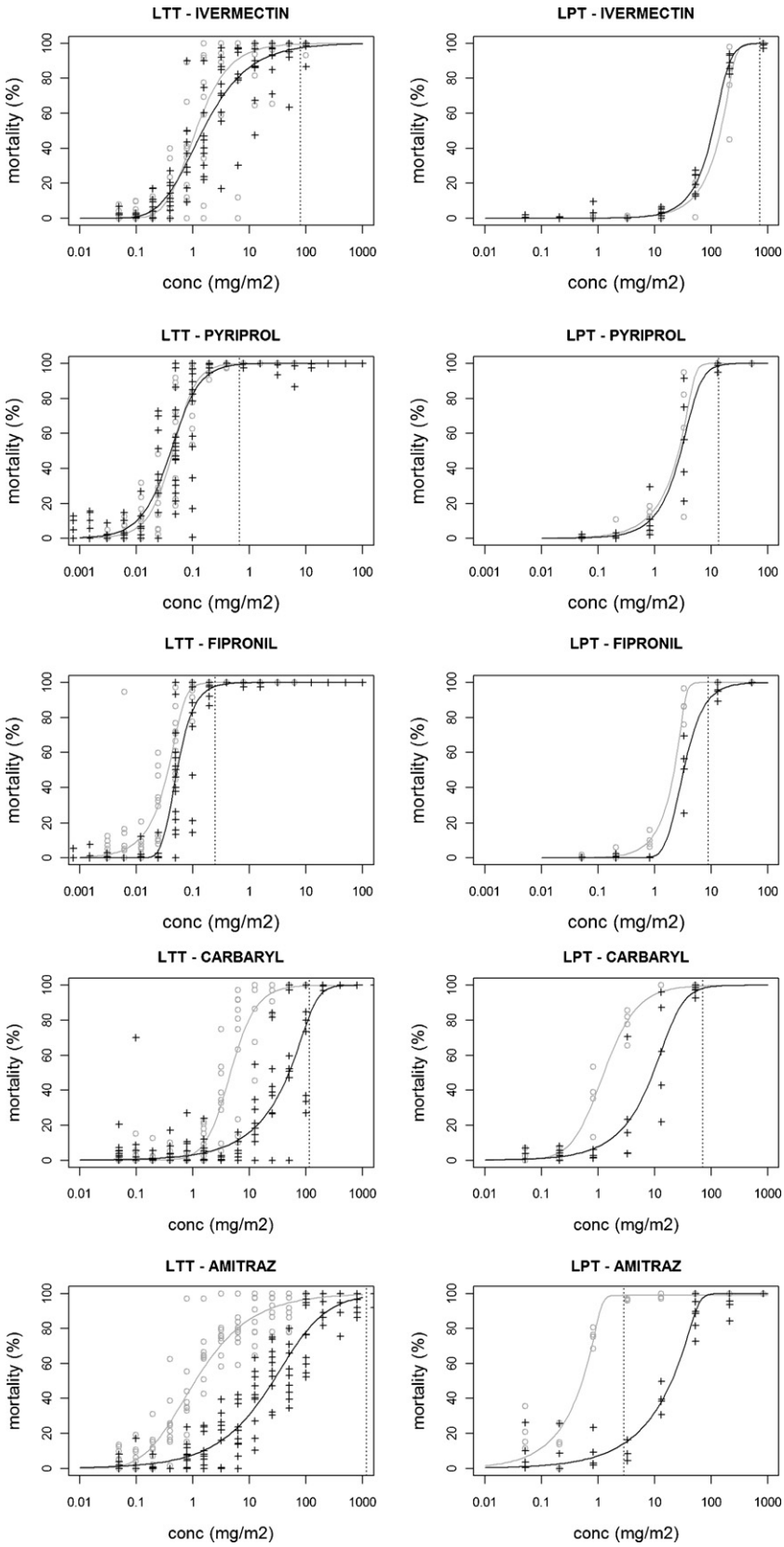

Fig. 1. (*Continued*).

Table 2
Comparison of the LTT and LPT results for the *R. microplus* Muñoz and Ultimo strains using 10 test compounds.

Class	Acaricide	Test	Tick strain	*n*	LC50[a]	(95% CI)	RR
OP	Coumaphos	LTT	Rm Muñoz	5400	2.39	(2.15–2.63)	15.0
			Rm Ultimo	10,750	35.77	(31.34–40.19)	
		LPT	Rm Muñoz	1617	8.79	na[b]	302.4
			Rm Ultimo	5034	2658	(2167–3148)	
	Diazinon	LTT	Rm Muñoz	3600	5.03	(4.59–5.47)	10.9
			Rm Ultimo	5400	54.92	(51.56–58.28)	
		LPT	Rm Muñoz	1695	9.04	(8.17–9.91)	22.1
			Rm Ultimo	1217	199.4	(137.4–261.4)	
SP	Flumethrin	LTT	Rm Muñoz	5400	0.012	(0.010–0.014)	157.8
			Rm Ultimo	11,750	1.91	(1.67–2.14)	
		LPT	Rm Muñoz	1479	0.50	(0.18–0.82)	106.6
			Rm Ultimo	2799	53.38	(44.34–62.42)	
	Cypermethrin	LTT	Rm Muñoz	5400	0.51	(0.42–0.59)	113.1
			Rm Ultimo	13,200	57.44	(49.75–65.14)	
		LPT	Rm Muñoz	1550	10.38	(0.06–20.71)	211.9
			Rm Ultimo	3325	2200	na	
ML	Moxidectin	LTT	Rm Muñoz	5400	0.37	(0.29–0.45)	0.9
			Rm Ultimo	5400	0.34	(0.30–0.38)	
		LPT	Rm Muñoz	1637	55.93	(52.35–59.51)	0.4
			Rm Ultimo	3203	22.52	(14.94–30.09)	
	Ivermectin	LTT	Rm Muñoz	5300	1.12	(0.84–1.40)	1.3
			Rm Ultimo	5400	1.47	(1.16–1.78)	
		LPT	Rm Muñoz	1007	147.63	(50.75–244.51)	0.7
			Rm Ultimo	3254	107.2	(73.0–141.4)	
PYZ	Pyriprol	LTT	Rm Muñoz	5350	0.044	(0.039–0.049)	0.9
			Rm Ultimo	10,750	0.040	(0.034–0.047)	
		LPT	Rm Muñoz	1066	2.67	(1.40–3.94)	1.1
			Rm Ultimo	2529	2.93	(2.12–3.74)	
	Fipronil	LTT	Rm Muñoz	5400	0.036	(0.032–0.040)	1.4
			Rm Ultimo	9000	0.052	(0.048–0.056)	
		LPT	Rm Muñoz	1532	2.27	(1.60–2.95)	1.4
			Rm Ultimo	1725	3.27	(2.72–3.83)	
CAR	Carbaryl	LTT	Rm Muñoz	5400	4.60	(3.80–5.39)	10.8
			Rm Ultimo	7600	49.82	(30.26–69.38)	
		LPT	Rm Muñoz	1316	1.27	(0.85–1.69)	7.5
			Rm Ultimo	2037	9.45	(4.12–14.78)	
FOR	Amitraz	LTT	Rm Muñoz	5400	1.09	(0.89–1.29)	24.0
			Rm Ultimo	7500	26.13	(20.02–32.25)	
		LPT	Rm Muñoz	1876	0.53	(0.42–0.64)	38.1
			Rm Ultimo	2396	20.05	(11.26–28.85)	

[a] mg/m^2.
[b] na, not available.

Table 3
LTT-, LPT- and FAO LPT-discriminating doses and the corresponding survival rates of the Ultimo strain.

Class	Acaricide	LTT		LPT		FAO-LPT[b]	
		DD[a] (mg/m^2)	% Survival	DD[a] (mg/m^2)	% Survival	DD (AI%)	% Survival
OP	Coumaphos	29.1	61.5	71.7	99.6	0.1 and 0.2	100 and 100
	Diazinon	55.5	49.0	174.8	62.2	0.1 and 0.2	91 and 54
SP	Flumethrin	0.09	99.7	3.2	100	0.0036 and 0.01	100 and 100
	Cypermethrin	19.6	78.3	43.4	100	0.2 and 0.5	100 and 100
ML	Moxidectin	13.2	0.1	257.8	3.9		
	Ivermectin	79.2	2.6	732.3	0.1		
PYZ	Pyriprol	0.66	0.6	13.8	1.2		
	Fipronil	0.25	2.1	8.3	11.4		
CAR	Carbaryl	113.9	17.6	71.4	1.7		
FOR	Amitraz	1183.4	5.2	2.7	86.2		

[a] DD = 2× LC_{99} of the susceptible Muñoz strain.
[b] According to FAO (2004).

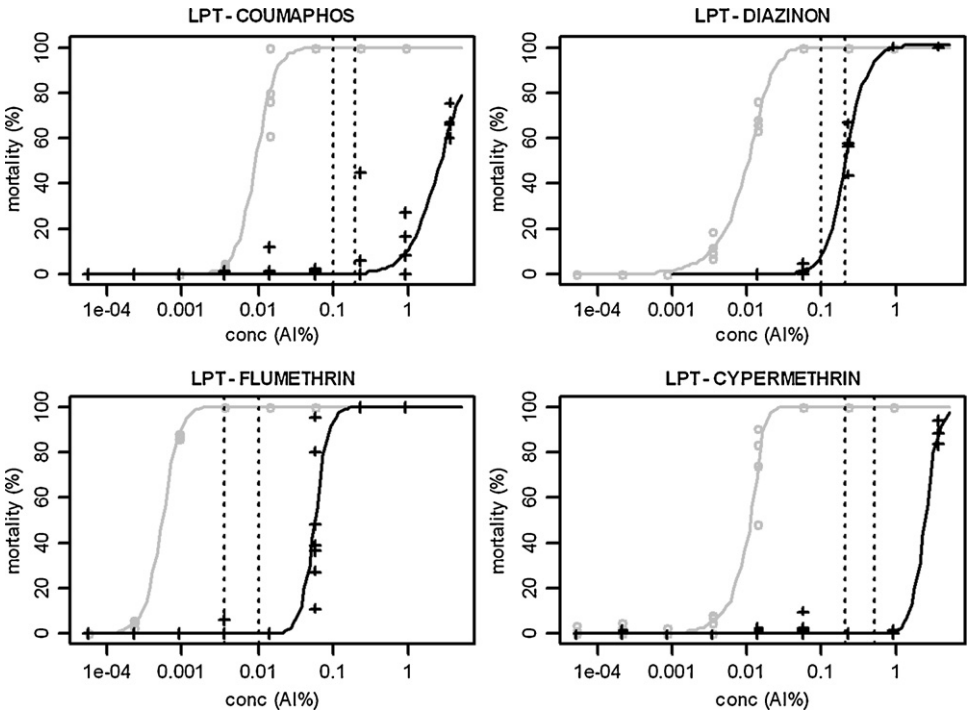

Fig. 2. Dose–response mortality obtained with the LPT after exposure of the susceptible Muñoz (○) and the resistant Ultimo (+) *R. microplus* strains to coumaphos, diazinon, flumethrin and cypermethrin. Dashed lines indicate the DDs recommended by the FAO.

Table 4
Results of the Larval Tarsal Test (LTT) for the susceptible Corapeake *R. sanguineus* strain.

Class	Acaricide	*n*	LC50[a]	(95% CI)
OP	Coumaphos	5400	2.04	(1.67–2.41)
	Diazinon	5400	10.62	(10.1–11.14)
SP	Flumethrin	8900	0.029	(0.027–0.031)
	Cypermethrin	5400	0.64	(0.44–0.84)
ML	Moxidectin	5400	2.43	(1.85–3.00)
	Ivermectin	5350	8.08	(5.39–10.77)
PYZ	Pyriprol	5400	0.25	(0.23–0.28)
	Fipronil	8900	0.114	(0.103–0.126)
CAR	Carbaryl	5400	5.64	(4.69–6.59)
FOR	Amitraz	5300	1.64	(0.69–2.58)

[a] mg/m^2.

4. Discussion

Three bioassays are widely used to identify and quantify *R. microplus* resistance against the most important acaricide classes. In 2004, the FAO recommended standard protocols of the AIT and LPT, including a modified version of the LPT for amitraz. The same year, White et al. (2004) developed the LIM. Here we present a new Larval Tarsal Test, the LTT, which is highly sensitive, allows testing of several compounds at the same time and is easy and quick to perform. We compared the LTT with the FAO-recommended larval test, LPT.

With both LTT and LPT, the complete dose–response mortality curve was obtained for all compounds, with the exception of coumaphos when tested with the LPT on the OP-resistant strain. The range of concentrations required to kill 0–100% of the population with the LTT covered 4–8 dilutions for most of the compounds, corresponding to a 8–128-fold concentration range for both susceptible and resistant strains. These ranges are comparable with those reported previously with other larval tests (Roulston et al., 1981; Miller et al., 2002; White et al., 2004). However, the ranges were slightly wider for cypermethrin and ivermectin and the one for amitraz was over 2000-fold, reflecting the flat slope of the dose–response curve for amitraz, which may prevent a good screening method for amitraz resistance in the field (Jonsson and Hope, 2007).

The LTT provided 2–150-fold lower LC_{50} values than the LPT for OP, SP, ML and PYZ using the *R. microplus* strains. In contrast LC_{50} values were lower using the LPT with carbaryl and amitraz. Several factors are likely to decrease the

Table 5
Average number of ticks and *in vivo* calculated efficacy (%) against adult and nymph stages of *R. microplus* Utlimo.

	Ctrl	OP		SP		FOR	
	Ticks	Ticks	Efficacy (%)	Ticks	Efficacy (%)	Ticks	Efficacy (%)
Adults	552.2	410.2	25.7	484.5	12.3	66.9	87.9
Nymphs	239.6	148.2	38.1	70.9	70.4	42.4	82.3

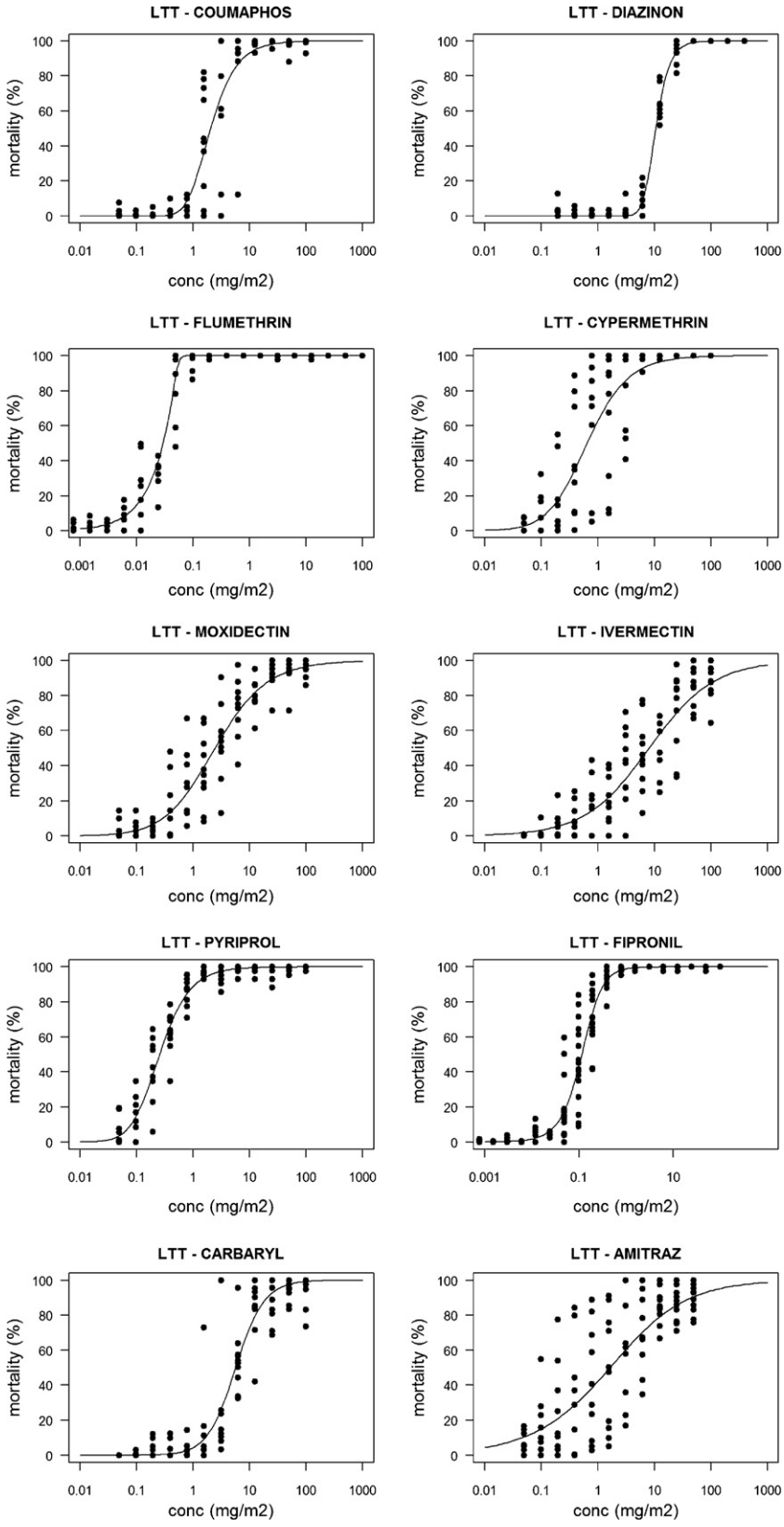

Fig. 3. Dose–response mortality obtained with the LTT for the susceptible Corapeake *R. sanguineus* strain. Data were analysed using a nonlinear regression model.

LC_{50} values of the LTT relative to the LPT. First, the incubation time in the LTT (2 weeks) is much longer than in the LPT (24 h). Second, larvae are exposed to the AI immediately after hatching in the LTT, whereas larvae used in the LPT are at least 7 days old. Finally, with the LPT, only a reduced proportion of the AI is in contact with the larvae since the compound is absorbed in the filter paper. With the LTT in contrast, the AI remains at the surface of the bottom of the wells. In contrast, the reduced area of the treated surface in the LTT could have an opposite influence on the test because larvae can avoid the treated surface by walking on the walls and on the top of the wells and are therefore not permanently in contact with the AI. It remains unclear why the LC_{50} values for carbaryl and amitraz were higher in the LTT and whether the different mode of action between acaricide classes may play a role.

Both bioassays provided comparable RRs within each class, except for coumaphos, for which the RR obtained by the LPT was 20-fold higher than with the LTT. Additionally, there were important differences between the acaricide classes for both LTT and LPT but these differences were similar for both bioassays.

In vivo characterisation of the Ultimo *R. microplus* strain showed high resistance to OP and SP and low resistance to amitraz. The Ultimo strain was originally characterised as resistant to SP and amitraz (Kunz and Kemp, 1994). This diminished resistance to amitraz supports previous evidence suggesting that resistance to amitraz is not maintained in populations in which selection is not applied (Foil et al., 2004; Jonsson and Hope, 2007). Ultimo is expected to be susceptible to ML since moxidectin (Cydectin® 0.5%, Pour-On) is used for treatment of animals at the end of studies and results in a complete elimination of all stages of the *R. microplus* Ultimo-ticks. The *in vivo* resistance profile of Ultimo against PYZ and CAR is unknown. Both *in vitro* bioassays provided results in agreement with the *in vivo* observations although some differences were observed for amitraz. Resistance ratios observed *in vitro* for amitraz were above 20 whereas *in vivo* resistance was only low. This phenomenon was previously reported by Nolan (1981) and Jonsson and Hope (2007) who observed high levels of resistance to amitraz measured with the LPT contrasting with normal efficacy observed in *in vivo* studies. The *in vitro* tests may therefore allow detecting resistance before it becomes visible in the field. For OP, a RR of 10 in the LTT appears to be an indicator of high *in vivo* OP resistance, although the OP compounds tested *in vivo* and *in vitro* were different. This is also true for the LPT when using diazinon, while the RR with coumaphos was significantly higher. For SP, *in vitro* RRs above 100 in both tests, LPT and LTT, reflected the high *in vivo* resistance. Lower RRs though probably also reflect *in vivo* resistance to SP as demonstrated with the LPT for deltamethrin (Barre et al., 2008) and permethrin (Davey and George, 1998). Here we provide first information on RRs which could be used as threshold values for the LTT and, in our hands, also for the LPT. However, it would be necessary to test additional strains *in vitro* and *in vivo* to determine robust threshold values.

Discriminating doses are valuable to reduce the amount of work needed to determine whether resistance is present. According to the FAO guidelines (2004), the percentage of ticks surviving treatment at the DD can be taken as the percentage resistance to the acaricide. However, DDs must be established with great caution (Jonsson et al., 2007). Here, the $2\times$ LC_{99} of the susceptible Muñoz strain was represented on the LTT and LPT graphs to see whether they could be considered as potential DDs and if interpretation of the results would have been consistent between both tests and with the FAO DDs for LPT. The use of both LTT- and LPT-DDs values would have revealed high resistance of the Ultimo strain to SP (78–100%) moderate or high resistance to OP (49–100%) and no resistance to ML and PYZ. In contrast, survival at the DDs of amitraz highly differed between both tests and would have led to opposite conclusions if the diagnosis was only based on survival rate of larvae at DDs. The very low survival rate obtained with the LTT (5%) is due to the flat slope of the dose–response curve obtained for amitraz with this test. Previous observations with the LPT revealed that a single DD cannot be determined for amitraz and that instead three concentrations should be chosen (FAO, 2004).

The potential LTT- and LPT-DDs presented in this paper would have therefore been suitable to detect resistance to OP and SP, which was confirmed *in vivo*, while the LTT-DD was not suitable to detect the low resistance to amitraz observed *in vivo*.

The use of the FAO-recommended DDs for the LPT would have also been suitable to assess the resistance of the *R. microplus* strains against OP and SP since all Ultimo larvae survived at the FAO LPT-DDs for coumaphos, flumethrin and cypermethrin, and over 50% of the Ultimo larvae survived at the DDs for diazinon. Additionally, the use of these DDs also confirmed the susceptibility of the Muñoz strain to OP and SP since no larvae survived at any of the DDs.

LTT results obtained with *R. sanguineus* showed that the LTT is also a suitable test to evaluate the susceptibility of the brown dog tick and could be applied to assess its resistance. *R. sanguineus* LC_{50} values were in the same range than those of the susceptible *R. microplus* Muñoz strain although some significant differences were observed. It therefore appears that the same intervals of concentrations are suitable for both *R. sanguineus* and *R. microplus* species. Comparison of LC_{50} values from two different species should only be made with great caution. For this reason, *in vivo* characterisation of the *R. sanguineus* strain would help to interpret values obtained with the LTT with more confidence. In addition, further studies should be conducted to assess the potential use of the LTT for other ixodid species.

We often, but not systematically, observed a higher mortality in the wells at the borders of the LTT control plates. To avoid biasing results due to this edge effect, DMSO only was distributed in the wells of the upper and lower rows of the microtiter plates and results were not included in the analyses. Wells of the control plate situated outside the borders were used to calculate the control mortality required in the Abbott's formula to obtain the corrected percent mortality. Abbott's correction of mortality was crucial in the LTT since the control mortality was higher than in the LPT. It typically ranged between 15% and 25% but also rose once up to 40% while this rate was mostly under 7% in the LPT. LTT control mortality was mainly due to non-hatching eggs. In the present study, none of the

LTTs was excluded, since the results were consistent, independently of the mortality in the control plates. This high non-hatching rate in control wells is probably due to the distribution process of eggs into the plates, which increase the eggs susceptibility to variation in the relative humidity (RH) and to desiccation. More recent observations showed that thorough control of RH can result in a considerable decrease of the rate of non-hatching eggs. However, a high variability was observed between the strains.

We decided to express concentrations in this paper in mg/m^2 instead of the usual AI% in order to take into account the differences between the surfaces treated in both tests. The 256-fold smaller surface treated in the LTT, in addition with the capacity of the LTT to detect LC_{50} values at lower doses, resulted in much lower quantities of acaricides required to assess compound activity. As an example, 1.76×10^{-4} mg of technical moxidectin was sufficient to kill 100% of the larvae with the LTT, while 6.34 mg were required in the LPT, corresponding to a factor of over 35,000.

The LTT offers important advantages in the ease of execution compared with the LPT. It overcomes the difficult handling of tick larvae by distributing eggs into the microtiter plates and thus avoiding all direct contacts with larvae. This can be of particular interest when assessing resistance of ticks collected in the field and potential vectors of pathogens. Additionally, the LTT requires less time to run a test despite being a two-step test. First, microtiter plates are prepared with the acaricidal compounds and eggs are distributed 2–3 weeks after females drop-off. Then, around three weeks later, mortality is evaluated. For a test aiming to evaluate 12 doses of 10 compounds, 3 replicates per dose, egg distribution can be done within 60 min once the microtiter plates are prepared with the AI, while mortality can be evaluated within 2 h. Distribution of eggs in one additional plate takes around 5 min while the time for its evaluation is around 20 min. With the LPT, in contrast, loading larvae in packets and evaluating the results of 3 replicates of the same number of compounds with 6 doses instead of 12 requires two days of full time work.

The difficulty to assess mortality in the LTT could be a critical point. Most often, surviving larvae climb up to the transparent sealing film. However, some of these larvae die after having reached the top of the wells, especially when treated with ivermectin. A reduced motility of larvae on the sealing film and difficulty to stimulate them was sometimes observed, even in control wells. Therefore, a reliable complementary criterion to assess mortality in such situation is to see whether the larvae have dried or if they still appear as well hydrated as those of the control wells, reflecting their survival. Combining the observation of the motility of the larvae and their general appearance seemed to be the best way to evaluate survival or mortality.

Both LPT and LTT require waiting 6 weeks after collection of the females before the results are available. This is a weakness of larval tests in comparison with adult tests such as the AIT, which provides information already within one week except for growth regulators which requires 5 weeks. When a rapid estimate of resistance is needed, e.g. to change without delay a tick-control strategy, then an adult test appears to be well-suited. However, adult tests require high numbers of engorged females and therefore, if a complete dose–response curve is required for one or more compounds, a larval test should be preferred in order to avoid being limited by the number of ticks available in the field.

Among larval tests, the LPT offers the strong advantage of being recommended by the FAO, which published a standardized protocol in 2004 providing by this way a valuable tool for comparison of results between laboratories. Unfortunately this test is labour-intensive and time-consuming. Nevertheless, the LPT is well-suited if a single compound is to be tested. The LIM, which is performed in microtiter plates, allows testing higher numbers of compounds than the LPT. The LTT, despite being realized in microtiter plates as the LIM, is based on a very different method which simplifies the procedure by avoiding handling larvae and reliably provides a sensitive evaluation of resistance. To be able to measure LC_{50} values of susceptible and resistant *R. microplus* strains with the LTT, we would recommend testing the following concentration intervals: 0.05–100 mg/m^2 for cypermethrin, moxidectin, ivermectin and amitraz; 0.4–800 mg/m^2 for carbaryl, coumaphos and diazinon; 0.003–6.25 mg/m^2 for flumethrin, fipronil and pyriprol. The same dose intervals would also be suitable for *R. sanguineus.*

The LTT is currently used for assessment of the resistance level of field strains originating from Brazil, Argentina, Australia and South Africa. All the compounds tested in this paper except carbaryl were selected for evaluation of the field strains. Efforts were made to modify the protocol to make it independent of specific laboratory infrastructures. Alternatives to the N_2 sample concentrator used for DMSO evaporation and to the seed counter used for the egg distribution into the microtiter plate wells are being evaluated.

In conclusion, the LTT is a promising bioassay which is suitable to assess resistance levels of *R. microplus* and *R. sanguineus* ticks. Further use of this test with field and laboratory strains will hopefully confirm its robustness.

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