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EVALUATION OF ACARICIDE RESISTANCE IN THE CATTLE TICK, *RHIPICEPHALUS (BOOPHILUS) MICROPLUS*, USING A NEW *IN VITRO* TEST AND MOLECULAR TOOLS

THESE

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par

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Resistance like life is poorly understood and like death is inevitable

M. Mitchell

Table of content

1. RÉSUMÉ

Rhipicephalus (Boophilus) microplus est une tique des bovins à un hôte qui se trouve dans les zones tropicales et subtropicales. Cet ectoparasite a un impact économique très important sur l'élevage bovin dans toutes les régions où il se trouve. Au Brésil, par exemple, les pertes dues à son impact direct et indirect ont été estimées à 2 milliards de dollars US en 2000. Le contrôle des populations de tiques des bovins repose essentiellement sur l'utilisation d'acaricides. L'utilisation intensive de ce moyen de lutte a eu pour conséquence le développement de résistances à la majorité des classes d'acaricides disponibles sur le marché. Un suivi local de la résistance aux acaricides est essentiel afin que les éleveurs puissent recevoir des informations sur les composés auxquels les populations de tiques présentes dans leur établissement sont résistantes et être guidés dans le choix de composés de remplacement à disposition. A une échelle globale, le suivi de la résistance permet d'observer sa progression afin d'essayer de ralentir son développement et d'allonger la durée d'utilisation des composés.

Les tests *in vitro* sont des méthodes très utiles pour détecter la résistance des tiques. La FAO recommande deux d'entre eux, l'un utilisant des larves, nommé Larval Packet test (LPT), l'autre utilisant des adultes, appelé Adult Immersion Test (AIT). Chaque test a ses avantages et ses inconvénients : le LPT est un test laborieux qui prend beaucoup de temps alors que le AIT nécessite une grande quantité de tiques. Ces désavantages limitent le nombre de composés et de doses pouvant être testés, limitant ainsi l'information obtenue. Pour surmonter ces difficultés, nous avons développé un nouveau test, nommé Larval Tarsal Test (LTT), qui permet de tester de nombreux composés en peu de temps et avec un minimum de tiques. Dans ce test, des œufs de tiques sont distribués dans des puits de plaques de microtritation préalablement traités avec les acaricides voulant être testés. Les œufs sont incubés jusqu'à l'éclosion des larves, qui sont ainsi exposées aux composés. La résistance des souches de tiques est alors évaluée en fonction de la mortalité des larves écloses. La capacité du LTT à détecter la résistance a été comparée au LPT, un des tests recommandés par la FAO. Pour cela, une souche de tiques sensible ainsi qu'une souche résistante de référence ont été exposées à neuf composés de cinq classes principales d'acaricides : les organophosphorés (OP), les pyréthroïdes de synthèse (SP), les amidines, les lactones macrocycliques (ML) et les phénylpyrazoles. Le LTT a permis d'obtenir de bonnes courbes de dose-réponse, il s'est montré aussi sensible que le LPT et, à mortalités équivalentes, a nécessité des doses de composés nettement plus basses que le LPT.

Ayant démontré que le LTT est un test adéquat, des populations de tiques provenant d'Argentine, d'Afrique du Sud et d'Australie ont été envoyées en Suisse pour évaluer leur résistance à l'aide du LTT. Ces tests ont confirmé l'intérêt du LTT à être utilisé pour détecter la résistance dans des

populations provenant du terrain. Afin que ce test puisse être effectué dans d'autres laboratoires, le LTT a été modifié de sorte que l'infrastructure nécessaire à sa réalisation soit simple. Suite à cela, le LTT a été utilisé dans deux laboratoires brésiliens, pour tester des populations de tiques locales. Quelques-unes de ces populations ont aussi été testées avec le LPT pour permettre des comparaisons supplémentaires. A nouveau, le LTT s'est monté très efficace en termes de temps et adéquat pour détecter la résistance aux acaricides des populations de terrain avec, cette fois-ci, une sensibilité même plus élevée que le LPT. Ces études ont également permis de fournir des données supplémentaires sur la situation de la résistance aux acaricides dans les pays étudiés. Ainsi, elles ont permis de détecter les premiers cas de résistance de la tique des bovins à l'amitraz en Argentine, elles ont montré l'ampleur de la résistance au fipronil au Brésil et mis en évidence l'importance de la résistance aux OP et aux SP dans les différents pays ainsi. De plus, elles ont permis de détecter quelques cas isolés de résistance ou de suspicion de résistance aux ML au Brésil, en Argentine et en Afrique du Sud.

Afin de compléter ce diagnostic *in vitro* de la résistance aux acaricides, une PCR multiplex a été mise au point pour détecter simultanément trois mutations ponctuelles connues comme étant impliquées dans la résistance aux SP chez *R. (B.) microplus*. Cette PCR a été utilisée pour tester les échantillons de tiques provenant du Brésil, d'Argentine, d'Afrique du Sud et d'Australie dont le phénotype avait été préalablement évalué avec le LTT, ainsi que quelques souches supplémentaires du Mexique. Il est apparu que les trois mutations ont des distributions géographiques distinctes et qu'elles résultent dans des phénotypes de résistance différents.

Mots clés : *Rhipicephalus (B.) microplus*, tique des bovins, résistance, acaricides, tests, Larval Tarsal Test, Brésil, Argentine, Afrique du Sud, Australie, PCR multiplex, mutation ponctuelle

2. ABSTRACT

Rhipicephalus (Boophilus) microplus is a one-host cattle tick which has a tropical and subtropical distribution. This major ectoparasite has a very important economic impact on cattle husbandry throughout its area of distribution. In Brazil, for example, the loss due to its direct and indirect effecs was estimated at 2 billion US dollars in 2000. The control of cattle tick populations relies mainly on acaricides and the intensive use of such products has led to the development of resistance to most of the acaricide classes available on the market. Monitoring of resistance is essential on a local scale, so that producers can obtain information on the resistance pattern of the tick population established in their farm and advice on using alternative compounds. In addition, on a global scale, monitoring of resistance may help to slow down the development of resistance and to extend compound lifespan.

Bioassays are very useful tools to monitor resistance. The Food and Agriculture Organisation of the United Nations (FAO) recommends two *in vitro* tests: the Laval Packet Test (LPT) and the Adult Immersion Test (AIT). Each test has its own advantages and disadvantages: the LPT is a laborious and time-consuming test while the AIT requires many ticks for testing. These two flaws limit the number of compounds and doses which can be tested, and therefore the information which can be obtained. Hence, we developed a new test named Larval Tarsal Test (LTT), to overcome these limitations. The LTT is a time effective test requiring small numbers of ticks which is based on the distribution of tick eggs in pre-treated wells of microtiter plates. The plates are incubated until larvae hatch and get exposed to the acaricidal compounds. Then, the evaluation of the susceptibility of the ticks is based on the assessment of larva mortality. The ability of the LTT to detect resistance was compared to the FAO-recommended LPT by testing a susceptible and a resistant reference laboratory strain with 9 compounds of 5 major acaricide classes: organophosphates (OP), synthetic pyrethroids (SP), amidines, macrocyclic lactones (ML), and phenylpyrazols. The LTT provided satisfactory dose-response curves, was as sensitive as the LPT and required much lower doses of the acaricides to obtain equal mortality levels.

Having demonstrated the suitability of the LTT, this test was used to evaluate the acaricide resistance pattern of field populations from Argentina, South Africa and Australia, which were shipped to Switzerland for testing. This study proved the ability of the LTT to detect resistance in field populations and the test was subsequently modified to simplify the equipment required and to be carried out in other laboratories. The LTT was then carried out in two Brazilian laboratories testing tick field populations from that country and, some of these populations were also tested with the LPT for additional comparison. Again, the LTT proved to be a time-effective and appropriate test to detect acaricide resistance in field populations with, this time, an even higher sensitivity than the LPT. Furthermore, these studies provided additional data on the resistance in the four sampled

countries. This study detected the first cases of resistance to amitraz in Argentina, found widespread fipronil resistance in Brazil, widespread OP and SP resistance in the different countries, and occasional cases of resistance or suspected resistance to ML in Brazil, Argentina and South Africa.

Finally, to complete this *in vitro* diagnosis of acaricidal resistance, a multiplex PCR was developed allowing the simultaneous detection of three single nucleotide substitutions known to confer resistance to SP in *R. (B.) microplus*. This assay was used to screen the tick samples originating from Brazil, Argentina, South Africa and Australia whose phenotype to SP had been determined by the use of the LTT, as well as some additional tick strains from Mexico. The three mutations were found to have distinct geographical distributions and to result in different resistance phenotypes.

Keywords : *Rhipicephalus (B.) microplus*, cattle tick, resistance, acaricides, bioassays, Larval Tarsal Test, Brazil, Argentina, South Africa, Australia, multiplex PCR, point mutation

3. INTRODUCTION

3.1. Description of the parasite

3.1.1. Systematics of ticks and *Rhipicephalus (Boophilus) microplus*

Ticks are hematophagous arthropods part of the class Arachnida. They belong to the suborder Ixodida which forms, with the mites, the order Acari. There are approximately 870 species of ticks described worldwide, which are divided into three families: the Ixodidae (hard ticks), including 685 species; the Argasidae (soft ticks), counting 185 species; and a third family, the Nuttalliellidae, which consists of a single species possessing characteristics between the Ixodidae and the Argasidae (ICTTD 2004b). The Ixodidae are characterized by the presence of chitinous plates on their body and are divided into 14 genera (ICTTD 2004a). *Boophilus* is a sub-genus of Ixodid ticks within the genus *Rhipicephalus*. The tick species studied in the present work was initially named *Boophilus microplus* (Canestrini 1888). Recent phylogenetic studies using molecular methods and morphological characters placed the 5 species of the genus *Boophilus* within the genus of *Rhipicephalus* (Murrell et al. 2000, Beati and Keirans 2001). We therefore refer to it in the present work as *R. (B.) microplus*. In many parts of the world *R. (B.) microplus* is known as “the cattle tick” (Walker et al. 2003), or as “Southern cattle tick” (in Texas) (ICTTD 2004b).

3.1.2. Life cycle

Rhipicephalus (B.) microplus has the typical life cycle of a one-host tick feeding on a single individual host (Figure 1). Its life cycle is divided into three stages: larva, nymph, and adult. Unfed hexapod larvae crawl on the vegetation and wait for passing hosts. Questing larvae use their front legs to grab to grazing cattle and crawl over their host to find a suitable place to attach and feed (ICTTD 2004a). They preferentially attach on the belly, flanks, dewlaps and shoulders of their host (Walker et al. 2003). The larvae feed for 6-8 days and then moult into an octopod nymph remaining attached on the host. Nymphs take a 7-9 day blood meal, and then moult into adults, male or female (ICTTD 2004b). Males have short repeated meals between which they search for females on the host (ICTTD 2004a). Mating occurs during the blood meal of the females. Once mated females have completed their blood meal, they detach from the host and drop to the ground. The last blood meal of the females allows them to produce the eggs which are laid in a single batch on the vegetation. Females die as soon as they finish oviposition. The total parasitic phase varies from 18 to 30 days for the females while males can remain sexually active on the host for up to 70 days (ICTTD 2004b).

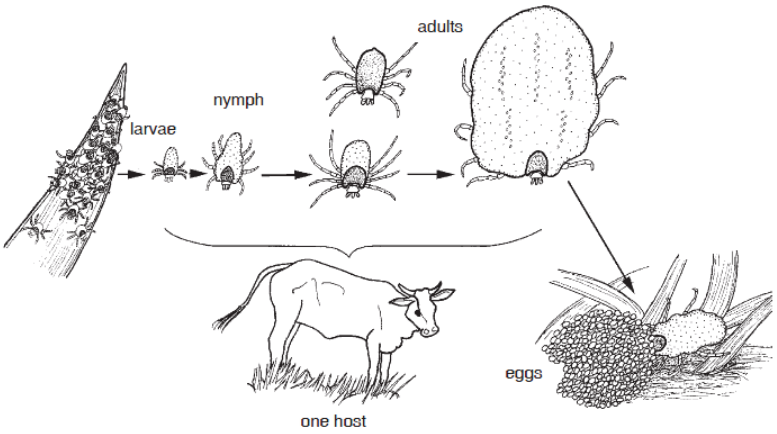

Figure 1: Schematic life cycle of a one-host tick. Source: ICTTD 2004a

The development of *R. (B.) microplus* on the host is almost independent from the climatic conditions while the rapidity of the pre-parasitic phase is influenced by the temperature and the humidity. Oviposition and hatching of the larvae are accelerated by warm weather while cold temperatures slow down or inhibit development. If the blood meal is complete and humidity and temperature conditions favourable (24-28°C), 50-60% of the body mass of the female is transformed into up to 2,000 to 4,000 eggs, among which 85-95% hatch into larvae. Unfed larva survive less than 30 days at high temperatures and up to over 120 days in cold conditions (ICTTD 2004b).

3.1.3. Ecology

R. (B.) microplus has a tropical and subtropical distribution (Figure 2). It is considered to be originally from south Asia (Pal and Wharton 1974) and to have spread to major cattle breeding areas like South America and Australia through commercial cattle transportation. In Africa, it has been suggested that *R. (B.) microplus* was first introduced in Madagascar with imported cattle and and then further into East and South Africa (Walker et al. 2003). On the American continent *R. (B.) microplus* is present from Uruguay and northern Argentina to the Mexican-Texan border. It has been eradicated from the southern part of its distribution in Argentina and Uruguay and from Texas (ICTTD 2004b). In Africa *R. (B.) microplus* is limited to southern and eastern Africa (South Africa, Zambia, Malawi, Tanzania and Kenya) and Madagascar (Walker et al. 2003), while in the Australian continent, it is distributed in the North and East of Australia and in New Caledonia. Its distribution on the Asian continent is not well known (Pal and Wharton 1974). Within its area of distribution, the presence of *R. (B.) microplus* depends on the distribution of its host and the climatic conditions. Cattle tick activity is adapted to

climatic seasonal variation to reduce the risk of desiccation. Large numbers of larvae are usually present on the pasture vegetation in late spring, and successive generations of larvae then occur through the summer and into autumn and early winter months (Walker et al. 2003).

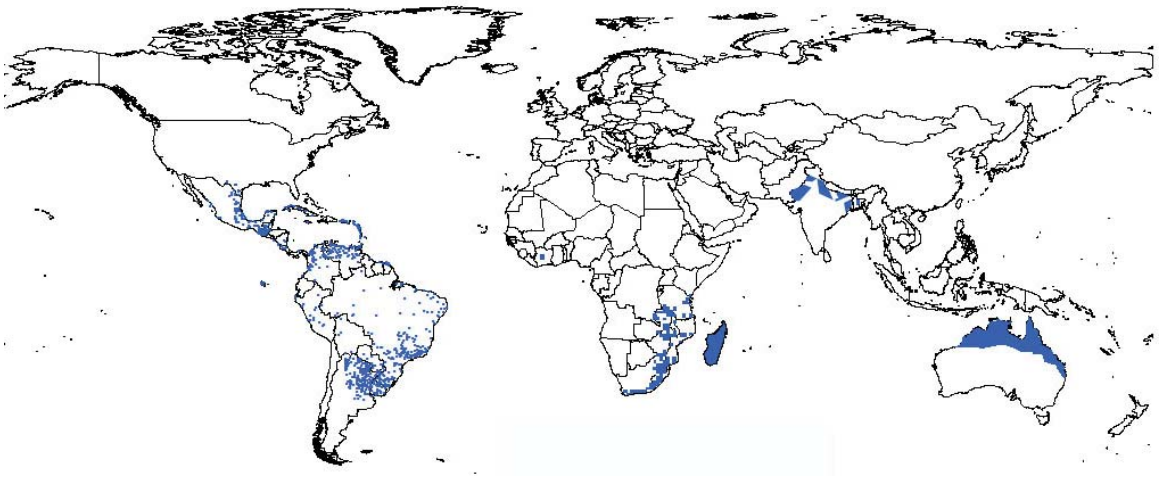

Figure 2: Distribution of *R. (B.) microplus* based on the maps and information available in ICCTD CD-roms (2004a, 2004b), Cutullé et al. (2009), Madder et al. (2007), and Kumar et al. (2011). The represented distribution in South America is historical because it has been eradicated in parts of southern Argentina and Uruguay. A map from Pal and Wharton (1974), with the distribution of *R. (B.) microplus* in Asia, is available in Appendice 1. Except for this map, very little information is available about the distribution of the cattle tick in Asia.

3.1.4. Host specificity

Although cattle are the principal host of *R. (B.) microplus,* this tick species can also be found on deer, horses, goats and dogs (ICTTD 2004b). Hence, if cattle are removed from their natural habitat, *R. (B.) microplus* is able to complete its life cycle on other hosts and maintain the population, which is a complicating factor in eradication programms.

3.1.5. Differential diagnosis of *R. (B.) microplus*

Morphologically, *R. (B.) microplus* is difficult to differentiate from *R. (B.) decoloratus* and *R. (B.) annulatus* and observation under a stereomicroscope is required to distinguish them. The criteria are the following ones (Walker et al. 2003): *R. (B.) microplus* hypostomal teeth are in 4+4 columns while they are in 3+3 columns in *R. (B.) decoloratus* (Figure 3). In addition, *R. (B.) microplus* has no protuberance-bearing setae on the internal margin of palp article 1 while this protuberance is

present in *R. (B.) decoloratus* (Figure 3). *R. (B.) microplus* and *R. (B.) annulatus* are also very similar. However, *R. (B.) microplus* males possess a caudal appendage which is absent in *R. (B.) annulatus*. In addition, the coxa I of the female of *R. (B.) microplus* presents two well-developed spurs while in *R. (B.) annulatus* this structure does not have well-developed internal spur (ICTTD 2004b) .

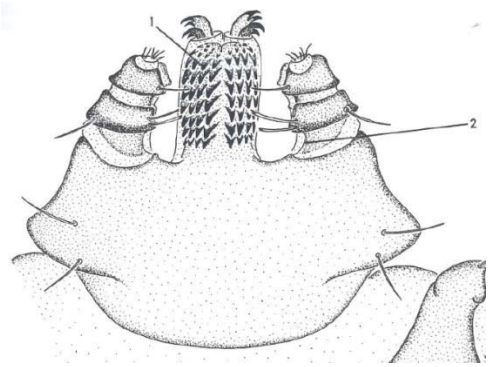

Figure 3: Morphological characteristics of *R. (B.) microplus*. 1: hypostomal teeth in 4+4 columns; 2: palp articles 1 internal margin has no protuberance-bearing setae. Source: Walker et al. 2003

3.1.6. Role as vector of tick-borne pathogens

R. (B.) microplus is an important vector of bovine babesiosis and of bovine anaplasmosis.

R. (B.) microplus transmits *Babesia bovis*, previously named *B. argentina*, and *Babesia bigemina*I, which cause bovine babesiosis, also named pyroplasmosis or red water fever (ICTTD 2004b). The babesia are intracellular protozoa and are part of the phylum Apicomplexa , class Aconoidasida, order Piroplasmidora, family Babesiidae, and genus *Babesia* (Telford et al. 1993). Babesia spp. are divided into large (2-5 µm) and small (1-2 µm) types, which include *B. bigemina* and *B. bovis*, respectively. Their life cycle is as follows (Kreier and Baker 1987, Telford et al. 1993): *B. bovis* is transmitted to cattle by infected larvae shortly after infestation while the transmission of *B. bigemina* is delayed until the ticks are nymphs or adults (FAO 1983). Babesia sporozoites are transmitted to the host through the saliva and penetrate red blood cells where they multiply to form merozoites. Infected red blood cells disrupt and release the merozoites, which in turn invade new red blood cells. Some merozoites differentiate into gamonts which, when the blood is ingested by the ticks, leave their host cells and transform into male and female gametes. Gametes fuse to form a zygote which matures first into an ookinete and later into sporokinetes. Some sporokinetes reach the tick salivary gland cells where they mature into sporonts and divide to form sporozoites. Sporozoites become infective within five days after the tick has attached to a host and are injected into the host before the ticks detach (Kreier and Baker 1987). During the migration through the tick, the ovaries are

infected by both *Babesia* spp., resulting in transovarial transmission of the pathogens from the engorged female to the eggs (ICTTD 2004b).

Babesiosis is a febrile disease characterized by anaemia, icterus and haemoglobinuria (excretion in the urine of the haemoglobin released during the lysis of the erythrocytes. This symptom was the reason for the name “red water fever”) which can be fatal (FAO 1983). In endemic areas, animals acquire immune protection if they are repeatedly bitten by infected ticks. This protection is not always complete because antigenically different strains of *Babesia* spp. can occur, but it is sufficient to protect cattle from death. However, if immune defences are broken down by other diseases or stress, the disease may be fatal (FAO 1983).

Bovine anaplasmosis, also called gall sickness, is caused by the Gram-negative bacteria *Anaplasma marginale* and *A. centrale* (ICTTD 2004a, ICTTD 2004b). These intracellular rickettsiae are transmitted to cattle when ticks are feeding. They invade the red blood cells of the cattle, develop within a vacuole and reproduce by binary fission to form up to eight cells. The red blood cell ruptures and releases the pathogens. The mode of development in the ticks is not known (Adam et al. 1971) and transovarial transmission has not been demonstrated (Suarez and Noh 2011).

The severity of the symptoms increases with age if the animals have not acquired immunity. The disease is generally mild in calves up to 1 year old, whereas the disease is acute and occasionally fatal in 2 to 3 years old cattle and even peracute and frequently fatal in older cattle (Mahoney 1977). Thus, in endemic situations, young calves develop an immunity which protects them in subsequent years while the disease is severe in susceptible cattle imported into infected areas and in case of epidemics (FAO 1983). The acute form of anaplasmosis is characterized by fever, anaemia, weakness, constipation, icterus, lack of appetite, depression, dehydration, laboured breathing, abortion and, as mentioned above, may lead to death (Mahoney 1977).

Bovine babesiosis and bovine anaplasmosis are not exclusively transmitted by *R. (B.) microplus*. Other *Boophilus*, *Haemaphysalis* and *Rhipicephalus* ticks can transmit bovine babesiosis (Telford et al. 1993), while anaplasmosis can be transmitted by other *Boophilus*, *Dermacentor*, *Rhipicephalus*, *Ixodes* and *Hyalomma* ticks (Adam et al. 1971) and mechanically by biting flies and contaminated needles (Suarez and Noh 2011). The successful treatment of babesiosis and anaplasmosis depends on early diagnosis of the infection and prompt administration of the appropriate drugs (FAO 1983).

Alternatively, and as a preventive measure, cattle can be vaccinated against both diseases (Adam et al. 1971).

3.2. Economic impact on cattle industry

Rhipicephalus (B.) microplus causes major losses to cattle industry through both direct and indirect negative effects. In Brazil for example, *R. (B.) microplus* is the most important ectoparasite of cattle and its economic impact on the Brazilian cattle industry (which has 169 million heads of cattle) was estimated at 2 billion US dollars in 2000, (Grisi et al. 2002). This figure includes losses due to decreased milk production and decreased weight gain, damage to the leather, increased mortality caused by tick-borne parasites, and treatment costs to control infestations.

3.2.1. Direct effects

Direct effects include cattle blood loss due to tick feeding. Each cattle tick that completes its lifecycle ingests an estimated 1-3 ml of blood. When cattle ticks feed in large numbers, they cause anaemia and loss of nutrients to cattle. In addition, the irritation caused by the ticks leads to a reduction in food intake by the cattle. All these factors impact negatively on weight gain and milk production (ICTTD 2004a). A study in Australia found that there is a loss of 0.6g of potential growth by cattle for each female tick that completes feeding (Walker et al. 2003). In Brazil, Horn (1983) estimated the decrease in weight gain to be 6 kg/animal/year.

Furthermore, ticks cause damage to the hide by making small scars in the skin, which reduces the value of the leather (ICTTD 2004a). Finally, tick infestations predispose animals to bacterial and fungal infections, as well as screw-worm attack of the wounds left by tick bites (FAO 1983).

3.2.2. Indirect effects and treatment costs

As mentioned in the previous section, *R. (B.) microplus* is a key vector of bovine babesiosis and bovine anaplasmosis which leads to lower weight gain and milk production and to increased mortality of animals. The mortality of cattle caused by these tick-borne pathogens was estimated to be 1.2% in Brazil (Horn 1983). In addition, ticks feeding in large numbers can suppress host immunity thereby exacerbating diseases (ICTTD 2004a).

Tick infestation control also leads to a considerable increase of production costs. A 1992 study in Argentina found that control costs, including the costs of the acaricides, the required man power, maintenance costs of the plunge dips used to apply the acaricides and government costs, represent 14.1% of total losses. The remaining costs were attributed to direct effects (63.6%), to the morbidity and mortality due to the tick-borne parasites (18%) and their control costs (2.6%) (Späth et al. 1994).

3.3. Tick control and resistance

The currently available tools for tick control consist of chemical acaricides used with different application methods and various formulations, tick-resistant animals, tick vaccines, tick-borne disease vaccines and rotations between livestock and crops (FAO 2004). However, among these different options, chemical control is by far the most important strategy to control *R. (B.) microplus* infestations.

3.3.1. History of acaricidal compound classes : their introduction on the market and emergence of resistance

Control of cattle ticks with chemical compounds started at the end of the nineteenth century with the use of arsenic. This class of compound was followed by the organochlorines (OC), organophosphates (OP), amidines, synthetic pyrethroids (SP), phenylpyrazols, macrocyclic lactones (ML), growth regulators and spinosyns. Each introduction of a new acaricide class has been followed by the emergence of resistance. In what follows, we present the different classes of compounds in their order of introduction on the acaricide market, and provide information about tick resistance.

3.3.1.1. Arsenicals

Arsenicals were the first compounds used for tick control, introduced in 1895 (Waltisbuhl et al. 2005). In 1896, an arsenic-based dipping vat was used in Queensland, Australia, for tick control by a local farmer (Angus 1996). This successful arsenic dip was rapidly adopted in other countries such as the United States, South Africa and Cuba and dipping became a widespread practice in Australia (Angus 1996). Dipping had to be very frequent due to the very short residual effect (24h) of arsenic (Mitchell 1996). After around 40 years of use, the first case of *R. (B.) microplus* resistance to arsenic was reported (LEGG 1947) in 1936.

3.3.1.2. Organochlorines

The first organochlorine (OC) compounds were introduced on the market as acaricides in 1939 (Graf et al. 2004) to control ticks resistant to arsenical. More compounds became available in the mid-1940s (Cobbett 1947; Maunder 1949). Organochlorines are divided into three main groups (Taylor 2001): chlorinated ethane derivatives, such as DDT (dichlorodiphenyltrichloroethane); the cyclodienes, which include dieldrin and toxaphene; the hexachlorocyclohexanes (HCH), such as lindane. Organochlorines had a high efficacy, a long residual activity and a large spectrum of action and had the advantage of being less toxic and cheaper than arsenicals. The first case of resistance to OC was observed in Brazil in 1952 (Freire 1953). A decade later, in 1962, the use of all OC was banned for tick control because of residues in meat, milk and the environment (Waltisbuhl et al. 2005), resulting from its low biodegradability and its affinity for fat tissues.

3.3.1.3. Organophosphates and carbamates

Organophosphates (OP) started to be used as ectoparasiticides in the mid-1950s (Andreotti 2010). They were used to control ticks which had become OC resistant. Major compounds of this class were ethion, chlorpyrifos, chlorfenvinfos and coumaphos. Organophosphates were less stable and less persistent than OC but some OP are very toxic to mammals (Cremlyn 1978, Taylor 2001). In contrast, they had the advantage of being biodegradable, and therefore not accumulating in the environment and to be much less lipophilic than OC. Residual effect of OP against tick reinfestation was two to three days (Mitchell 1996). Organophosphate resistance appeared first in Australia in the mid-1960s (Shaw and Malcolm 1964, Shaw 1966, Roulston et al. 1968) and is nowadays widespread across the entire distribution of *R. (B.) microplus* (Appendices 2a and 3). Furthermore, OP resistance was also observed in an outbreaking strain in Texas (Miller et al. 2005).

Carbamates are closely related to OP and the two main compounds used for tick control are carbaryl and propoxur. Carbaryl has low toxicity for mammals but may be carcinogenic and is often combined with other active ingredients (Taylor 2001).

3.3.1.4. Amidines

Amidines started to be used for tick control in the mid-1970s (Nolan 1981). Nowadays, amitraz is the main active ingredient used in this group. It has a narrow spectrum of action but is very effective

against ticks, with residual effectiveness against tick re-infestation for nine days. It has minimal toxicity to cattle and humans, is rapidly degraded in the environment and has no meat withholding period (Jonsson and Hope 2007).

Amitraz resistance appeared 4 to 10 years after its initial use in different parts of the world and was identified for the first time in the early 1980s in Australia (Nolan 1981). Since then, it has also been reported in Mexico, South America, South Africa and New Caledonia (Appendices 2c). However, in 2007, Jonsson and Hope (2007) reported that amitraz was still one of the most popular acaricides for the control of cattle ticks in Australia, Latin America and Southern Africa.

3.3.1.5. Synthetic pyrethroids

Synthetic pyrethroids were introduced in the mid to late 1970s (Graf et al. 2004) and have been widely used. Synthetic pyrethroids are very effective insecticides and acaricides, are not very toxic to mammals and are highly biodegradable.

In the late 1980s, resistance was observed in Australia (Nolan et al. 1989) and Brazil (Leite 1988, Laranja et al. 1989). Nowadays, SP resistance is extremely common and has been shown to be widespread in all the countries where resistance studies have been carried out including Mexico, Central and South America, South Africa, Australia and New Caledonia (Appendices 2b). As for the OP, SP resistance was also observed in an outbreaking strain in Texas (Miller et al. 2007).

3.3.1.6. Macrocyclic lactones

Macrocyclic lactones were introduced on the market in 1981 (Geary 2005). They are divided into two categories (Taylor 2001): the avermectins, such as ivermectin, doramectin, abamectin, eprinomectin; and the milbemycins, including moxidectin and milbemycin oxime. Macrocyclic lactones are active systemically against ticks and have a longer residual activity than SP and are active against a wide range of arthropods and nematodes (Taylor 2001). However, long withholding periods for meat and milk may limit the use of this class in cattle (Andreotti 2010). Resistance to avermectins was first reported in Brazil in 2001 (doramectin and ivermectin) (Martins and Furlong 2001), and was later also reported to ivermectin in Mexico (Perez-Cogollo et al. 2010). Macrocyclic lactone resistance has not been reported elsewhere yet (Appendices 1d and 4).

3.3.1.7. Phenylpyrazols

Fipronil is the only phenylpyrazol compound to be used in livestock for the control of cattle ticks and its use started in the mid-90s (Davey et al. 1998). Fipronil has a long residual activity (Taylor 2001) and persists up to five weeks in the field (Davey et al. 1999). Resistance to fipronil was reported for the first time in 2007 in Uruguay (Cuore et al. 2007), and later in Brazil (Castro-Janer et al. 2010a, Castro-Janer et al. 2010b) (Appendices 1e and 5).

3.3.1.8. Growth regulators

Growth regulators are one of the newest acaricide class, with the first representative compound of the class, fluazuron, available on the acaricidal market, since 1994 in Australia. These compounds have a completely different mode of action compared to the previously cited classes. Based on their mode of action, they are divided into benzoylphenyl ureas, which are chitin synthesis inhibitors, triazine/pyrimidine derivates, acting as chitin inhibitors and juvenile hormone analogues (Graf 1993). Fluazuron is a benzoylphenyl ureas which provides long-term protection against *R. (B.) microplus* (6-12 weeks) (Bull et al. 1996). In contrast, the other benzoylphenyl ureas compounds have a relatively low efficacy against ticks. Fluazuron cannot be used for dairy cows because it is highly lipophilic and therefore accumulates in body fat tissues and milk.

Resistance has not yet been reported in the literature but a case has been presented in a congress poster session in 2010 (Jackson and Stutchburry 2010) and has been observed in Brazil (João R. Martins, personal communication).

3.3.1.9. Spinosyns

Spinosad is a naturalyte containing a mixture of two metabolites produced by fermentation by the actinomycete *Saccharopolyspora spinosa* (Davey et al. 2001, Miller et al. 2011). It has been shown to be effective against *R. (B.) microplus* larvae and nymphs (Davey et al. 2001) and is registered for tick control in some countries in Latin America, such as Brazil (Jonsson et al. 2010b). Spinosad is rapidly biodegraded in soil, has low cross resistance with other chemicals and has reduced risk for workers (Davey et al. 2001).

3.3.2. Current distribution of the resistance to acaricides

3.3.2.1. Maps of resistance distribution at the country level

As mentioned previously, resistance of *R. (B.) microplus* to OP, SP and amitraz is widespread worldwide. In addition, resistance to ML and fipronil, although less common, has also emerged,. The situation as of June 2012 is represented for each acaricide class at the country level on maps available in Appendices 1a to 1e. Figure 4 is a summarising map indicating the cumulated resistance to these five classes of acaricides. These maps are based on peer-reviewed publications, but also on conference proceeding articles and FAO reports. They include literature published in English, Spanish and Portuguese.

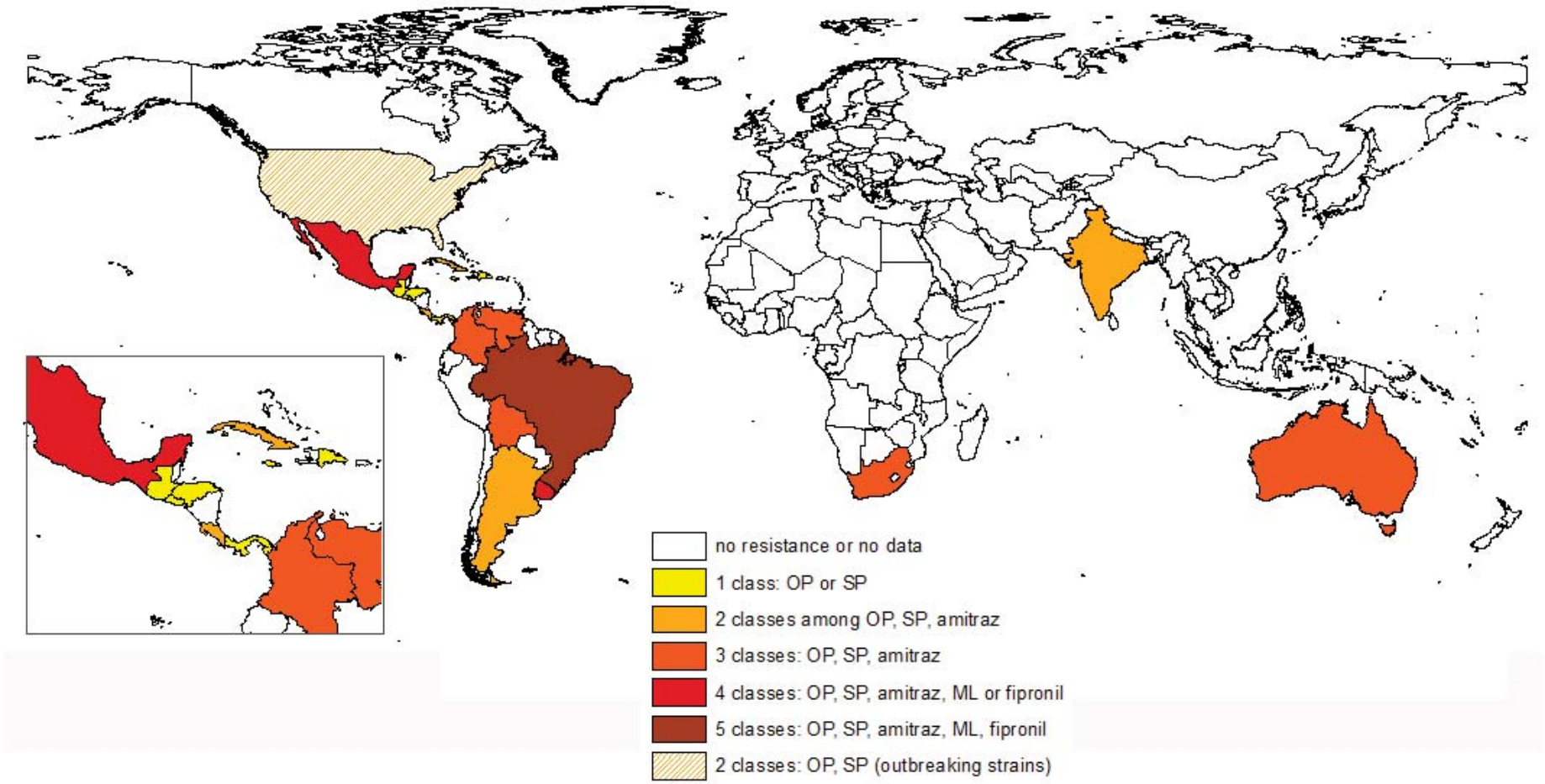

Figure 4: Cumulated resistance to organophospates (OP), synthetic pyrethroids (SP), amitraz, macrocyclic lactones (ML) and fipronil. The number of classes against which resistance has been reported is indicated with a colour code. The colour indicates that there is at least one report of resistance in the country. The map is therefore not quantitative. Established based on the information available by June 2012.

3.3.2.2. Literature overview

Appendices 3 to 5 provide bibliographic references in tabulated form for resistance of *R. (B.) microplus* to OP, ML and fipronil reported in peer-reviewed journals, conference proceedings and FAO reports. The original publications reporting the resistance were always searched, but when it could not be obtained, the references of the articles mentioning them are given.

3.3.3. Mode of actions of acaricides

3.3.3.1. Reminder of the basics of the transmission of nerve impulses in the nervous system.

Most acaricides act on the nervous system of arthropods either on the axon of the presynaptic neuron, on neurotransmitters or on post-synaptic receptors. As an introduction to the description of the mode of action of the different acaricide classes, we provide a short review about the fundamental mechanisms of the transmission of nerve impulses in the nervous system.

The transmission of information along the axon is based on action potentials, a phenomenon driven by the exchange of ions between the outside and the inside of the cell membrane. At rest, the membrane of a neuron has a voltage of -70 mV and is therefore polarized. Stimulation of the neuron causes the membrane to depolarize. If the threshold value of -55 mV is reached, the voltage of the membrane rises abruptly up to +40 mV. This increase of the voltage, called depolarization, is caused by the opening of voltage-gated sodium channels, allowing the entry of Na^+ ions into the cell. As soon as the peak is reached, depolarization is followed by the opening of K^+ channels that permits the exit of K^+ ions from the cell. This influx of potassium ions causes the voltage to fall very quickly, usually exceeding the resting level, before stabilizing around -70 mV. The voltage decrease phase is called hyperpolarization.

The arrival of an action potential at the pre-synaptic terminal results in the opening of Ca^{2+} channels and entry of Ca^{2+} ions into the synaptic knob. Calcium triggers the exocytosis of vesicles containing neurotransmitters, such as glutamate, gamma-aminobutyric acid (GABA), acetylcholine, monoamine or octopamine. The neurotransmitter released into the synaptic cleft binds to receptors on the membrane of the postsynaptic cell. These receptors are ligand-gated ion channels, and they open when a specific neurotransmitter (“ligand”) binds to the receptor. Depending on the neurotransmitters involved, the opening of the post synaptic receptor results in depolarization of the

postsynaptic cell, allowing the transmission of the nerve impulse to the next neuron, or in contrast, results in hyperpolarization (via influx of Cl^-) of the postsynaptic cell.

3.3.3.2. Arsenicals

Arsenical compounds act on oxidative phosphorylation, thus disrupting ATP production.

3.3.3.3. Organochlorines

Organochlorine mode of action differs between the three organochlorine sub-groups.

Chlorinated ethanes hold sodium channels open, resulting in delayed repolarization of the axonal membrane (Saunders and Harper 1994). This state renders the nerve vulnerable to repetitive discharge from small stimuli that would normally not cause an action potential in a fully repolarized neuron. The chlorinated cyclodienes appear to have at least two modes of action: inhibition of the GABA stimulated chloride channel and interference with the Ca^{2+} flux. This results in the inhibition of the post-synaptic potential, leading to a state of partial depolarization of the post-synaptic membrane and vulnerability to repeated discharge (Saunders and Harper 1994). Lindane, from the HCH class, has a similar mode of action, binding to the GABA receptor and resulting in an inhibition of GABA-dependent Cl^- flux into the neuron (Saunders and Harper 1994).

3.3.3.4. Organophosphates

Toxicity of OP is due to the inhibition of the activity of acetylcholinesterase. Organophosphates mimic the structure of acetylcholine and bind to acetylcholinesterase, thereby preventing this enzyme from hydrolysing acetylcholine (Taylor 2001). If acetylcholine is not degraded, it accumulates in the synaptic cleft and muscle end plates, resulting in neuromuscular paralysis (Saunders & Harpers, 1994).

3.3.3.5. Amidines

The mode of action of amidine acaricides has not yet been determined, despite many efforts. Two target sites have been proposed in arthrophods (see Jonsson and Hope 2007 for a review):

monoamine oxidase; and octopamine receptors. However, no direct evidence for the action of amitraz on these two potential target sites has been established.

3.3.3.6. Synthetic pyrethroids

The voltage-gated sodium channel is the target site of pyrethoids, resulting in extended depolarisation and eventual paralysis (Vijverberg et al. 1982). Synthetic pyrethroids are divided into two groups (Types I and II) and both act on the sodium voltage-gated channel of the pre-synaptic neuron leading to delayed repolarization (Taylor 2001).

3.3.3.7. Macrocyclic lactones

Target site of ML are also believed to be the GABA- and glutamate-gated chloride channels (Taylor 2001). It was first demonstrated that ML stimulates the release of GABA from nerve endings and enhances the binding of GABA to its receptor on the post-synaptic neuron. The enhanced GABA binding results in an increased flow of Cl^- into the cell leading to hyperpolarization. This leads to a paralysis and the subsequent death of the arthropod (Campbell and Benz 1984, Shoop et al. 1995).

3.3.3.8. Fipronil

Fipronil is a phenylpyrazolic compound whose mode of action is to block transmission of signals by binding to the GABA-gated and glutamate-gated chloride channels (Zhao et al. 2004) and consequently inhibits the flux of Cl^- into the nerve cell resulting in hyperexcitation of the arthropod nervous system (Postal et al. 1995, Taylor 2001).

3.3.3.9. Growth regulators

Benzoylphenyl ureas, such as fluazuron, are chitin inhibitors. Chitin is a complex aminopolysaccaride and a major component of the arthropod cuticule. During each moult, chitine molecules are assembled with proteins into microfibrils (Cohen 1993). The exact mechanism with which benzoylphenyl ureas inhibits the chitin synthesis is not fully understood. The compound has no effect on the enzyme chitin synthetase, but it may interfere with the assembly of the chitin chains into microfibrils (Cohen 1993). When immature arthropod stages are exposed to these compounds, they are not able to complete ecdysis and as a consequence die during the moulting process.

3.3.3.10. Spinosad

Spinosad acts on the nicotinic acetylcholine receptors of the postsynaptic cells (Millar and Denholm 2007) and also acts on GABA receptors (Jonsson et al. 2010b). Resistance to spinosad has not yet been reported in the literature (Miller et al. 2011).

3.3.4. Mechanisms of resistance in *R. (B.) microplus*

3.3.4.1. Introduction

Resistance can arise through several mechanisms which are generally classified into three main categories: target site insensitivity, increased metabolic detoxification, and reduced cuticular penetration (Guerrero et al. 2012a).

Target-site and metabolic resistance are common in *R. (B.) microplus* and have been widely studied for some classes of compounds, such as the SP. Target site resistance occurs when a single nucleotide substitution in the gene coding for the target molecule of an acaricide results in an amino acid change which confers a lower susceptibility to the acaricidal compound (Guerrero et al. 2012a).

Metabolic resistance occurs when arthropods develop an increased ability to detoxify or sequester an acaricide. Three main enzyme families are known to be involved in this type of resistance: cytochrome P450s, esterases, and glutathione S-transferases. Chemicals known as synergists are often used to identify the type of enzymes involved in metabolic resistance. Piperonyl butoxide (PBO), triphenyl phosphate (TPP) and diethylmaleate (DEM) are the three most commonly used synergists and are considered to be specific inhibitors for cytochrome P450s, esterases, and glutathione S-transferases, respectively (Guerrero et al. 2012a). Synergist studies are usually carried out using the Larval Packet Test, comparing the survival of resistant ticks to a chemical in presence or absence of a specific synergist (Crampton et al. 1999, Miller et al. 1999, Li et al. 2003, Li et al. 2008). An increased toxicity of a chemical compound in presence of a synergist is an indicator of the probable involvement of the corresponding enzyme class in the degradation of the acaricidal compound (Guerrero et al. 2012a). Synergist studies are useful guides to determine if metabolic

resistance mechanisms are present but do not provide a definitive determination of the mechanism of metabolic resistance.

Finally, penetration resistance is the alteration in the ability of an acaricide to penetrate the target organism. Although it has been reported in *R. (B.) microplus* (Schnitzerling et al. 1983), this mechanism has not been much studied.

3.3.4.2. Interest of studying mechanisms of resistance

Studying mechanisms by which pests become resistant to pesticides is important for several reasons. First, if the biochemical or molecular mechanisms of resistance are known, then tools can be developed to rapidly detect the emergence of resistance and be integrated into a resistance management programme. Second, when resistance to a certain compound emerges, knowledge about the resistance mechanism will inform the choice of a suitable replacement compound which will not be affected by the resistance developed against the previous compound thereby avoiding cross-resistance. Third, if the mechanism of resistance is known, pesticide lifetime may be extended once resistance emerges by combining the pesticide with an appropriate synergist. Finally, resistant individuals are valuable for studying the mode of action of the pesticide (Scott 1990).

3.3.4.3. Resistance mechanisms

Resistance mechanisms to OP and amitraz have been studied but not yet elucidated. With respect to OP, more than one acetylocholinesterase enzyme may be targeted and be involved in resistance (Baffi et al. 2008, Temeyer et al. 2010). Several studies attributed *R. (B.) microplus* OP resistance to target site insensitivity although no mutation has been identified yet (Guerrero et al. 2012a) while other authors provided evidence of metabolic resistance (Villarino et al. 2003, Li et al. 2003, Saldivar et al. 2008). Regarding amitraz, target site resistance has been suspected although it has not be demonstrated (Guerrero et al. 2012a) and synergist studies showed that metabolic resistance plays a role in amitraz resistance in some strains (Knowles and Roulston 1973, Li et al. 2004).

Resistance mechanisms to SP are better understood: both target site- and metabolism-based pyrethroid resistance have been identified in *R. (B.) microplus*. Generally, when both mechanisms coexist, target site resistance is the most important one.

Three single nucleotide substitutions have been shown to confer pyrethroid resistance: first, a mutation located in the domain III of the *R. (B.) microplus* sodium channel gene resulting in a

phenylalanine to isoleucine amino acid substitution was identified by He et al. (1999) in Mexican tick populations. More recently, two additional mutations have been identified in domain II of Australian populations resulting in leucine to isoleucine and glycine to valine amino acid substitutions (Morgan et al. 2009, Jonsson et al. 2010a). These three mutations are associated with different resistance phenotypes and have different geographic distributions. The domain III mutation is widespread throughout Mexico but apparently limited to this country and confers very high resistance to flumethrin, cypermethrin and permethrin, while the two domain III mutations confer lower levels of resistance. The mutation described by Morgan et al. (2009) was shown to provide resistance to the same acaricide spectrum as the domain III1 mutation and to be widespread throughout the world (see Chapter 5) while the mutation described by Jonnson et al. (2010) provides resistance to flumethrin but not to cypermethrin and was found only in Australia.

The molecular aspects of metabolic pyrethroid resistance are not yet well-defined in *R. (B.) microplus*. Overproduction of an esterase that hydrolysed permethrin, designated CzEst9, was observed by Jamroz et al. (2000) and Pruett et al. (2002). In addition, synergist studies with PBO have indicated that cytochrome P450s also play a role in pyrethroid resistance in some strains (Miller et al. 1999) but the molecular mechanisms have not been studied yet.

3.4. Diagnosis of resistance

Monitoring of cattle tick resistance is essential on a local and global scale. First of all, tick control failure is not always due to resistance but can be caused by other factors such as faulty equipment, inappropriate dose or expired and therefore ineffective chemicals. Hence, when tick control failure is observed, suspected resistance should be confirmed before selecting a new acaricide. Once resistance is confirmed, farmers need to be advised on using alternative chemicals for controlling the resistant populations. To do so, the susceptibility of the resistant ticks to potential replacement chemicals has to be tested. Tests should include the evaluation of tick susceptibility to previously used compounds to which the ticks may have also developed resistance. In addition, since cross-resistance is known to occur between compounds of the same class, but not systematically, more than one representative of each class should be tested. For example, in the 1970s, there was little cross-resistance among OP compounds was usually not reported for all the compounds of the class (Roulston et al. 1981) and many additional years of successful OP use was achieved by testing for specific OP resistance and using an alternative OP compound (Kemp et al. 1998). As we can see from this example, monitoring of resistance is an essential tool in pest management. In addition, global

monitoring of ticks should facilitate detecting resistance at an early stage which is essential to slowing its spread by avoiding further selection of resistant individuals.

When resistance is suspected, it can be tested by re-treating cattle (*in vivo*) with the same acaricide after ensuring application procedures and doses are correct (Kemp et al. 1998). However, this procedure is costly, and does not provide information on alternative acaricides. Therefore, *in vitro* tests seem more appropriate, because they are less costly and provide more information on which acaricides can be used if resistance is detected. Hence, resistance can be evaluated using *in vitro* bioassays, but also, through the use of biochemical or molecular tests, to detect metabolic- and target site-based resistance, respectively.

3.4.1. Bioassays

Bioassays are based on *in vitro* exposure of ticks (larvae or engorged females) to a single dose or to several increasing doses of an acaricidal compound. The type of contact with the active ingredient (AI) and its duration differ among the tests. An ideal bioassay should meet many requirements (FAO 2004). The diagnostic test should be sensitive enough to identify resistance early in its emergence and should cover the full range of chemical groups that are in use. The test should be simple and inexpensive, require a low number of engorged female ticks and be time-effective. In addition, it should provide rapid and reliable results, and be suitable for standardization among laboratories in many countries. However, none of the available tests meet all these requirements.

In order to have unified standards, the Food and Agriculture Organisation of United Nations (FAO) adopted and recommended the use of a bioassay named larval packet test (LPT) since 1975 (Kemp et al. 1998), and provided a standardised protocol, available in Kemp 1999. This test was considered by the FAO to be the most repeatable and was therefore selected as the test of choice for surveys and for definitive confirmation of resistance despite some limitations due to the laborious nature of this test (FAO 2004). In order to facilitate regional acaricide resistance monitoring and management, an FAO Regional Reference Laboratory for the diagnosis of tick resistance was created in Mexico in 2000 (Kemp et al. 1998). Further reference laboratories were expected to be established in Colombia and Uruguay. However, in 2004, despite its efforts, the FAO pointed out that “a lack of standardized techniques for diagnosing acaricide resistance appears to be the main difficulty in creating and maintaining a tick resistance monitoring system” (FAO 2004). A survey carried out by a FAO Working Group on Parasite Resistance (WGPR), which existed at that time, revealed that the laboratory

method most widely used to diagnose resistance was not the LPT but an adult test called Adult Immersion Test (AIT) (FAO 2004). Therefore the FAO decided to provide a protocol for the AIT and recommended it as a preliminary screening test for resistance because it is easy to use and provide results rapidly.

To evaluate resistance, ticks can be exposed to several increasing doses of acaricides to establish a dose-response curve from which the doses inducing 50% or 90% mortality (LC_{50}, LC_{90}) can be calculated and compared to a susceptible reference strain to determine the corresponding resistance ratios (RR) (Figure 5). Alternatively, to reduce the amount of work and ticks needed to determine whether resistance is present, a dose-response curve can be established for the susceptible reference strain and used to determine a discriminating dose (DD), which should allow identification of resistant isolates (FAO 2004). This DD is calculated as twice the LC_{99} (Jonsson et al. 2007) or twice the $LC_{99.9}$ (FAO 2004) of the susceptible strain. Ticks surviving at this DD are considered resistant. The use and the way to calculate these DD has been criticised, though (Jonsson et al. 2007).

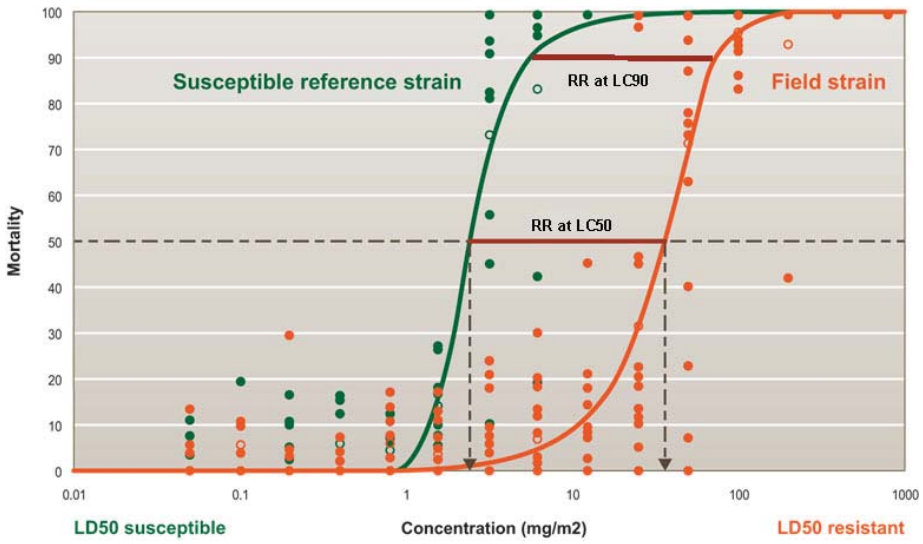

Figure 5: Example of dose-response curves obtained for a field population (orange curve) in comparison to a susceptible reference strain (green curve). Resistance ratios (RR) are calculated by dividing the LC_{50} (or LC_{90}) of the field strain by the LC_{50} (or LC_{90}) of the reference strain.

Below, the protocols of the AIT and LPT are described, as well as another test, the Larval Immersion Test (LIT) which also plays an important role in the diagnosis of acaricide resistance despite not being recommended by the FAO.

3.4.1.1. Adult Immersion Test

The AIT was first developed by Drummond (1973). The principle of the initial Drummond AIT was to treat engorged female ticks with a range of dilutions of an acaricide and to assess the effect of a treatment on egg laying and egg haltching, comparing treated and untreated ticks.

In this protocol, groups of 10 engorged females are weighed, immersed in the different acaricide solutions for 30 seconds, removed from the immersion solutions, placed on a paper towel to dry, and incubated at 27-28ºC and 80-95% RH. After 2 weeks, eggs produced by the ticks in each treatment group are weighed, and incubated to estimate the percentage of hatched larvae.

In this test, efficacy of acaricides is determined by comparing the estimated reproduction (ER) of each group of treated ticks with that of the control ticks. Estimated reproduction is an estimate of the number of larvae produced by each female which is calculated as follows:

$$ER = \frac{weight\ of\ eggs\ laid\ (g)}{number\ of\ females} \times estimated\ hatch\ (\%) \times\ 20000\ (\#\ eggs\ per\ g)$$

Secondly, the ER of each group of treated ticks is compared with that of its control group. The percentage control is calculated as follows: $Control\ (\%) = \frac{ER\ control\ ticks - ER\ treated\ ticks}{ER\ control\ ticks} \times 100$

And the resistance as follows: $Resistance\ (\%) = 100 - Control\ (\%)$

In 2004, when the FAO recommended the use of the AIT, it also proposed a modified AIT protocol as a preliminary screening test for resistance, allowing the test to be completed within 7 days rather than 4 to 5 weeks and requiring fewer engorged female ticks. In this modified protocol, a single discriminating dose is tested instead of several doses, eggs are not weighed and egg laying is observed but not larval hatching. In this protocol, groups of 10 engorged females are immersed in the acaricide solution for 30 minutes, removed from the immersion solutions, dried on paper towel, and incubated. After 7 days, the number of ticks having laid eggs is counted.

In this case, acaricide efficacy is based on the successful inhibition of oviposition. Therefore, ability of the ticks to oviposit after treatment is the criteria used to assess resistance. Hence, the percentage resistance is calculated as:

$$Resistance\ (\%) = \frac{number\ of\ treated\ ticks\ laying\ eggs}{number\ of\ untreated\ ticks\ laying\ eggs} \times 100$$

While providing this simplified protocol, the FAO provided DD values set as the double of the $LC_{99.9}$ of susceptible reference strains. However, this simplified AIT and these DD have been strongly criticized (Jonsson et al. 2007). Indeed, in a study aiming to assess the repeatability of the test and its ability to discriminate between resistant and susceptible ticks, the authors showed that there was a dramatic variation between the DD they obtained using different susceptible strains and the DD proposed by the FAO. In addition, they showed that the application of these DD did not allow discriminating resistant from susceptible tick isolates. Hence, the authors to conclude that the application of DD to the AIT does not provide an effective screening test. Furthermore, the calculation of the DD as the double of the $LC_{99.9}$ as recommended by the FAO (2004) has been criticised by Robertson et al. (2007) who challenged the benefit of these estimations and recommended not to use so high LC values.

Despite the protocols provided by the FAO aiming to contribute to the standardization of the AIT, many different protocols are currently in use, making the comparison of the results difficult. The variations are usually related to the nature of the acaricide (technical grade or commercial), the immersion time (30 sec to 30 min: 30 sec. (Drummond et al. 1973), 1 min (Soberanes-Céspedes et al. 2002), 2 to 30 min (Kumar et al. 2011), 30 min (FAO 2004), and the solvent used for dilution (mix of 25% water, 65% xylene, and 10% Triton-X (Drummond et al. 1973); 0.1% Triton-X100 (Mansucript 1); 40% acetone (Oliveira et al. 2000); water (Mendes et al. 2011)).

3.4.1.2. Larval Packet Test

The LPT was first developed by Stone and Haydock (1962). In this test, tick larvae are exposed to chemically impregnated filter papers and their subsequent mortality is quantified after 24 hours.

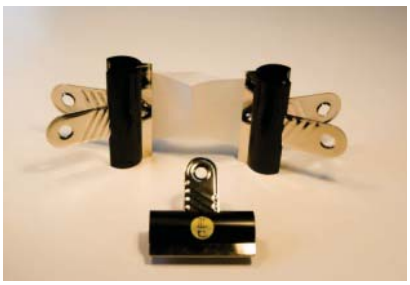

Figure 6: open-ended packet for LPT

Technical grade acaricides are dissolved in two parts trichloroethylene and one part olive oil and this formulation is serially diluted in trichloroethylene:olive oil. A volume of approximately 0.7 ml of each dilution is applied to a filter paper (approx. 7.5–10 cm) and trichloroethylene is allowed to evaporate under a fume hood for 2 h. Treated papers are then folded in half and the sides sealed with bulldog clips forming an open-ended packet (Figure 6). A small cluster of approximately one hundred 7-21 day old tick larvae is picked up from a tube using a fine paintbrush and is inserted into each packet, which is then sealed with a third bulldog clip. Packets are incubated at 80-95% RH and 27-28°C for 24 h and the number of dead and live larvae are recorded. The mortality criterion is the inability of the larvae to walk. Larvae that move their legs but do not walk are counted as dead.

Control packets with the diluent only are prepared for each series to be tested. Control mortality is usually very low, and if greater than 10%, the test should be rejected and repeated (FAO 2004). The larval mortality in the control is used to correct the percentage mortality observed in the test packets by applying Abbott's formula (Abbott 1987):

$$corrected\ \%\ mortality = \frac{\%\ test\ mortality - \%\ control\ mortality}{100 - \%\ control\ mortality} \times 100$$

Several doses are tested for each compound with the goal to test concentrations low and high enough to obtain 0% and 100% mortality, respectively. The LC_{50} or LC_{90} are estimated from the dose-response curves and compared to a susceptible reference strain. Resistance ratios are calculated relative to the reference strain.

$$resistance\ ratio\ 50\ (RR50) = \frac{LC50\ of\ the\ tested\ tick\ isolate}{LC50\ of\ the\ susceptible\ reference\ strain}$$

For amitraz, Miller et al. (2002) developed a modified LPT protocol which improves the dose-mortality relationship and decreases the amount of deviation of the data from their log-probit model. This protocol contains two changes in comparison to the FAO standard protocol: formulated

amitraz instead of technical amitraz is diluted in trichloroethylene: olive oil; nylon fabric is used instead of filter paper. These modifications were also recommended by the FAO (2004).

To decrease the amount of work, the FAO also recommended the use of DD for the LPT and provided various DD for OP and SP (FAO 2004). In addition, following the adoption of the LPT by the FAO as the preferred means of measuring resistance in ticks, the FAO developed an Acaricide Resistance Testing Kit. This kit was initially produced and distributed by the FAO Regional Reference Laboratory created in Mexico in 2000 and contained standardized materials such as the test papers impregnated with acaricides at the FAO-recommended DD and procedures facilitating comparison of data obtained from different parts of the world (FAO 2004).

Although this kit is not distributed anymore and the application of the DD proposed by the FAO is not common practice, the standardized LPT protocol is widespread and, in contrast to AIT, is carried out with little variation between the different laboratories.

3.4.1.3. Larval Immersion Test

The Larval Immersion Test (LIT) was first developed by Shaw (1966) and later modified by Sabatini et al. (2001). In this test, tick larvae are immersed in acaricide dilutions and then incubated for 24 hours before the assessment of mortality.

In the protocol by Sabatini et al. (2001), technical acaricides are diluted in absolute ethanol containing 2% of Triton X-100 and are serially diluted to obtain immersion solutions containing 1% ethanol and 0.02% Triton X-100. Two ml of each immersion solution are distributed in 5 ml tubes. Approximately 500 larvae are transferred to each tube using a paintbrush and immersed for 10 min. After this time, larvae are taken out of the tube, allowed to dry on paper towel and ~100 larvae are transferred to a filter paper packet (see the LPT protocol). Packets are incubated at 27-28°C and 85-95%RH for 24 hours before being opened for evaluation. Larva motility is the criterion used to assess mortality of larvae, as in the LPT.

Control larvae are immersed in the diluent only and mortality of the controls is used to correct the mortality observed in the treated larvae by applying Abbott's formula as in the LPT. As in the LPT, several doses per compound are tested to obtain a full dose-response mortality curve from which the LC_{50} or LC_{90} are calculated, and compared to a susceptible strain to generate the corresponding resistance ratios.

The LIT is carried out following the protocol described by Sabatini et al. (2001) in recent publications reporting the use of the this bioassay (Klafke et al. 2006, Castro-Janer et al. 2011, Klafke et al. 2012) or with a difference in the dilution solution (Castro-Janer et al. 2009, Castro-Janer et al. 2011). The LIT is currently mainly used for the detection of resistance to ivermectin and fipronil and was shown to perform better than the LPT for the detection of resistance to these two compounds (Castro-Janer et al. 2009, Klafke et al. 2012).

3.4.2. Biochemical tools

Metabolic resistance can be diagnosed by measuring the activity of certain enzymes. For example, biochemical studies confirmed the importance of esterase in the metabolic resistance to OP and SP which had been suspected by synergist studies (Jamroz et al. 2000, Pruett et al. 2002, Villarino et al. 2003, Baffi et al. 2007, Baffi et al. 2008). Two categories of esterases are usually studied for potential OP and SP hydrolytic activity, namely the carboxylesterases and the acetylcholinesterases. Potential involvement of esterases in metabolic resistance is investigated through studies measuring hydrolytic activity of these enzymes. To do so, total soluble proteins are extracted from larvae and esterase activity is quantified by gel electrophoresis (Jamroz et al. 2000, Soberanes-Céspedes et al. 2005, Baffi et al. 2008) or colorimetry (Rosario-Cruz et al. 2005). Regarding electrophoresis, proteins can be separated using a non-denaturing polyacrylamide gel (Jamroz et al. 2000, Baffi et al. 2008) or under denaturing conditions (Soberanes-Céspedes et al. 2005). In the latter case, proteins are re-natured after the migration. Esterase activity of the proteins is then detected and visualized by incubating the gel with α-naphthyl acetate and a substrate. In addition, inhibitors can be used to determine the level of activity attributable to acetylcholinesterases or carboxylesterases separately. Hence, gels can be pre-incubated with eserine sulphate to inhibit acetylcholinesterase activity or with thiphenyl phosphate to inhibit carboxylesterase activity. The comparison of esterase activity with and without the inhibitors allows quantification of the activity of the specific esterase class. The colorimetry method is based on the capacity of esterase to hydrolyse α-naphthyl acetate to α-naphthol product, which is detected by colorimetry (Dary et al. 1990).

3.4.3. Molecular tools

There are two prerequisites for the development of molecular tools to diagnose target site resistance. First, the molecule targeted by the compound must be known. Second, mutations conferring resistance to the compound must have been identified in the target. In *R. (B.) microplus*,

these conditions are satisfied only for the pyrethroids. As explained in a previous section, three single nucleotide substitutions have been identified in the voltage-gated sodium channel gene of *R. (B.) microplus* (He et al. 1999, Morgan et al. 2009, Jonsson et al. 2010a). PCR assays have been developed to detect the presence of two of these mutations: Guerrero et al. (2001) designed a PCR assay for the detection of the domain III mutation identified by He et al. (1999) and Morgan et al. (2009) developed a PCR assay for the domain II mutation they identified. For both assays, allele-specific forward primers have been designed so that they contain the wild type or mutated allele at their 3'-end. The reverse primer is a non-diagnostic primer. PCR amplifications are carried out in two separate reactions to detect both the pyrethroid susceptible (wild-type diagnostic primer) and the pyrethroid resistant alleles (mutant diagnostic primer) to identify RR, RS and SS genotypes of individual ticks. Amplified products of 68 bp (Guerrero et al. 2001) and 102 bp (Morgan et al. 2009) are then observed after electrophoresis on agarose gels. These diagnostic PCR assays have been used to investigate the presence of mutations in the sodium channel genes in Mexico (Rosario-Cruz et al. 2005, Rosario-Cruz et al. 2009), Brazil (Andreotti et al. 2011, Domingues et al. 2012), Australia (Chen et al. 2009) and Texas (Miller et al. 2007).

3.5. A sustainable approach of cattle tick control to delay resistance development

The availability of tools to determine acaricide resistance in ticks is a prerequisite for advising farmers how to treat cattle successfully over the short term and how to reduce the risk of further development of resistance over the long term. As shown by the history of acaricide development, the introduction of each new acaricide class was followed by the development of resistance. Therefore, whenever a new class of compounds is introduced on the market, the question is not whether, but when resistance will appear. Hence, to delay the emergence and spread of acaricide resistance, the implementation of strategies to delay resistance in the field is crucial. It is essential to apply good quality acaricidal compounds in a correct manner and at the right dose. Several factors are considered to slow down the emergence of resistance, such as: (1) the reduction of the frequency of treatments. This requirement, however, conflicts with the needs of producers to have a high level of tick control (FAO 2004). (2) limiting the number of ticks exposed to chemical treatments by using a threshold approach. In such an approach, treatments are only applied when a predetermined number of engorged females is reached on each animal (FAO 2004). (3) rotation of acaricides with different modes of action (Thullner et al. 2007). This strategy must be done with great care to avoid the selection of multi-resistant strains. Rotation every two years is advised, or after follow-up with efficiency tests (da Rocha et al. 2011b). (4) the use of combination products; the combination should demonstrate an additive or synergistic increase in acaricide efficacy. In addition to these potential

approaches, monitoring via bioassays, biochemical or molecular tests is essential for early detection of acaricide resistance and to adapt treatment strategies. Furthermore, when possible, chemical control of tick populations should be integrated in a pest management program aiming to decrease the frequency of treatment application and to delay the onset of resistance. Non-chemical methods which can be combined with chemical control are: (1) the use of tick-resistant cattle breeds. Optimal cattle breeds can be achieved by crossing breeds with natural tick resistance and more productive cattle breeds, thereby creating a hybrid with good resistance against ticks and good production. (2) the use of cattle tick vaccines; despite moderate field efficacy, they are very useful in reducing the basal tick burden on cattle (Guerrero et al. 2012b). (3) Rotation between crops and livestock, which involves removal of all livestock from pastures for a period of time long enough to ensure the death of most of free-living ticks (FAO 2004). (4) Biological control, which is the least developed option for cattle ticks, but the use of the fungus *Metarhizium anisopliae*, already commercialised as an insecticide, seems to be successful against *R. (B.) microplus* in laboratory bioassays (FAO 2004) (Elisa Cimitan, personnal communications). Whatever the chosen strategy, education of the farmers is crucial so that they understand the importance and benefit of the methods which should be implemented. Studies have shown that the incorrect use of chemicals is common (Amaral et al. 2011, da Rocha et al. 2011b), accelerating the development of resistance (Santos 2009). Furthermore, recent surveys of milk producers in Brazil showed that they had little knowledge about the tick life cycle, the acaricide mode of action, and were not aware of the mechanisms leading to the development of resistance (da Rocha et al. 2011a, da Rocha et al. 2011b). Therefore, to ensure the sustainable use of currently effective acaricides, it is essential to educate farmers with respects to ticks, tick control, tick resistance and integrated pest management.

3.6. Concluding remark

As shown in the previous chapters, the availability of *in vitro* methods and of molecular tools to determine the resistance status of ticks is of great importance from both a scientific and an economic viewpoint. However, although the available bioassays are very helpful, each of them has flaws which limit the number of compounds and doses that can be tested. The molecular tests, on the other hand, are very specific but are currently rarely used for routine diagnosis. Hence, there is a need to develop new tests which are easy to perform, require only basic infrastructure and provide a maximum of information on acaricide resistance with a limited number of ticks.

3.7. Objectives

Goals

The goals of this work were (i) to develop a new bioassay to evaluate acaricide resistance (ii) to apply this test for the assessment of acaricide resistance in field populations (iii) to investigate the presence of three single nucleotide substitutions conferring resistance to SP and to correlate it to phenotypic resistance.

Specific objectives

- To adapt a pre-existing screening test into a bioassay for evaluation of acaricide resistance in *R. (B.) microplus*.
- To validate this new larval test, named Larval Tarsal Test (LTT), with two laboratory strains by comparing it to the FAO-recommended larval test (LPT)
- To adapt the LTT to be conducted with simplified equipment
- To evaluate the pattern of acaricidal resistance of field populations originating from Brazil, Argentina, South Africa, and Australia, using the LTT.
- To develop a multiplex diagnostic PCR assay to allow the simultaneous detection of three known *R. (B.) microplus* sodium channel gene mutations that are associated with target site pyrethroid resistance
- To investigate the presence of these three mutations in field and laboratory populations originating from Brazil, Argentina, South Africa, Mexico and Australia and to correlate their frequency to phenotypic resistance.

4. RESULTS

4.1 Chapter 1

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

Veterinary Parasitology, 2011, Volume 182, Pages 269-280

4.2 Chapter 2

In vitro diagnosis of the first case of amitraz resistance in *Rhipicephalus microplus* in Santo Tomé (Corrientes), Argentina

Submitted to Veterinary Parasitology as Short Communication on May 24th 2012

4.3 Chapter 3

Determination of Acaricide Resistance in *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) Field Populations of Argentina, South Africa and Australia with the Larval Tarsal Test

Submitted to the Journal of Medical Entomology on June 8th 2012

4.4 Chapter 4

Use of the Larval Tarsal Test to determine acaricide resistance in *Rhipicephalus (Boophilus) microplus* Brazilian field populations

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4.5 Chapter 5

Distribution patterns of three sodium channel mutations associated with pyrethroid resistance in *Rhipicephalus (Boophilus) microplus* populations from North and South America, South Africa and Australia

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4.6 Chapter 6

Acaricide Resistance Mechanisms in *Rhipicephalus (Boophilus) microplus*

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4.1. Chapitre 1 : A new *in vitro* test to evaluate the resistance level against acaricides of the cattle tick, *Rhipicephalus (Boophilus) microplus*

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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).

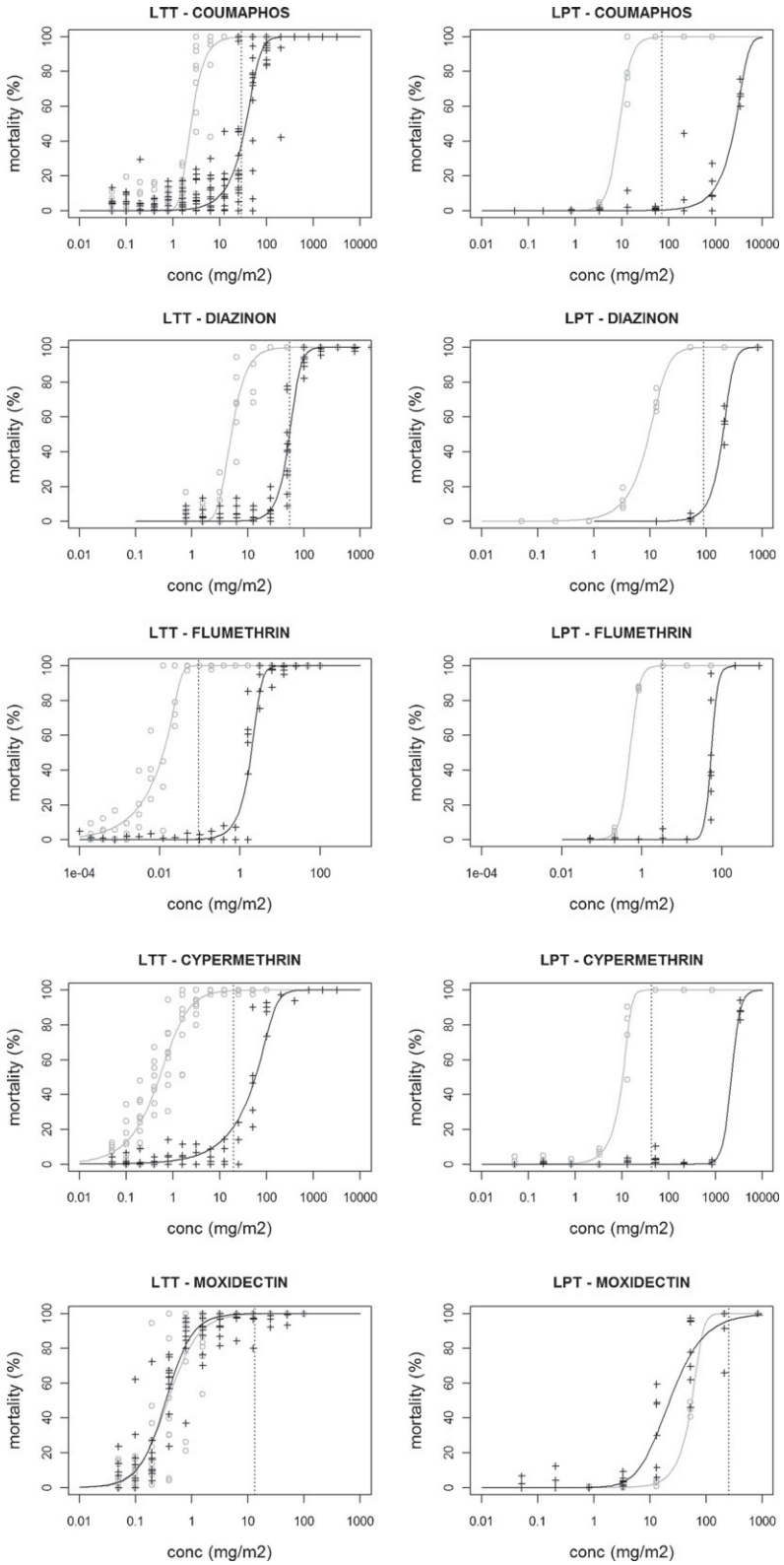

Fig. 1. Dose–response mortality for the susceptible Muñoz (○) and the resistant Ultimo (+) *R. microplus* strains obtained with the LTT (left side) and the LPT (right side). Data were analysed using a nonlinear regression model for all compounds. Dashed lines indicate the concentrations corresponding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

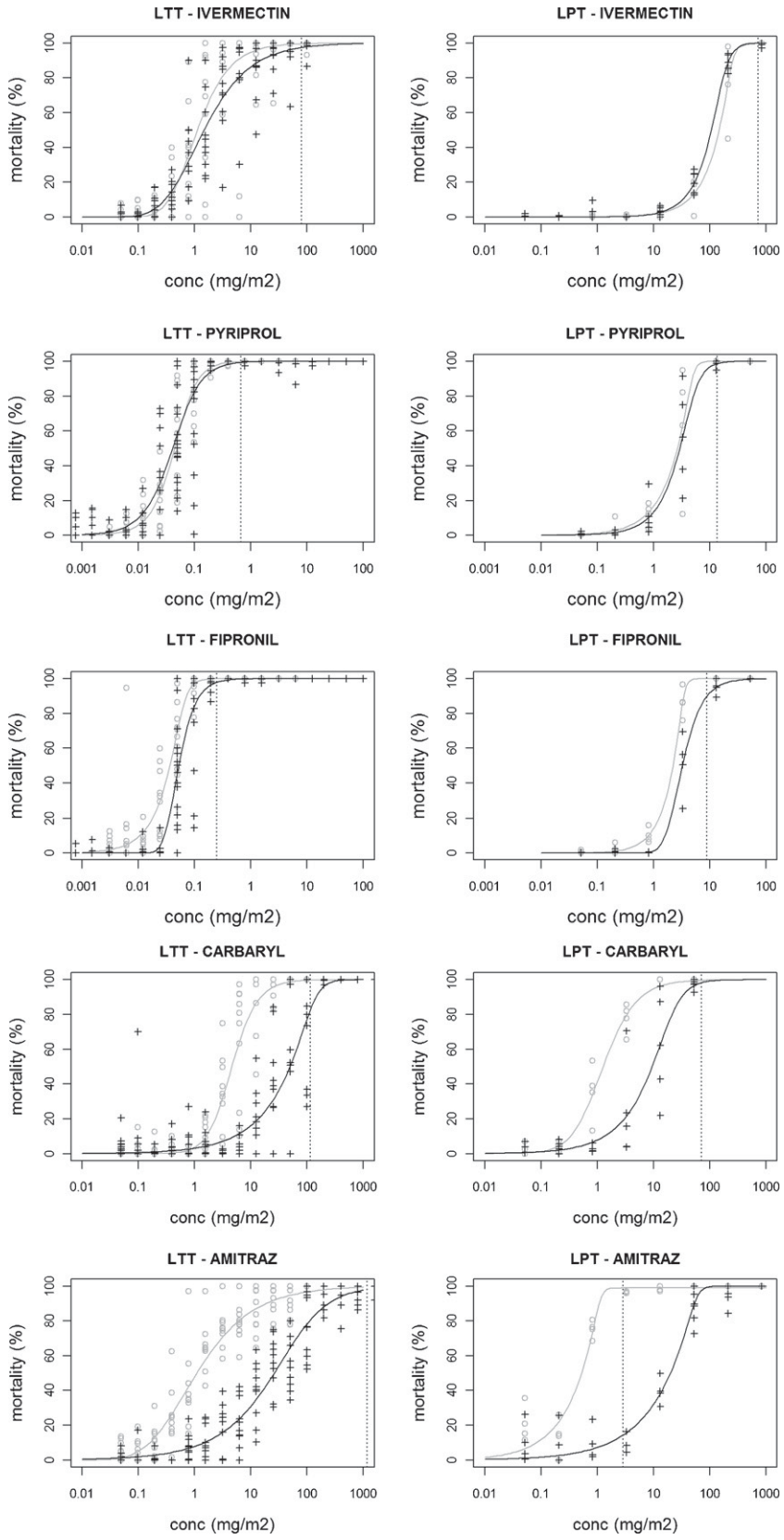

Fig. 1. (*Continued*).

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).

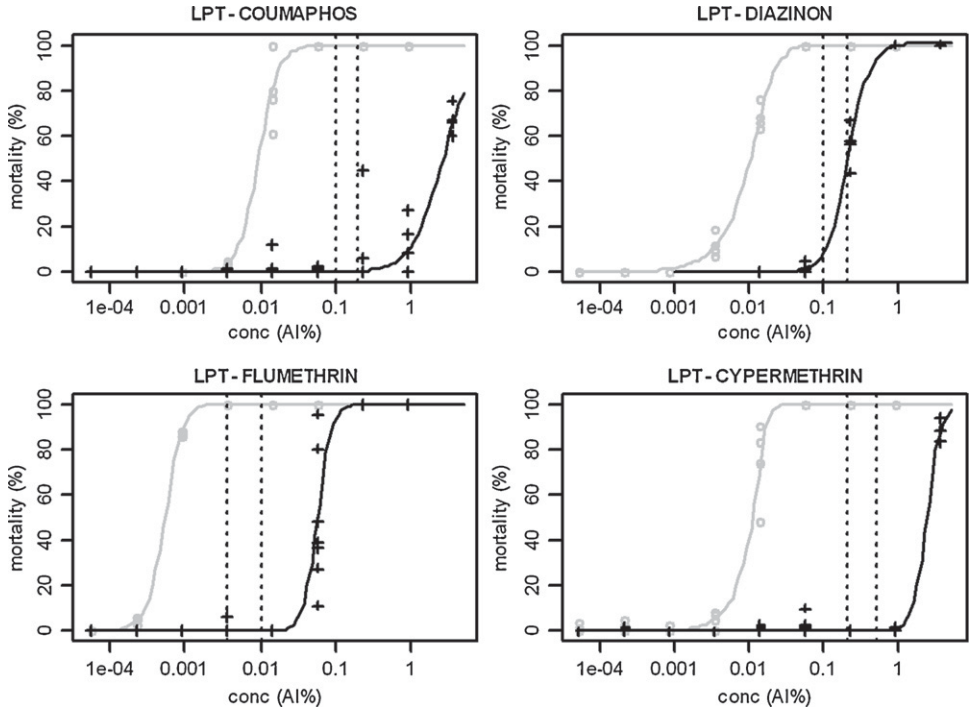

Fig. 2. Dose–response mortality obtained with the LPT after exposure of the susceptible Muñoz (○) and the resistant Ultimo (+) *R. microplus* strains to coumaphos, diazinon, flumethrin and cypermethrin. Dashed lines indicate the DDs recommended by the FAO.

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

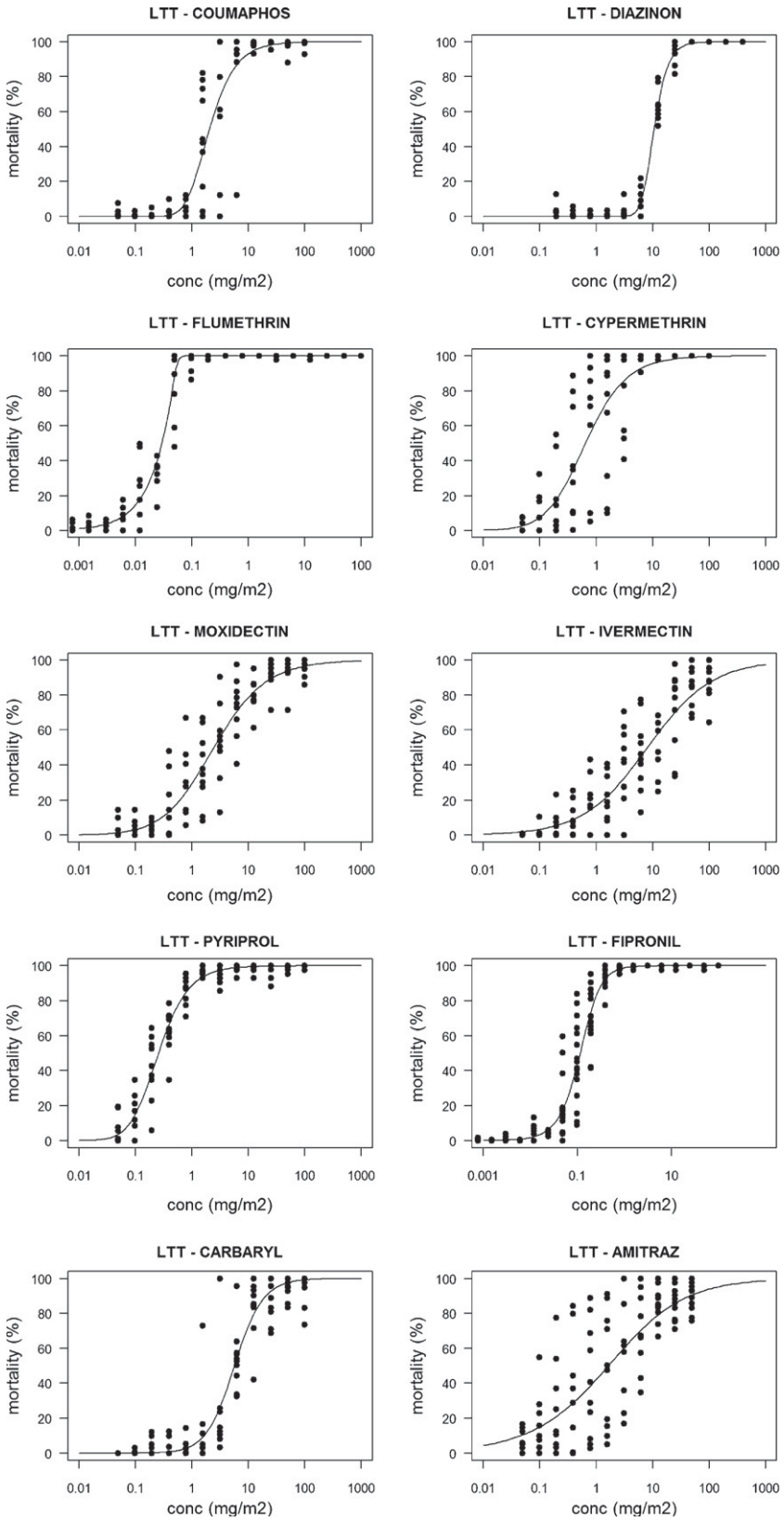

Fig. 3. Dose–response mortality obtained with the LTT for the susceptible Corapeake *R. sanguineus* strain. Data were analysed using a nonlinear regression model.

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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4.2. Chapitre 2: *In vitro* diagnosis of the first case of amitraz resistance in *Rhipicephalus microplus* in Santo Tomé (Corrientes), Argentina

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In vitro diagnosis of the first case of amitraz resistance in *Rhipicephalus microplus* in Santo Tomé (Corrientes), Argentina

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Abstract

In Argentina, the cattle tick *Rhipicephalus microplus* has already developed resistance to organophospates and synthetic pyrethroids. However, no cases of amitraz resistance have ever been recorded in this country despite its heavy use. A recent failure of amitraz to control ticks in a farm located in Santo Tomé, province of Corrientes, resulted in the collection of samples for acaricide resistance diagnosis. The modified Drummond adult immersion test (AIT) and the larval tarsal test (LTT) were performed separately in Argentina and Switzerland to evaluate efficacy of amitraz and other acaricides. The AIT showed that oviposition in the Santo Tomé field isolate was not inhibited when it was challenged to 250 and 500 ppm amitraz, and 50 ppm deltamethrin. However, oviposition was reduced by 90.6% when this field isolate was challenged to a combination of 400 ppm ethion and 100 ppm cypermethrin. To confirm the results obtained with the AIT, 2 additional tick samples were collected and shipped to Switzerland for resistance diagnosis of amitraz, cypermethrin and flumethrin, using the LTT. With this bioassay, the resistance ratios of the 2 field isolates were 32.5 and 57.0 for amitraz and between 5.9 and 27.2 for the synthetic pyrethroids. Both *in vitro* bioassays confirmed amitraz and synthetic pyrethroid resistance in the Santo Tomé samples. These results account for the first evidence of amitraz resistance in *R. microplus* in Argentina.

Keywords: cattle tick, *Rhipicephalus microplus*, amitraz, resistance, Argentina

Introduction

The cattle tick *Rhipicephalus microplus* can be found in the tropical and subtropical central northeastern regions of Argentina, between 22º and 34º south latitudes (Mattos and Signorini, 1989), associated to the biomes of Chaco and Pampa (Estrada-Peña et al., 2006). In Argentina, a variety of acaricides have already been used and as a consequence, *R. microplus* has developed resistance to organophosphates (Pérez Arrieta et al., 1980) and synthetic pyrethroids (Caracostantogolo et al., 1996) in the province of Corrientes. In this province, the most common way to control tick infestations is the application of amitraz by plunge dipping (Guglielmone et al., 2007). Nevertheless, no cases of amitraz resistance have been yet notified in Argentina. Resistance to amitraz has been already described in other countries such as Australia (Nolan, 1981), South Africa (Strydom and Peter, 1999), Brazil (Li et al., 2004), Colombia (Benavides et al., 2000) and Mexico (Soberanes et al., 2002).

A recent failure of amitraz to control cattle ticks in the field was observed in a farm located in Santo Tomé, province of Corrientes. Following this observation, all the infested animals were treated twice with amitraz, with a 9-day interval between the treatments. These treatments were supervised by local staff from SENASA (The National Animal Health and Agri-food Quality Service of Argentina) who corroborated the lack of control using this acaricide. In addition, samples of ticks were collected and submitted for *in vitro* testing to a governmental laboratory, as this is usually the case when animal health authorities in Argentina want to confirm suspect cases of resistance. The most common bioassays used to diagnose acaricide resistance are the larval packet test (LPT) (Stone and Haydock, 1962), the larval immersion test (LIT) (Shaw, 1966), and the adult immersion test (AIT) (Drummond et al., 1973). More recently, a new bioassay named larval tarsal test (LTT) has been developed by Lovis et al. (2011). This test is less laborious than the LPT, and compared to the AIT, has the advantage of requiring a small number of engorged ticks. However, 6 weeks are required to obtain the results, as with the LPT. Thus, none of these bioassays are perfect and they all combine advantages and disadvantages in terms of simplicity, sensitivity, accuracy, and promptness to obtain the results. In the present study, the AIT and the LTT were used to confirm the presence of resistant *R. microplus* populations in Argentina.

Material and methods

Two series of experiments were performed. The first series was carried out in Argentina (The National Institute of Agricultural Technology (INTA)) where efficacy of amitraz, deltamethrin, ethion and cypermethrin against the Santo Tomé field isolate was evaluated in two separate AITs. The second series was performed in Switzerland (Novartis Animal Health Research Centre (CRA)) where resistance to amitraz, cypermethrin, and flumethrin was tested with the LTT.

Ticks

Four samples of ticks were collected between July and November 2010 from a farm located in Santo Tomé, province of Corrientes, where lack of efficacy of treatment with amitraz had been observed. The Muñoz strain (Li et al., 2005) was used as susceptible reference strain for the LTT while the INTA A26 strain was used as susceptible reference strain for the AIT. The INTA A26 was originally collected from a farm in Corrientes in 2009, and maintained since then in the laboratory of parasitology at INTA Castelar. Both susceptible strains are free of *Babesia* and *Anaplasma*.

Acaricides

Commercial acaricides such as Azadieno Plus® (Merial, amitraz 12.5%), Ruster® (Gleba, deltamethrin 2.5%), and Pöhja mix® (Laboratorio Vetué, ethion 40%; cypermethrin 10%) were diluted with 0.1% Triton-X100 (BDH) and used for the AIT. In contrast, for the LTT, technical grade amitraz (Sigma-Aldrich, active ingredient (AI) 99.4%), cypermethrin (Novartis, AI >90%) and flumethrin (Sigma-Aldrich, AI 97%) were diluted with dimethyl sulfoxide (DMSO, Fluka) and used for testing.

Adult Immersion Test

Adult immersion tests were performed in Argentina as described by Drummond et al. (1973) with some modifications related to the immersion time (2 minutes instead of 30 seconds) and the acaricide diluent (0.1% Triton-X100 diluted in distilled water instead of 25% water, 65% xylene, and 10% Triton-X100). Engorged female ticks (EFT) were rinsed in tap water, dried on paper towels, and randomised by size to form as many groups as commercial acaricides to be tested. Groups of ticks were immersed 2 minutes in the different acaricide solutions or in Triton-X100 (control group). Afterwards, ticks were recovered from the immersion solutions, dried on paper towels, and

incubated for 14 days at 27-28ºC and 80-85% relative humidity (RH) for subsequent egg collection once oviposition was completed. Effectiveness of a treatment was determined by the reduction in the oviposition in the treated groups compared to the oviposition in the control group at day 15.

$$\text{Efficacy (\%)} = \left(\frac{\text{Control egg weight mean} - \text{Treated egg weight mean}}{\text{Control egg weight mean}}\right) \text{x } 100$$

Two AITs were performed. In the first AIT, the inhibition of oviposition at different concentrations of amitraz was compared between the Santo Tomé field isolate and the susceptible reference strain INTA A26. The second AIT assessed the oviposition performance of the Santo Tomé field isolate immersed in 250 ppm amitraz, 50 ppm deltamethrin, and 400 ppm ethion combined with 100 ppm cypermethrin in comparison to a control group.

Larval Tarsal Test

The LTT was conducted in Switzerland as described by Lovis et al. (2011) using two samples of the Santo Tomé isolate (ST23 and ST24). Briefly, tick eggs were distributed into the wells of 96-well microtiter plates pre-coated with increasing concentrations of acaricides, and DMSO for the control wells. The plates were then sealed and incubated at 28-29ºC and 70-80% RH to allow egg hatching. Three weeks after the distribution of the eggs, larval mortality was assessed in each well by the absence of motility and general appearance of the larvae. Each test was replicated three times, and resistance was determined by the calculation of resistance ratios (RR) (quotient between the concentration inducing 50% mortality (LC_{50}) of the Santo Tomé field isolate and the LC_{50} of the susceptible Muñoz strain).

Statistical analyses

Statistical analysis of the AIT data was performed with Statistix 8. A Kruskal-Wallis test was used to compare Santo Tomé egg weight means between the different treatment groups in the first and second AIT. Oviposition in the non-treated Santo Tomé and INTA A26 ticks was compared with a Wilcoxon Rank Sum Test (Mann-Whitney U Test). Data analysis of the LTT results was performed on the R software (version 2.12.0) using the drc package (version 2.0-1), specific for modelling dose-response curves (Ritz and Streibig, 2005). Lethal doses inducing 50% mortality, RR and their 95% confidence intervals (CI) were calculated as previously described by Lovis et al. (2011).

Results

Adult Immersion Test

The results of the comparison of the egg weights between the Santo Tomé field isolate and the susceptible INTA A26 strain after immersion in increasing concentrations of amitraz are described in Table 1. Both the field isolate and the susceptible strain laid eggs in absence of amitraz, and there were no significant differences between them (p=0.11). Oviposition in the Santo Tomé ticks was not affected by 250 and 500 ppm amitraz whereas it was completely inhibited in the INTA A26 ticks by any of the two amitraz concentrations. There were no statistical differences (p=0.58) among egg weights of the Santo Tomé isolate in the first AIT.

Table 1. First AIT. Egg weight comparison between the Santo Tomé field isolate and the susceptible laboratory strain (INTA A26) after engorged females were immersed for 2 minutes in 250 ppm and 500 ppm amitraz. Egg weights are expressed as mean ± standard deviation.

Amitraz concentration (ppm)	Egg weight (g)	
	Santo Tomé (n = 10)	INTA A26 (n = 10)
0	0.125^{a}±0.034	0.150^{a}±0.032
250	0.089^{a}±0.044	0±0
500	0.105^{a}±0.025	0±0

Equal superscripts in the same row indicate no statistical differences between egg weights among strains (p=0.11)

Equal superscripts in the same column indicate no statistical differences between egg weights in Santo Tomé isolate (p=0.58)

In the second AIT (Table 2), where the Santo Tomé field isolate was challenged with several acaricides, 250 ppm amitraz and 50 ppm deltamethrin provided efficacies of 1.5% and 3.0% in preventing oviposition, respectively. In contrast, an efficacy slightly over 90% was obtained when testing with the combination of organophosphate and synthetic pyrethroid.

Table 2. Second AIT. Acaricide efficacy determination of the Santo Tomé field isolate. Engorged females were immersed 2 minutes in 250 ppm of amitraz, 50 ppm of deltamethrin, and in the combination of 400 ppm of ethion + 100 ppm of cypermethrin. Egg weights are expressed as mean ± standard deviation.

Acaricide concentration (ppm)	Santo Tomé (n = 14)	
	Egg weight (g)	Acaricide efficacy (%)
0	0.112^{a}±0.018	–
250 amitraz	0.110^{a}±0.017	1.5
50 deltamethrin	0.109^{a}±0.014	3.0
400 ethion + 100 cypermethrin	0.011^{b}±0.025	90.6

Different superscripts indicate statistical differences between egg weights among groups ($p<0.05$)

Larval Tarsal Test

A full dose-response curve was obtained with the LTT for the 2 Santo Tomé samples. Both samples demonstrated resistance to amitraz with RR of 57.0 (95% CI: 41.9-72.0) and 32.5 (95% CI: 24.1-40.8) (Table 3). Resistance to both cypermethrin and flumethrin was also observed with RR between 5.9 (95% CI: 3.0-8.8) and 27.2 (95% CI: 15.4-39.1).

Table 3. Larval Tarsal Test. Comparison of LC_{50} values and RR for amitraz, cypermethrin and flumethrin between the susceptible reference strain (Muñoz) and the two Santo Tomé isolates (ST23, first collection; ST24, second collection).

Acaricide	Tick	LC_{50}* (95% CI)	RR (95% CI)
Amitraz	Muñoz	1.09 (0.89 – 1.29)	–
	ST23	62.06 (45.55 – 78.57)	57.0 (41.9 – 72.0)
	ST24	35.40 (27.17 – 43.64)	32.5 (24.1 – 40.8)
Cypermethrin	Muñoz	0.51 (0.42 – 0.60)	–
	ST23	6.62 (2.92 – 10.33)	13.0 (6.6 – 19.4)
	ST24	13.78 (8.62 – 18.94)	27.2 (15.4 – 39.1)
Flumethrin	Muñoz	0.012 (0.01 – 0.014)	–
	ST23	0.072 (0.036 – 0.108)	5.9 (3.0 – 8.8)
	ST24	0.268 (0.186 – 0.35)	21.8 (14.3 – 29.2)

* Concentrations are in mg/m^2

Discussion

We diagnosed amitraz and synthetic pyrethroid resistance in the Santo Tomé field isolate using two different *in vitro* assays: the AIT and the LTT. These two tests diagnose resistance using different parasitic stages. The main advantage of the AIT is to be easy to perform and to provide a diagnosis of resistance within two weeks, while the LTT requires six weeks before the results are available. On the other hand, the main disadvantage of the AIT is the difficulty to obtain a sufficient number of engorged females to carry out the test. This limitation applies to both the field ticks to be tested and the laboratory susceptible ticks. To overcome this limitation, the AIT was recommended to screen for resistance using a single dose, the discriminating dose (DD) (FAO, 2004), which would perfectly differentiate between susceptible and resistant individuals by killing only the susceptible individuals (ffrench-Constant and Roush, 1990). The DD is determined as 2 x $LC_{99.9}$ or 2 x LC_{99} of a susceptible reference strain to a specific compound (FAO, 2004; Jonsson et al., 2007). However, due to the difficulty to estimate the DD, because of natural biological variation for example (Robertson et al., 2007), it is preferable to test several doses to establish a full dose-response curve and to calculate lethal concentrations (LC_{50}, LC_{90}) and their corresponding resistance ratios whenever possible.

The use of DD has been questioned by Jonsson et al. (2007) when they evaluated the performance of the AIT as an acaricide resistance screening test. In their experiments, in Australia and USA, they found impossible to apply any of the DDs suggested by the FAO (2004), especially for amitraz. Similarly, Miller et al. (2007), who evaluated the ability of three larval bioassays ('Soberanes', 'Miller' and 'White' techniques) to determine amitraz susceptibility, showed the difficulty to obtain a satisfactory dose-response relationship to amitraz and to determine a DD that perfectly discriminates between susceptible and resistant individuals in these bioassays. Despite their limitations, the AIT and larval bioassays are still valuable tools to detect resistance in tick isolates.

The intrinsic simplicity of the AIT may paradoxically counteract against it. As the technique was adapted for the evaluation of different acaricides and was modified by several authors, there is yet no AIT standard protocol. Modifications are usually related to the nature of the acaricide (technical grade or commercial), the immersion time (from 30 sec (Drummond et al., 1973) to 30 min (FAO, 2004)), and the agents (Gonçalves et al., 2007) used to dissolve the acaricides. While Oliveira et al. (2000) determined 5 min as the minimal immersion time to establish the LC_{50} for technical amitraz diluted in 40% acetone, Soberanes et al. (2002) was able to detect resistance to amitraz by immersing engorged females in water-diluted commercial amitraz (12.5%) for 1 min. More recently,

Kumar et al. (2011) found no significant differences in mortality when they compared immersion times varying from 2 to 30 minutes for diazinon, cypermethrin and malathion. This contrasts with Sabatini et al. (2001) who found that the mortality increased proportionally to the immersion time from 30 sec to 30 min when diluting commercial abamectin in water. The present study provides evidence of significant acaricide effects using 2 min as the immersion time for amitraz, deltamethrin and a combination of an organophosphate and synthetic pyrethroid. All the examples cited above account for the need of a standardisation of the variables that affect the performance of the AIT.

In the present study, no DD based on the susceptible INTA A26 was available. Therefore, in the first AIT, amitraz was tested at the recommended label concentration (250 ppm) and its double (500 ppm) to corroborate the failure of amitraz observed in the farm. The production of eggs at these two concentrations by the field isolate but not by the susceptible ticks was a clear indication of resistance in this assay. For the second AIT, in which various acaricides (including amitraz) were tested at their recommended label concentration, no susceptible INTA A26 ticks were available for testing. Hence, efficacy of the acaricides was tested only on the field isolate and compared to a control group. As expected, and in agreement with the result of the first AIT, the efficacy of amitraz was very low. The egg masses laid by the Santo Tomé ticks immersed in 250 ppm amitraz in the first and second AIT showed no statistical differences (0.089±0.044 vs. 0.110±0.017; p=0.26). Similarly to amitraz, the inefficacy of 50 ppm deltamethrin to inhibit oviposition in the field isolate was also evident. Only the acaricide combination of an organophosphate and a synthetic pyrethroid constituted an acceptable alternative of treatment reducing oviposition by 90 percent.

To confirm the AIT results, two additional samples of ticks were collected in the Santo Tomé farm and shipped to Switzerland where the LTT was performed (Table 3). Amitraz and synthetic pyrethroids resistance was diagnosed in the two Santo Tomé field isolates, corroborating the AIT results. The significant difference observed between the LC_{50} of the two samples for amitraz and flumethrin probably results from the natural biological variation of the samples collected in the farm.

To conclude, both the AIT and LTT confirmed amitraz and synthetic pyrethroid resistance in the Santo Tomé field isolate. Following the *in vivo* and *in vitro* results, the SENASA implemented in the Santo Tomé farm an Integrated Control Programme (ICP) based on the seasonal population dynamics of *R. microplus* (Ivancovich et al., 1984). This programme consists of the monitoring of a strategic rotational treatment scheme between the pour-on application of fluazuron, a chitin synthesis inhibitor, and the plunge dipping in a mixture of ethion and cypermethrin. Finally, as this emergence

of amitraz resistance in Santo Tomé, Corrientes, has raised concern to the Animal Health Authorities, the SENASA is planning a survey that will involve a local sampling to further investigate this situation and the possible spread of resistance to neighbouring farms.

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4.3. Chapitre 3: Determination of Acaricide Resistance in *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) Field Populations of Argentina, South Africa and Australia with the Larval Tarsal Test

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Lovis et al.: Acaricide Resistance Determined by Larval Tarsal Test

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Determination of Acaricide Resistance in *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) Field Populations of Argentina, South Africa and Australia with the Larval Tarsal Test

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Abstract

Infections with ticks have an important economic impact on the cattle industry worldwide and resistance to acaricides has become a widespread phenomenon. In order to optimise their treatment strategy the farmers need to know if and against which classes potential acaricide-resistance does occur. Bioassays are used to assess the resistance level and pattern of *Rhipicephalus (Boophilus) microplus* populations. The objective of the present study was to evaluate the capacity of the recently described Larval Tarsal Test (LTT) to assess the susceptibility of field populations originating from Argentina (8), South Africa (3) and Australia (2) to nine acaricidal compounds from five major classes: organosphosphates (OP), synthetic pyrethroids (SP), macrocyclic lactones (ML), pyrazols (PYZ) and amitraz. The resistance ratios at concentration inducing 50% and 90% mortality were used to detect established and emerging resistance. This study confirmed the newly reported presence of amitraz resistance in populations from Argentina. In addition, resistance to SP appeared to be widespread (7/8, 88%) in the selected Argentinean farms. In South Africa one of three populations was found to be resistant to SP and to a PYZ compound (pyriprol). Furthermore, resistance to OP and SP was observed in Australia. Finally, the LTT proved to be a suitable test to evaluate the susceptibility of *R. (B.) microplus* field populations to the most relevant acaricidal classes.

Key words: Larval Tarsal Test, *Rhipicephalus (Boophilus) microplus*, Argentina, South Africa, Australia, acaricide resistance

Resumen

Las infestaciones por garrapatas tienen un importante impacto económico en la industria del ganado de todo el mundo y la resistencia a los acaricidas se ha convertido en un fenómeno generalizado. Con el fin de optimizar la estrategia de los tratamientos, los ganaderos necesitan saber en contra de cuáles clases de acaricidas ocurre esa potencial resistencia. Se utilizan bioensayos para evaluar el patrón y nivel de resistencia de *Rhipicephalus (Boophilus) microplus*. El objetivo del presente estudio fue evaluar la capacidad de la recientemente descripta prueba del tarso de las larvas (LTT) para evaluar la susceptibilidad de poblaciones de campo procedentes de Argentina (8), Sudáfrica (3) y Australia (2) a nueve compuestos acaricidas de cinco clases principales: organofosforados (OP), piretroides sintéticos (SP), lactonas macrocíclicas (ML), pirazoles (PYZ) y amitraz. Para detectar resistencia establecida y emergente, se calcularon niveles de resistencia basados en concentraciones que inducen mortalidad a 50% y 90%. Este estudio confirma la nueva denuncia de la presencia de resistencia al amitraz en las poblaciones de garrapatas de Argentina. Además, la resistencia a SP parece estar muy difundida (7/8, 88%) en los establecimientos argentinos seleccionados. En Sudáfrica, en una de las tres poblaciones, se encontró que era resistente a SP y a un compuesto PYZ (pyriprol). Además, resistencia a OP y SP fue observada en Australia. Finalmente, la LTT ha demostrado ser un ensayo adecuado, para evaluar la susceptibilidad de poblaciones de campo de *R. (B.) microplus* a las clases acaricidas más relevantes.

Introduction

Ticks are the major limiting factor to cattle husbandry in many tropical and sub-tropical areas and cause important economic losses (Graf et al. 2004). Among them, the southern cattle tick *Rhipicephalus (Boophilus) microplus* (Canestrini) has developed resistance to all the available classes of acaricides with the exception of the growth regulators and the naturalytes (FAO 1998, FAO 2004, Castro-Janer et al. 2011). North-Argentina is at the southern limit of its distribution in Latin America, and cattle ticks tend to spread towards south despite the current eradication program (d'Agostino 2010). In 1992, 9 million cattle were estimated to be infested with *R. (B.) microplus* ticks (Guglielmone 1992) and total losses were estimated to be over 150 million US dollars (Späth et al. 1994). However few reports of acaricide resistance in Argentina are available in the literature. Resistance to organophosphates (OP) was first reported in the 1970s (Grillo Torrado and Gutiérrez 1970, Grillo Torrado and Pérez Arrieta 1977) while synthetic pyrethroids (SP) resistance was first identified in 1996 in Argentina (Caracostantógolo et al. 1996). The first case of amitraz resistance was very recently described in the province of Corrientes (Cutullé et al. , submitted). There is also only little information available on acaricide-resistance of *R. (B.) microplus* in South Africa. Its resistance to OP was first reported in 1979 (Baker et al. 1979). Later, resistance to SP and to amitraz have also been identified in *Boophilus* spp., firstly without distinction of the species (FAO 1998, Strydom and Peter 1999, de Bruin 1999) and in 2008 in *R. (B.) microplus* (Ntondini et al. 2008). In Australia, *B.(R.) microplus* was introduced accidentally with imported cattle, probably before 1870 (Angus 1996, Graf et al. 2004) and spread since then to the northern and the eastern part of the country (Cutullé et al. 2009). Acaricide resistance in Australia is well documented. Resistance to OP appeared in the mid-1960s (Shaw and Malcolm 1964, Shaw 1966, Roulston et al. 1968) and was widespread by the mid-1970s (Roulston et al. 1981). Resistance to SP appeared in the late 1980s (Nolan et al. 1989) and increased rapidly (FAO 1998, Jonsson et al. 2000). In comparison, amitraz resistance, which appeared in the early 1980s (Nolan 1981), spread in Australia much more slowly (FAO 1998, Jonsson et al. 2000, Jonsson and Hope 2007). To our knowledge, no resistance to macrocyclic lactones (ML) or pyrazol (PYZ) compounds has ever been reported in any of these three countries.

Various bioassays are used to evaluate tick susceptibility, such as the adult immersion test (AIT) (FAO 2004), the larval packet test (LPT) (FAO 2004) and the larval immersion test (LIT) (Shaw 1966, Sabatini et al. 2001). More recently a new bioassay, the larval tarsal test (LTT), was developed and compared to the LPT and was shown to be equally sensitive to detect resistance to coumaphos, SP and amitraz (Lovis et al. 2011). The advantage of the LTT is to allow testing a large number of compounds and doses in little time and with a small number of engorged females.

The objective of the present study was to apply the LTT to field populations originating from different countries and to evaluate its capacity to detect resistance in the field. Hence tick populations from Argentina, South Africa and Australia were collected and their susceptibility to nine compounds from five major classes (OP, SP, ML, PYZ and amitraz) was evaluated with the LTT.

Materials and Methods

Tick Strains

The Muñoz strain was used as the susceptible reference strain. This strain was collected during an outbreak in Zapata County, Texas, USA, in 1999. It was then established and reared without acaricide selection at the Cattle Fever Tick Research Laboratory (CFTRL), Edinburg, Texas. Some larvae of the F48 generation were transferred to the Novartis Animal Health Research Center (CRA), St-Aubin, Switzerland in 2010 to establish a colony. Ticks used for the bioassays were from F49 and F50 generations.

Engorged females were collected in Australia, South Africa and Argentina and were shipped to Switzerland for *in vitro* testing at CRA. Australia: In February 2009, engorged females of *R*. (*B*.) *microplus* were collected in two beef cattle farms from Mount-Urah and Curra municipalities, Queensland, Australia (Fig. 1) and shipped to the CRA to establish a colony which was maintained without acaricide selection. F3 and F4 generations were used for *in vitro* testing of the Urah and Curra strain, respectively. South Africa: In February and April 2010, three samples of 10-40 engorged females of *R*. (*B*.) *microplus* were obtained from South Africa. *R*. (*B*.) *microplus* was morphologically differentiated from *Boophilus decoloratus* at collection using a stereomicroscope (Walker et al. 2003). Two samples were collected from cattle hold on communal lands where cattle belong to several owners (Pleetenberg Bay area, Western Cape (ST11) and Eglington, Hluvukani area, Mpumalanga (ST15). The third population (ST12) originated from a familial beef cattle farm located in Pleetenberg Bay, Western Cape (Fig. 1). Argentina: In November 2010, eight *R*. (*B*.) *microplus* samples were collected from seven beef cattle farms of the province of Corrientes, North-East Argentina where farmers were complaining about some lack of treatment efficacy. The samples contained 13-41 engorged females collected from 6-15 infested cows and were originating from the following four municipalities: Loreto (ST27), Saladas (ST21, ST22), Santo Tomé (ST24, ST26, ST29, ST30), Virasoro (ST25) (Fig. 1). Ticks from ST21 and ST22 were collected in the same farm, but on

cattle which had been treated differently and were therefore considered as two separate populations. Populations originating from South Africa and Argentina were intended to be tested *in vitro* at arrival in Switzerland without being beforehand maintained on calves.

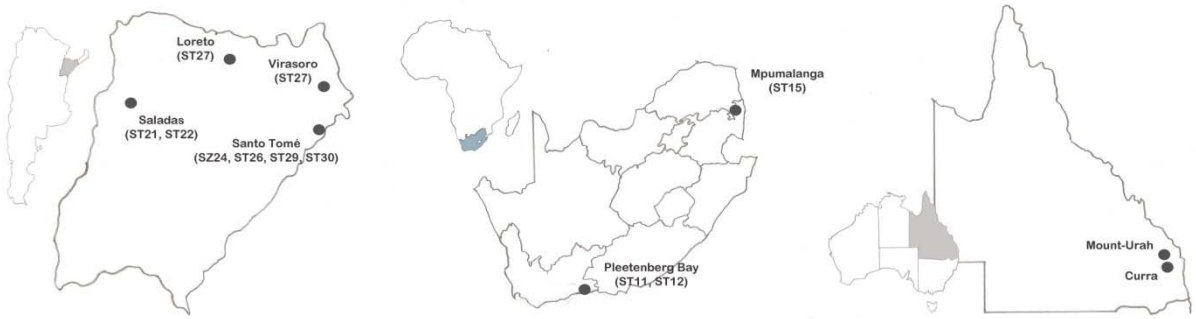

Figure 1. Locations of the populations collected in Argentina (Province of Corrientes), South Africa (Provinces of Western Cape and Mpumalanga) and Australia (State of Queensland)

For the shipment, engorged females were placed in glass tubes with meshed lids hold in a soft structure and kept in a polystyrene box containing a piece of cloth soaked with distilled water to ensure sufficient humidity. The polystyrene box was placed in a foamed box protected by a cardboard. At arrival in the CRA, ticks were moved to larger containers and maintained at 28+/-1 °C and 80% relative humidity (RH) to complete oviposition. Eggs were used for *in vitro* testing around a week before hatching (F1 generation). For two strains (ST11 and ST12), the use of perforated Falcon tubes instead of glass tubes negatively impacted the preservation of the eggs laid during shipment. At arrival in the CRA, the remaining healthy eggs were allowed to hatch and larvae were used to infest a calf. The resulting engorged females produced a sufficient number of healthy eggs (F2 generation) for *in vitro* testing.

Acaricides

Technical grade amitraz, coumaphos (OP), cypermethrin (SP), diazinon (OP), fipronil (PYZ), flumethrin (SP), ivermectin (ML), moxidectin (ML) and pyriprol (PYZ) were used in this study. Details on these compounds are available in Lovis et al. (2011) (Table 1). Technical grade compounds were dissolved in dimethyl sulfoxide (DMSO; Fluka, Switzerland) to prepare stock solutions at 20,000 parts per million (ppm).

Larval Tarsal Test (LTT)

The LTT was carried out as described previously (Lovis et al. 2011). Briefly, 20 µl of a coating solution containing 100% ethanol and 0.25% of olive oil (Sigma-Aldrich, Fluka, Switzerland) was dispensed into each well of flat bottom 96-well plates (NUNC, Catalogue No. 260836, Denmark) and ethanol was allowed to evaporate overnight under a fume hood. Then, a top dose of each acaricidal compound was prepared in DMSO from the stock solution and 12 twofold dilutions were subsequently prepared. A volume of 5 µl of each dilution was dispensed on the bottom of the corresponding wells of the microtiter plates, i.e. a concentration of 566 ppm corresponded to 100 mg/m^2. The upper and lower rows as well as one of the inner rows of the plates always contained 5 µl of DMSO only. This set-up allowed testing five compounds per plate and each concentration was tested in triplicates on three separate plates. The inner rows containing only DMSO were used as a control. DMSO was evaporated either by using an N_2 sampler concentrator (Techne DB-3 Dri-Block, Witec AG, Switzerland) or a centrifugal vacuum concentrator (SC21017 SpeedVac® Plus, ThermoSavant).

Plates were used for testing within three days after preparation. Around 50 eggs were distributed per well using a seed counter (elmor, Switzerland). Plates were incubated for 24 hours at 28+/-1 °C and ~95% RH before being sealed with a transparent sealing film (VIEWseal, Greiner bio-one, Switzerland). Sealed plates were then placed at 28+/-1 °C and 70-80% RH. Plates were removed from the environmental chamber around two weeks after hatching and larval mortality was evaluated by counting dead or alive larvae using a stereomicroscope. Larval motility and global appearance were used as criteria to assess mortality.

The Argentinean populations were all tested with the nine selected compounds except ST30 which was tested with only one compound of each class due to a limited number of ticks available. The Australian and South African populations were tested with the same nine compounds except diazinon which was not tested. All the compounds were tested at the same concentration range (0.05-100 mg/m^2) for the two Australian strains. This range was adapted for the Argentinean and South African populations and the following concentrations were tested: fipronil, flumethrin, pyriprol: 0.003-6.25 mg/m^2; moxidectin: 0.05-100 mg/m^2; ivermectin: 0.05-100 or 0.2-400 mg/m^2; cypermethrin: 0.2-400 mg/m^2; amitraz: 0.1-200 or 0.4-800 mg/m^2; coumaphos, diazinon: 0.4-800 mg/m^2.

Statistical Analysis

Data were entered in Excel software (Microsoft Office 2003) and transferred to Intercooled STATA release 11.0 (StataCorp, College Station, TX, USA). All mortality values were normalized by the mortality of the DMSO control wells using Abbott's formula (Abbott 1987). Outer wells of the microplates with increased mortality due to edge effects in plates were removed.. The R software (version 2.12.0) was used for statistical analysis using the drc package (version 2.0-1), specific for modelling dose-response curves (Ritz and Streibig 2005). Dose-mortality data were modelled using a five-parameter log-logistic function (drm command) with the lower and upper limits locked at 0 and 100, respectively. Doses inducing 50% mortality (LC_{50}), LC_{90} and LC_{99} and their 95% confidence intervals (CI) were estimated with the ED command using the delta option. Resistance ratios based on the LC_{50} (RR50) and on the LC_{90} (RR90) were calculated in reference to the susceptible Muñoz strain using the SI command and the Delta interval for their 95% CI. Resistance ratios were considered significant if their 95% CI did not include 1. Three classes based on RR values were created to emphasize the increasing resistance intensity. Populations were considered susceptible to a specific compound when the RR was smaller or equal to 4, moderately resistant for $4<RR\leq10$ and highly resistant for RR greater than 10. Potential discriminating doses (DD) were calculated as $2xLC_{99}$ of the susceptible reference strain (Jonsson et al. 2007). The survival rates of the field strains at the DD were predicted using the PR command. Discriminating doses were not generated for amitraz as the use of a single DD is not recommended for this compound (FAO 2004, Jonsson et al. 2007, Lovis et al. 2011).

Results

Doses inducing 50% and 90% mortality as well as their 95% CI are displayed in Table 1. The susceptible Muñoz strain was used as reference for comparison with the field populations and RR50, RR90 as well as their 95% CI are available in Table 1. In addition, table 1 includes the survival rates of the field populations at the DD. Data are divided into sections (1a to 1e) corresponding to the acaricide classes.

The 95% CI of LC90 and of RR90 were wider than those calculated for LC50 and RR50. Resistance statuses were therefore established based on RR50, and then compared in the discussion to those based on RR90. Some discrepancy was observed between the ability of the two estimates to detect resistance in case of absence of parallelism between the dose-response curves of the field populations and the reference strain as illustrated in Fig. 2 for flumethrin and amitraz.

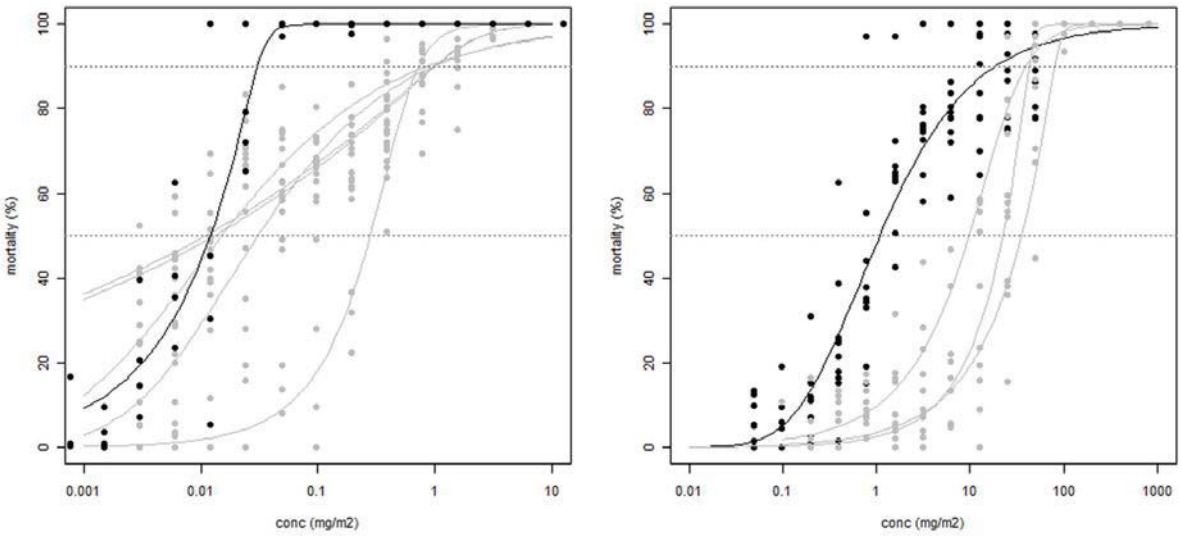

Figure 2. Dose-response curves of some Argentinean field populations (grey) in comparison to the susceptible reference Muñoz strain (black) (A) ST22, ST25-29 when tested with flumethrin. (B) ST24, ST25 and ST30 when tested with amitraz. The grey dotted horizontal lines indicate 50% and 90% mortalities

Resistance Status by Country Based on RR50

In Argentina, resistance to SP was the most common. Cypermethrin resistance was detected in all the populations except one (7/8, 88%) with RR50 ranging from 4.2 (2.5-5.9) to 57.0 (37.5-76.5). In addition, two of these populations also demonstrated resistance to flumethrin based on RR50. Resistance to amitraz was observed in three of the eight populations (38%) with RR50 ranging between 9.0 (5.9-12.1) and 32.5 (24.1-40.8). One case of moderate resistance to OP (diazinon, RR50=5.4, 4.7-6.1) was recorded, while all the populations were shown to be susceptible to coumaphos, ML and PYZ. Analysis of the three populations originating from South Africa revealed that two of them (ST11, ST12) were susceptible to all compounds while the third one (ST15) was considered as highly resistant to SP (RR50=101.5, 72.4-130.6) and moderately resistant to pyriprol (RR50=9.9, 6.0-13.9). Finally the two Australian populations showed similar resistance profiles to the

nine tested compounds, both being moderately resistant to coumaphos and highly resistant to SP while they were susceptible to all other compounds.

Discriminating Doses

Survival rates at the DD of the resistant populations (based on RR50) were all greater than 10%, ranging from 16.1 to 100%. A single exception was observed with ST27 when tested with diazinon (5.2% survival at DD). Survival rates at the DD of the susceptible populations (based on RR50) were below 10% with the following five exceptions out of 108 tests: ST30 tested with coumaphos (15.2% survival at DD); ST22, ST25, ST26 and ST27 tested with flumethrin (survival rates between 26.1 and 34.5% at DD).

Table 1. Lethal concentrations 50 and LC_{90} with their 95% CI obtained for the 8 Argentinean (ST21-ST30), 3 South African (ST11-ST15) and 2 Australian (Curra, Urah) field strains of *R. (B.) microplus* as well as their RR in comparison to the susceptible reference strain (Muñoz) and their survival rates at the DD when tested with coumaphos and diazinon. Concentrations are expressed in mg/m2.

		COUMAPHOS									DIAZINON								
country	strain	LC50	(95% CI)	RR at LC50	(95% CI)	LC90	(95% CI)	RR at LC90	(95% CI)	PR[a]	LC50	(95% CI)	RR at LC50	(95% CI)	LC90	(95% CI)	RR at LC90	(95% CI)	PR[a]
	Muñoz	2.4	(2.15-2.64)			5.4	(3.88-6.85)				5.0	(4.59-5.47)			10.8	(8.62-13.07)			
ARG	ST21	4.6	(3.56-5.54)	1.9	(1.5-2.4)	13.3	(5.08-21.49)	2.5	(0.8-4.1)	2.2	9.8	(8.65-10.89)	1.9	(1.7-2.2)	17.9	(13.59-22.16)	1.7	(1.2-2.1)	0.3
	ST22	6.6	(5.12-8)	2.8	(2.2-3.3)	17.0	(5.31-28.73)	3.2	(1.2-5.1)	3.6	13.0	(11.84-14.21)	2.6	(2.3-2.9)	20.0	(11.1-28.99)	1.9	(1.1-2.7)	0.1
	ST24	3.5	(2.69-4.39)	1.5	(1.1-1.9)	21.6	(8.26-34.98)	4.1	(1.3-6.8)	7.4	8.9	(7.85-9.89)	1.8	na	17.2	(13.76-20.56)	1.6	na	0.3
	ST25	4.1	(3.41-4.81)	1.7	(1.3-2.1)	14.3	(7.74-20.93)	2.7	(1.1-4.3)	3.5	10.6	(10.22-11.03)	2.1	(1.7-2.4)	14.0	(12.23-15.72)	1.4	(0.8-1.9)	0.0
	ST26	6.1	(4.5-7.66)	2.7	na	14.2	(5.21-23.13)	2.4	(2.1-2.8)	0.7	15.4	(13.77-17.11)	3.6	(3.1-4)	22.4	(16.68-28.06)	2.3	(1.8-2.7)	0.1
	ST27	4.6	(3.55-5.6)	1.9	(1.4-2.4)	8.7	(4.12-13.24)	1.6	(0.6-2.7)	0.1	27.3	(24.77-29.9)	5.4	(4.7-6.1)	46.8	(35.05-58.51)	4.3	(2.9-5.8)	5.2
	ST29	3.1	(2.6-3.67)	1.3	(1-1.6)	9.7	(5.03-14.4)	1.8	(0.7-2.9)	1.7	6.5	(5.72-7.3)	1.3	(1.1-1.4)	10.9	(6.15-15.57)	1.0	(0.6-1.4)	0.0
	ST30	5.1	(3.98-6.2)	2.1	(1.5-2.7)	49.9	(18.64-81.23)	9.2	(1.6-16.8)	15.2	na	na	na	na	na	na	na	na	na
SAFR	ST11	4.7	(3.38-6.11)	2.0	(1.1-2.9)	9.4	(4.12-14.71)	1.7	(-0.4-3.8)	0.0	na	na	na	na	na	na	na	na	na
	ST12	5.6	(4.48-6.66)	2.3	(1.8-2.9)	9.7	(5.98-13.36)	1.8	(0.9-2.8)	0.0	na	na	na	na	na	na	na	na	na
	ST15	5.6	(4.01-7.25)	2.4	(1.8-3)	21.1	(4.83-37.37)	3.9	(1.3-6.5)	6.4	na	na	na	na	na	na	na	na	na
AUS	Curra	15.2	(9.43-21.05)	6.4	(4.3-8.5)	85.7	(-5.57-177)	16.0	(1.5-30.5)	29.7	na	na	na	na	na	na	na	na	na
	Urah	13.5	(9.74-17.22)	5.6	(3.9-7.3)	40.0	(25.9-54.17)	7.5	(4-10.9)	24.1	na	na	na	na	na	na	na	na	na

Colour code: RR≤4 are represented on a light grey background; 4<RR≤10 are represented on a dark grey background; RR>10 are represented on a black background

na: not available

[a] predicted survival rates at the potential discriminating doses (2xLC_{99} of the susceptible reference Muñoz strain)

Table 2. Lethal concentrations 50 and LC_{90} with their 95% CI obtained for the 8 Argentinean (ST21-ST30), 3 South African (ST11-ST15) and 2 Australian (Curra, Urah) field strains of *R. (B.) microplus* as well as their RR in comparison to the susceptible reference strain (Muñoz) and their survival rates at the DD when tested with cypermethrin and flumethrin. Concentrations are expressed in mg/m2.

		CYPERMETHRIN									FLUMETHRIN								
country	strain	LC50	(95% CI)	RR at LC50	(95% CI)	LC90	(95% CI)	RR at LC90	(95% CI)	PR[a]	LC50	(95% CI)	RR at LC50	(95% CI)	LC90	(95% CI)	RR at LC90	(95% CI)	PR[a]
	Muñoz	0.51	(0.42-0.6)			2.2	(0-2.2)				0.01	(0.01-0.014)			0.030	(0.023-0.038)			
ARG	ST21	0.87	(0.63-1.1)	1.7	(1-2.4)	4.7	(3.4-6)	2.1	(0.9-3.3)	0.0	< 0.003[b]	na	< 1	na	0.006	(0.004-0.009)	0.2	na	0.0
ARG	ST22	3.5	(2.1-4.9)	6.8	(3.7-10)	48.3	(10.4-86.1)	21.7	(2.5-40.9)	20.5	0.01	(0.001-0.025)	1.2	(0.3-2)	1.01	(0.27-1.76)	36.5	(2.7-70.3)	34.5
ARG	ST24	13.8	(8.6-18.9)	27.2	(15.4-39.1)	68.0	(28.3-108)	30.2	(8.8-51.6)	40.5	0.27	(0.186-0.35)	21.8	(14.3-29.2)	1.38	(0.67-2.1)	46.3	(19.5-73.1)	67.2
ARG	ST25	3.4	(1.5-5.2)	6.6	(4-9.3)	36.6	(-2.7-75.9)	16.4	(3.2-29.5)	16.7	0.03	(0.019-0.043)	2.5	(1.7-3.4)	0.98	(-0.08-2.04)	31.3	(5-57.7)	31.9
ARG	ST26	2.9	(2.2-3.6)	5.6	(3.4-7.9)	51.0	(20.4-81.6)	23.2	(2.3-44.2)	18.2	0.02	(0.01-0.02)	1.3	(0.8-1.7)	0.85	(0.16-1.54)	27.8	(2.2-53.5)	26.1
ARG	ST27	2.2	(1.7-2.6)	4.2	(2.5-5.9)	43.4	(18.2-68.7)	19.4	(1.3-37.4)	16.1	0.01	(0.002-0.02)	0.9	(0.2-1.6)	0.97	(0.28-1.67)	32.2	(8.8-55.6)	33.4
ARG	ST29	29.1	(21-37.1)	57.0	(37.5-76.5)	68.7	(39.7-97.7)	30.8	(13.3-48.4)	67.4	0.28	(0.229-0.333)	23.0	(16.7-29.3)	0.73	(0.44-1.02)	24.0	(11.4-36.6)	83.1
ARG	ST30	10.6	(3.2-18)	21.5	(10.1-32.9)	274.1	(-146-694)	116.2	(-7.2-240)	41.2	na	na	na	na	na	na	na	na	na
SAFR	ST11	1.1	(0.66-1.62)	2.3	(1.2-3.3)	4.6	(1.6-7.7)	2.1	(0.5-3.6)	0.3	< 0.05[c]	na	< 4	na	< 0.05[c]	na	< 1.6	na	0.0
SAFR	ST12	0.85	(0.66-1.05)	1.7	(0.9-2.5)	3.0	(1.9-4.2)	1.4	(0.2-2.5)	0.1	< 0.05[c]	na	< 4	na	< 0.05[c]	na	< 1.6	na	0.0
SAFR	ST15	51.5	(42.6-60.4)	101.5	(72.4-131)	98.0	(83.8-112)	43.7	(24.8-62.5)	79.4	0.61	(0.496-0.729)	51.9	(36.9-66.8)	1.45	(1.11-1.783)	46.1	(29.9-62.3)	87.1
AUS	Curra	4.4	(3.6-5.3)	8.7	(5.3-12)	84.2	(38.4-130)	38.3	(-0.1-76.6)	23.7	0.27	(0.215-0.329)	23.0	(14.3-31.8)	1.63	(1.07-2.19)	51.5	(22-80.9)	67.2
AUS	Urah	17.2	(11.7-22.7)	33.9	(23.7-44.1)	109.8[d]	(62.9-157)	48.8	(24.6-73.1)	47.9	0.52	(0.418-0.613)	43.4	(32.1-54.6)	1.83	(1.34-2.32)	58.3	(38.2-78.5)	78.0

Colour code: RR≤4 are represented on a light grey background; 4<RR≤10 are represented on a dark grey background; RR>10 are represented on a black background

[a] predicted survival rates at the potential discriminating doses (2xLC_{99} of the susceptible reference Muñoz strain)

[b] mortality of 77% at the lowest dose tested (0.003 mg/m2); c no survival at the lowest dose tested (0.05 mg/m2)

[d] estimate based on extrapolation (highest dose tested 100 mg/m2)

Table 3. Lethal concentrations 50 and LC_{90} with their 95% CI obtained for the 8 Argentinean (ST21-ST30), 3 South African (ST11-ST15) and 2 Australian (Curra, Urah) field strains of *R. (B.) microplus* as well as their RR in comparison to the susceptible reference strain (Muñoz) and their survival rates at the DD when tested with ivermectin and moxidectin. Concentrations are expressed in mg/m2.

		IVERMECTIN									MOXIDECTIN								
country	strain	LC50	(95% CI)	RR at LC50	(95% CI)	LC90	(95% CI)	RR at LC90	(95% CI)	PR[a]	LC50	(95% CI)	RR at LC50	(95% CI)	LC90	(95% CI)	RR at LC90	(95% CI)	PR[a]
	Muñoz	1.12	(0.84-1.4)			5.6	(1.6-9.7)				0.37	(0.29-0.45)			1.47	(0.75-2.19)			
ARG	ST21	2.03	(0.98-3.07)	1.8	(0.8-2.9)	8.1	(-1.6-17.9)	1.4	(-0.9-3.7)	0.2	0.39	(0.19-0.6)	1.1	(0.6-1.6)	1.96	(0.75-3.18)	1.4	(0.5-2.3)	0.0
	ST22	1.38	(0.88-1.88)	1.2	(0.6-1.9)	3.2	(2-4.3)	0.5	(-0.3-1.3)	0.0	0.89	(0.48-1.29)	2.4	(1.9-2.9)	10.08	(-0.78-20.95)	6.6	(3-10.2)	8.1
	ST24	0.97	(0.81-1.13)	0.9	(0.4-1.4)	2.5	(1.5-3.6)	0.4	(-0.3-1.2)	0.0	1.25	(0.84-1.67)	3.4	(2.3-4.5)	3.35	(2.05-4.65)	2.3	(1.1-3.5)	0.0
	ST25	0.38	(0.22-0.53)	0.3	(0.1-0.6)	2.4	(-0.6-5.5)	0.4	(-0.4-1.2)	0.2	0.32	(0.17-0.47)	0.9	(-0.1-1.9)	1.06	(0.28-1.85)	0.7	(-0.5-1.9)	0.0
	ST26	0.78	(0.61-0.96)	0.7	(0.3-1.1)	1.8	(1.1-2.5)	0.3	(-0.2-0.8)	0.0	0.40	(0.34-0.46)	1.1	(0.7-1.5)	0.86	(0.65-1.07)	0.6	(0.2-1)	0.0
	ST27	0.39	(0.3-0.49)	0.4	(0.2-0.6)	0.74	(0.6-0.9)	0.1	(-0.1-0.3)	0.0	0.17	(0.11-0.24)	0.5	(0.2-0.8)	1.25	(0.21-2.28)	0.8	(-0.4-1.9)	0.2
	ST29	0.91	(0.68-1.14)	0.8	(0.4-1.3)	3.8	(1-6.6)	0.7	(-0.4-1.8)	0.2	0.29	(0.26-0.32)	0.8	(0.6-1)	0.58	(0.43-0.73)	0.4	(0.2-0.6)	0.0
	ST30	0.77	(0.51-1.03)	0.7	(0.2-1.2)	5.4	(0.2-10.5)	0.9	(-0.9-2.8)	0.6	na	na	na	na	na	na	na	na	na
SAFR	ST11	2.14	(1.47-2.81)	1.9	(1.4-2.5)	4.0	(1-7)	0.7	na	0.0	0.81	(0.61-1)	2.2	(1.6-2.8)	1.49	(1.09-1.89)	1.0	(0.6-1.5)	0.0
	ST12	1.15	(0.69-1.61)	1.0	(0.6-1.5)	4.7	(-1.1-10.4)	0.8	(-0.2-1.8)	0.2	0.67	(0.55-0.8)	1.8	(1.3-2.4)	1.61	(1.27-1.94)	1.1	(0.6-1.6)	0.0
	ST15	2.82	(0.72-4.93)	2.5	(1.3-3.7)	39.4	(-36.6-115.5)	6.8	(-0.9-14.4)	6.2	0.25	(0.22-0.29)	0.7	(0.5-0.9)	0.52	(0.31-0.74)	0.4	(0.1-0.6)	0.0
AUS	Curra	0.53	(-0.01-1.07)	0.5	(0-0.9)	17.5	(-4.7-39.8)	3.3	(-0.3-6.8)	0.9	0.09	(0.06-0.12)	0.3	(0.1-0.4)	0.84	(0.2-1.48)	0.6	(0-1.2)	0.8
	Urah	0.49	(0.27-0.71)	0.4	(0.1-0.8)	5.0	(2.9-7.2)	0.9	(0.4-1.3)	0.0	0.16	(0.1-0.22)	0.4	(0.2-0.7)	0.72	(0.49-0.95)	0.5	(0.2-0.8)	0.0

Colour code: RR≤4 are represented on a light grey background; 4<RR≤10 are represented on a dark grey background; RR>10 are represented on a black background

na: not available

[a] predicted survival rates at the potential discriminating doses (2xLC_{99} of the susceptible reference Muñoz strain)

Table 4. Lethal concentrations 50 and LC_{90} with their 95% CI obtained for the 8 Argentinean (ST21-ST30), 3 South African (ST11-ST15) and 2 Australian (Curra, Urah) field strains of *R. (B.) microplus* as well as their RR in comparison to the susceptible reference strain (Muñoz) and their survival rates at the DD when tested with fipronil and pyriprol. Concentrations are expressed in mg/m2.

		FIPRONIL									PYRIPROL								
country	strain	LC50	(95% CI)	RR at LC50	(95% CI)	LC90	(95% CI)	RR at LC90	(95% CI)	PR[a]	LC50	(95% CI)	RR at LC50	(95% CI)	LC90	(95% CI)	RR at LC90	(95% CI)	PR[a]
	Muñoz	0.04	(0.032-0.04)			0.07	(0.051-0.092)				0.04	(0.039-0.049)			0.12	(0.09-0.15)			
ARG	ST21	0.04	(0.034-0.053)	1.2	(1-1.5)	0.09	(0.049-0.128)	1.2	(0.7-1.8)	0.2	0.03	(0.023-0.038)	0.7	(0.5-0.9)	0.06	(0-0.12)	0.5	(0.1-1)	0.0
	ST22	0.04	(0.026-0.059)	1.2	(0.9-1.5)	0.13	(0.037-0.219)	1.8	(0.9-2.7)	0.4	0.07	(0.05-0.086)	1.6	(1.1-2)	0.18	(0.09-0.27)	1.5	(0.8-2.3)	0.1
	ST24	0.04	(0.032-0.038)	1.0	(0.8-1.1)	0.07	(0.052-0.077)	0.9	(0.5-1.3)	0.2	0.04	(0.02-0.054)	0.9	(0.4-1.3)	0.12	(0.04-0.21)	1.0	(0.3-1.8)	0.5
	ST25	0.03	(0.021-0.03)	0.7	(0.5-0.9)	0.06	(0.041-0.079)	0.8	(0.5-1.2)	0.0	0.03	(0.023-0.043)	0.8	(0.5-1)	0.13	(0.06-0.2)	1.1	(0.5-1.7)	0.6
	ST26	0.06	(0.048-0.062)	1.5	(1.2-1.9)	0.10	(0.088-0.118)	1.4	(1-1.9)	0.0	0.05	(0.043-0.056)	1.1	(0.9-1.4)	0.14	(0.09-0.18)	1.2	(0.5-1.8)	0.3
	ST27	0.04	(0.025-0.044)	1.0	(0.7-1.3)	0.09	(0.052-0.136)	1.3	(0.6-2.1)	1.0	0.02	(0.018-0.027)	0.5	(0.4-0.6)	0.06	(0.03-0.09)	0.5	(0.2-0.8)	0.1
	ST29	0.05	(0.042-0.061)	1.4	(1.1-1.8)	0.14	(0.087-0.201)	2.0	(0.9-3.1)	3.4	0.11	(0.088-0.129)	2.5	(1.8-3.1)	0.22	(0.15-0.3)	1.9	(1-2.8)	0.1
	ST30	0.03	(0.027-0.042)	1.0	(0.7-1.2)	0.11	(0.079-0.14)	1.5	(0.9-2.1)	0.0	na	na	na	na	na	na	na	na	na
SAFR	ST11	0.06	(0.052-0.063)	1.6	(1.3-1.9)	0.10	(0.092-0.116)	1.5	(1.1-1.8)	0.0	0.1	(0.069-0.124)	2.2	(1.6-2.8)	0.22	(0.07-0.37)	2.0	(0.9-3)	0.0
	ST12	0.03	(0.022-0.045)	1.0	(0.7-1.2)	0.08	(0.046-0.116)	1.2	(0.8-1.5)	0.0	0.05	(0.034-0.057)	1.0	(0.7-1.4)	0.15	(0.07-0.23)	1.3	(0.3-2.2)	0.6
	ST15	0.14	(0.126-0.159)	3.9	(3.3-4.6)	0.21	(0.179-0.235)	2.9	(2.1-3.7)	0.8	0.44	(0.155-0.728)	9.9	(6-13.9)	2.45	(-0.81-5.72)	21.4	(3.5-39.3)	36.7
AUS	Curra	0.05	(0.043-0.055)	1.4	(1.1-1.6)	0.12	(0.082-0.159)	1.7	(1-2.4)	2.3	0.11	(0.09-0.136)	2.5	(2-3)	0.15	(0.06-0.24)	1.3	(0.5-2)	0.0
	Urah	0.03	(0.026-0.028)	0.7	(0.7-0.8)	0.06	(0.055-0.073)	0.8	(0.6-1.1)	0.5	0.03	(0.023-0.028)	0.6	(0.5-0.7)	0.06	(0.04-0.07)	0.6	(0.4-0.8)	0.0

Colour code: RR≤4 are represented on a light grey background; 4<RR≤10 are represented on a dark grey background; RR>10 are represented on a black background

na: not available

[a] predicted survival rates at the potential discriminating doses (2x LC_{99} of the susceptible reference Muñoz strain)

Table 5. Lethal concentrations 50 and LC_{90} with their 95% CI obtained for the 8 Argentinean (ST21-ST30), 3 South African (ST11-ST15) and 2 Australian (Curra, Urah) field strains of *R. (B.) microplus* as well as their RR in comparison to the susceptible reference strain (Muñoz). Concentrations are expressed in mg/m2.

		AMITRAZ							
country	strain	LC50	(95% CI)	RR at LC50	(95% CI)	LC90	(95% CI)	RR at LC90	(95% CI)
	Muñoz	1.1	(0.9-1.3)			18.5	(9-28)		
ARG	ST21	0.9	(0.7-1.1)	0.9	(0.7-1.1)	1.9	(1.1-2.7)	0.1	(0.1-0.1)
	ST22	1.7	(1.5-1.9)	1.6	(1.2-2)	4.1	(3.2-5)	0.2	(0.1-0.4)
	ST24	35.4	(27.2-43.6)	32.5	(24.1-40.8)	79.8	(50.3-109.3)	4.3	(2-6.6)
	ST25	9.8	(7.2-12.3)	9.0	(5.9-12.1)	39.0	(21.3-56.7)	2.1	(0.7-3.6)
	ST26	1.8	(1.5-2)	1.6	(1.2-2)	7.8	(5.1-10.5)	0.4	(0.2-0.7)
	ST27	1.0	(1-1.1)	1.0	(0.6-1.3)	3.9	(3.3-4.4)	0.2	(0.1-0.4)
	ST29	0.8	(0.4-1.1)	0.7	(0.5-0.9)	2.6	(0.4-4.9)	0.1	(0-0.2)
	ST30	22.4	(18-26.8)	20.5	(15.2-25.8)	42.5	(30.2-54.8)	2.3	(1.1-3.5)
SAFR	ST11	1.0	(0.8-1.1)	0.9	(0.6-1.2)	3.1	(2-4.3)	0.2	(0-0.3)
	ST12	1.2	(0.8-1.6)	1.1	(0.9-1.4)	6.9	(1-12.9)	0.4	(0.1-0.6)
	ST15	3.8	(2.7-4.8)	3.5	(2.4-4.6)	21.4	(6.3-36.6)	1.2	(0.2-2.1)
AUS	Curra	1.8	(1.1-3.5)	1.6	(0.9-2.4)	18.3	(8.1-85.8)	1.0	(0.3-1.7)
	Urah	2.3	(1.1-2.5)	2.1	(1-3.2)	46.9	(9.7-26.8)	2.7	(0.1-5.2)

Colour code: RR≤4 are represented on a light grey background; 4<RR≤10 are represented on a dark grey background; RR>10 are represented on a black background

Discussion

The concentrations of the acaricidal compounds which were tested in this study were suitable to calculate LC50 and LC90 of susceptible and resistant populations in 97% of the tests. The remaining 3% consisted of: ST11, ST12 and ST21, for which the tested doses of flumethrin were not sufficiently low to allow determining the LC50 and LC90 and the estimates could not be extrapolated from the model; Urah, for which the LC90 to cypermethrin was slightly above the highest tested dose and the estimate was therefore extrapolated from the model (Table 1).

In general, the RR50 was the method of choice to determine the resistance status because their 95% CI was smaller compared to RR90. However, RR90 is complementary to RR50 when the dose-response curves of the field populations and the reference strain are not parallel. A smaller slope of the field population, which leads to RR90 greater than RR50, is an indicator that resistance is developing and that the population is heterogeneous, with susceptible and resistant individuals (FAO 2004). Therefore RR90 has to be considered to detect emerging resistance. In the present survey, the comparison of RR90 and RR50 to distinguish susceptible from resistant field populations reveals that most of the differences would appear for flumethrin. Indeed, due to the smaller slopes of the field populations (Fig. 2a), four of the five Argentinean populations diagnosed susceptible to flumethrin based on RR50 were considered highly resistant based on RR90, with RR90 values between 27.8 (2.2-53.5) and 36.5 (2.7-70.3), indicating emerging flumethrin resistance in these populations. Interestingly, these four populations would also have been considered resistant based on their survival rate at the DD. According to the FAO guidelines (2004) the percentage of ticks surviving at the DD can be taken as the percentage of resistance to the acaricide in the population. Therefore, the interpretation of the results based on the DD corroborates the observation of emerging resistance based on RR90.

Similarly to flumethrin, one population appeared resistant to moxidectin in Argentina when using RR90 (ST22, RR90=6.6, 3.0-10.2) and another population appeared resistant to ivermectin in South Africa (ST15, RR90=6.8, -0.9-14.4) although the latter case was not significant due to lack of replicates. Even though these last two cases are isolated, since all the other tick populations were susceptible to ML, they should be considered with care. Indeed, RR90 should help detecting emerging resistance and therefore suggest here that ML resistance might be appearing. However, no history of ML-treatment was reported in the farms from which these ticks were collected. We are

therefore lacking a direct correlation between *in vitro*-finding and *in vivo*-situation and to our knowledge no resistance to ML has ever been reported in the literature in Argentina and South Africa.

Conversely, for amitraz, RR90 were much lower than RR50 and two of the three resistant populations based on RR50 would have been considered susceptible based on RR90. Unlike with flumethrin, slopes of the response to amitraz were greater among the resistant populations compared to the reference strain (Fig. 2b). Therefore, amitraz resistance would have been missed considering RR90, indicating that RR90 are inadequate values to identify amitraz resistance.

In Argentina, resistance to SP was detected in seven out of eight populations (88%). Although farms were selected based on reports of lack of treatment efficacy, it shows that SP resistance is widespread in Northeast Argentina (Province of Corrientes). The current use of SP for tick-treatments was reported in only three of these farms (ST22, ST29 and ST30). However, an earlier use of this class cannot be excluded in the other farms. In contrast, resistance to OP was found in a single Argentinean population (ST27). This class of compounds was not reported to be used for treatment by the farmers but OPs have been used in the province prior to SPs, and lack of efficacy was reported at that time (Grillo Torrado and Gutiérrez 1970). The LTT identified a high resistance to amitraz in ST24 and confirmed the lack of efficacy observed *in vivo* prior to tick collection. Resistance to amitraz was also observed in ST25 and ST30, which were originating from farms with a history of amitraz treatment, either directly (ST25) or in the close neighbourhood (ST30, being close to ST24). This survey supports the very recent first report of amitraz resistance in Argentina (Cutullé et al. , submitted). Amitraz resistance in Argentina is worrying because this compound currently plays a major role in the eradication program of ticks in this country. In Argentina, tick control is regulated by the SENASA (Animal Health Authorities) through a law (12.566) and a decree (7623/54) which divide the northern part of the country (endemic area) in three areas (SENASA 1938): an infested area, where there is no obligation of eradication; a tick free area, where the percentage of infested fields should not exceed 1%; and between the two, an eradication area, where treatments are compulsory, aiming to reach eradication. This tick control program has more than 70 years and is based on treatments in dipping vats at a 21-day interval (resolution 27/1999) (SENASA 1999). This led to the development of resistance to most of the existing compounds used in dipping vats. Since 1999 injectable (macrocyclic lactones) and pour-on (fluazuron) compounds are allowed to be used in combination with plunge dipping or alone (Julio Reggi, personal communication). However, amitraz is currently the main active ingredient used for treatment due to the lack of efficacy of the other

compounds used for dipping vats, hence the important impact of the development of amitraz resistance.

In South Africa, resistance of *Boophilus* spp to OP, SP and amitraz is well known (FAO 1998, Strydom and Peter 1999), but ticks were not identified at the species level in these previous studies. A national survey carried out in randomly selected commercial farms showed resistance prevalence of 33%, 22% and 7% to OP, SP and amidines, respectively (FAO 1998). In the current study, ticks were not collected in commercial farms but in a small familial farm and in two herds grouping cattle of different owners and pasturing on communal lands. In these herds the state veterinary authorities offer a weekly amitraz dip, and cattle owners are free to bring their animals for the treatments or to perform any other treatments. Therefore, we do not know exactly to which active ingredients the cattle on which the ticks were collected were exposed. None of the three populations demonstrated resistance to OP or amitraz while one population (ST15) demonstrated resistance to SP and pyriprol and a suspicion of ivermectin resistance. The observation of pyriprol resistance, reinforced by a RR50 to fipronil very close to our cut-off value of 4.0 (RR50=3.9, 3.3-4.6) would suggest that some PYZ compounds have been used for treatments, however pyriprol is not meant to be used for cattle but is exclusively recommended to treat tick infestations in dogs and the probability that these formulations have been used for cattle is very low. However, it cannot be excluded that other products of the PYZ class, such as phytosanitary products, have been used on animals or for plant protection.

In Queensland, Australia, prevalence of resistance to OP was reported to be between 12% and 96% depending on the regions in 1981 (Roulston et al. 1981). In the same state, prevalence of resistance to flumethrin and to amitraz was estimated to 76% and 10%, respectively in 2000 (Jonsson et al. 2000). The two Australian populations analysed in the present survey also originated from Queensland and demonstrated the two most common resistance in their country, i.e. OP and SP resistance, while they were susceptible to amitraz and all the other tested compounds.

In the present study, the cut-off value of 4 was selected to distinguish resistant from susceptible ticks based on RR. Lower threshold values have been previously used (Chevillon et al. 2007, Mendes et al. 2007) while in other studies, RR were separated into 3 classes among which RR of the intermediate class were considered as indicators of tolerance or of incipient resistance (Bianchi et al. 2003, Castro-Janer et al. 2011, Klafke et al. 2012). The cut-off value of 4 should avoid over-diagnosing resistance. Inversely, one could argue that resistance may be missed. However, if we had selected a threshold

value of 3 instead of 4, resistance statuses would have been identical in 96% of the cases and only four additional cases would have been considered resistant. Therefore, we are confident that this threshold value offers a good compromise to differentiate susceptible from resistant populations.

The use of DD to determine whether resistance is present is widespread with adult tests such as the AIT, but has been criticised (Jonsson et al. 2007). It offers the benefit to reduce the number of engorged females required for testing and the amount of work since a single dose of each compound is tested. Although the benefit of testing a single dose per compound with the LTT is very much reduced, we were interested to see whether the use of DD would be suitable to detect resistance in the present study. A survival rate of ticks at the DD of 10% was used to differentiate resistant from susceptible tick populations. Resistance statuses based on DD using this cut-off value were in agreement with those based on RR50 or RR90 and the use of DD therefore appeared to be adequate to diagnose established and emerging resistance in these field populations (Table 1b).

To conclude, the LTT allowed the detection of resistance in field populations of *R. (B.) microplus* ticks originating from Argentina, South Africa and Australia. Resistance status were based on RR50, using a cut-off value of 4 to differentiate susceptible from resistant populations. In addition, RR90 were also considered to detect emerging resistance. Finally, survival rates at DD were compared to the resistance statuses based on RR50 and RR90. Additional cases of amitraz resistance in Argentina were identified as well as the first case of resistance to pyriprol in South Africa. In addition, emerging resistance to ML was suspected in an Argentinean and South African farm. For this study, ticks were imported to Switzerland for testing. However, in the future, we would like the LTT to be performed in laboratories of the countries of collection. To do so, a detailed description of the test using simplified equipment will be very soon published (Lovis et al., submitted).

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4.4. Chapitre 4: Use of the Larval Tarsal Test to determine acaricide resistance in *Rhipicephalus (Boophilus) microplus* Brazilian field populations

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Use of the Larval Tarsal Test to determine acaricide resistance in *Rhipicephalus (Boophilus) microplus* Brazilian field populations

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Short title: The Larval Tarsal Test to assess acaricide resistance of cattle ticks in Brazil

Abstract

Acaricide resistance of the cattle tick *Rhipicephalus (Boophilus) microplus* is widespread in most of the countries where this parasite is present. Bioassays are used to diagnose the level and pattern of resistance in tick populations. In the present study, we describe a detailed protocol of the Larval Tarsal Test (LTT) using simplified equipment and data on the resistance of 17 tick field populations originating from 5 Brazilian states. Nine acaricidal compounds from 5 major classes were tested: organophosphates (OP), synthetic pyrethroids (SP), macrocyclic lactones (ML), phenylpyrazols (PYZ) and amidines. For comparison, four of the tick populations were also tested with the Larval Packet Test (LPT) with one compound per class. The most common resistances were to SP, amitraz and OP, with frequencies of 94%, 88% and 82%, respectively. Resistance to PYZ was also found to be widespread (65%), suggesting a rapid development of fipronil resistance in Brazil. One case of ML resistance and 2 cases of suspected ML resistance were identified with the LTT. The LTT led to higher resistance ratios to all compounds than the LPT, reflecting its high sensitivity to detect resistance. Finally, the LTT allowed testing a larger number of compounds and doses with reduced labour in comparison to the LPT and turned out to be a reliable bioassay to detect resistance in field populations.

Key words: Larval Tarsal Test, *Rhipicephalus (Boophilus) microplus*, Brazil, acaricide resistance, tick

Introduction

The one-host cattle tick *Rhipicephalus (Boophilus) microplus* is a pest of major economic importance in tropical and subtropical countries. Treatments nearly exclusively rely on acaricides and multi-drug resistance has become widespread (Alonso-diaz et al., 2006; Jonsson and Hope, 2007; Martins et al., 2008; Castro-Janer et al., 2011). In Brazil, *R. (B.) microplus* is the most important ectoparasite of cattle and its economic impact on the Brazilian cattle industry was estimated at 2 billion US dollars per year (Grisi et al., 2002). This amount includes losses due to the increased mortality caused by tick-borne parasites, losses due to decreased milk production and decreased weight gain, damage to the leather, and treatment costs to control infestations. In Brazil resistance successively emerged to arsenic in 1950 (Freire, 1953), to organophosphates (OP) in 1974 (Amaral et al., 1974) and to synthetic pyrethroids (SP) in 1988 (Leite, 1988; Laranja et al., 1989). At the end of the 1990s, Farias (1999) pointed out that the widespread resistance to SP was a big issue in Brazil, especially considering that 90% of the acaricides available on the market at that time belonged to SP. In parallel, amitraz became an important alternative to control OP and SP-resistant populations but resistance to this compound was already reported in 1999 (Furlong, 1999; Farias, 1999). As a result, macrocyclic lactones (ML) were extensively used, and the first case of avermectin-resistance was observed in 2001 (Martins and Furlong, 2001), followed by other cases of ivermectin-resistance (Klafke et al., 2006; Klafke et al., 2012). Recently, resistance to fipronil, a phenylpyrazol (PYZ) compound, was also detected (Castro-Janer et al., 2010). Nowadays, reports of resistance to OP, SP and amitraz are very common in Brazil (Farias et al., 2008; Martins et al., 2008; Mendes et al., 2011; Andreotti et al., 2011) while resistance to ivermectin and fipronil is still limited (Castro-Janer et al., 2010; Klafke et al., 2012). The newest acaricide classes are the benzoylureas (growth regulators) and the spinosyns, against which resistance has not been reported in the literature yet.

Farmers are currently facing many issues to control multi-drug resistant tick populations. It is essential that they obtain some information on the resistance profile of these populations in order to help them choosing the most suitable compounds to enhance treatment efficacy. In this context, bioassays are used to determine the resistance of tick populations to specific acaricides. Various *in vitro* tests are available, each of them with their own advantages and disadvantages. The FAO currently recommends the Adult Immersion Test (AIT) and the Larval Packet Test (LPT) (2004). The Larval Immersion Test (LIT) (Shaw, 1966) modified by Sabatini et al. (2001) is also currently used, mainly to test ivermectin and fipronil, and was shown to be more suitable to identify resistance to these 2 compounds (Castro-Janer et al., 2009; Klafke et al., 2012). In 2011, a new bioassay, the Larval

Tarsal Test (LTT), was described and compared to the LPT (Lovis et al., 2011). The LTT is performed in microplates pre-treated with acaricides in which eggs are distributed, avoiding the handling of larvae and thus allowing testing a larger number of compounds and doses. The distribution of the eggs in the plates and the evaluation of the tests with the LTT required approximately ten-fold less time than the loading of the larvae in the packets and the evaluation of the tests with the LPT (Lovis et al., 2011).

Resistance status of tick populations can be determined by exposing ticks to a unique dose based on the data of a susceptible reference strain, and survival to this discriminating dose (DD) is considered as an indicator of resistance (FAO, 2004). In contrast, ticks can be exposed to several doses of acaricides in order to establish the doses which induce 50% or 90% mortality and compare them to a susceptible reference strain to determine the corresponding resistance ratios (RR50 and RR90). If the dose-response curves of the field populations and the reference strain are parallel, then these two values are similar. However, in absence of parallelism, two scenarios can be observed and the comparison of RR50 with RR90 reflects them: either the slope of the field population is smaller than the reference strain, which leads to a RR90 greater than the RR50, or the slope of the field population is greater. Thereby, RR50 may be close to 1, whereas RR90 are much higher, allowing the detection of resistance. The comparison of RR50 and RR90 and observation of the slope of the response provides valuable information on emerging resistance.

In this paper, we provide a detailed protocol using simplified equipment for the LTT and evaluate this test for the detection of acaricidal resistance in field tick populations. The LTT was carried out in two laboratories in Brazil using 17 tick populations originating from 5 states of Brazil with 9 acaricidal compounds from 5 major classes (OP, SP, ML, PYZ and amidines). In addition, the resistance status of 4 field populations was also tested with the LPT using 5 compounds for comparison with the LTT.

Material and methods

Ticks

Susceptible strains

The Mozo strain was used as susceptible reference strain for OP, ML and PYZ while the Muñoz strain was used as susceptible reference strain for SP and amitraz. The Mozo strain was from the Instituto de Pesquisas Veterinárias Desidério Finamor (IPVDF), Eldorado do Sul, Brazil, obtained in November 2010 from the Centro de Investigaciones Veterinarias Miguel C. Rubino where it had been reared without acaricide pressure since collection from the field in 1973 in Uruguay. Some resistance to SP and amitraz was observed in the IPVDF isolate. The Muñoz strain was from the Novartis Animal Health Research Center (CRA), St-Aubin, Switzerland, obtained in 2010 from the Cattle Fever Tick Research Laboratory (CFTRL), Edinburg, Texas, where it had been reared without acaricide selection since collection from the field in 1999 in Zapata County, Texas, USA.

Field populations

In January and February 2011, *R. (B.) microplus* engorged females were collected in 17 Brazilian beef cattle ranches where farmers had observed some lack of treatment efficacy. Tick samples were originated from the following 5 states: São Paulo (7), Rio Grande do Sul (RS, 4), Mato Grosso do Sul (MS, 4), Paraná (PR, 1), Espírito Santo (ES, 1). Samples included at least 20 fully engorged females collected from a minimum of 6 cows.

Preparation of ticks

Engorged females were brought to the Instituto Biológico (IB), São Paulo, Brazil or to the IPVDF and kept at 28+/-1°C and 65-85% relative humidity (RH) to complete oviposition. Two to 3 weeks after collection of the females, eggs were used for testing with the LTT or were transferred to glass vials closed with humidified cotton plugs for the LPT. Larvae used for the LPT (ST40, ST41, ST42 and ST44) were 14-21 days old. The Mozo strain was tested at the IB and the IPVDF while the Muñoz strain was tested at the CRA (data not shown). They were stored at the same conditions than the field populations.

Acaricides

Technical grade chlorpyriphos (OP) (Sigma–Aldrich, Fluka, Germany), amitraz, coumaphos (OP), cypermethrin (SP), fipronil (PYZ), flumethrin (SP), ivermectin (ML), moxidectin (ML) and pyriprol (PYZ) were used with the LTT. Details on the latter compounds are available in Lovis et al. (2011) (Table 1). Compounds were dissolved in dimethyl sulfoxide (DMSO; Fluka) to prepare stock solutions of 20,000 parts per million (ppm). For the LPT, technical grade chlorpyriphos (Sigma–Aldrich), cypermethrin (Sigma–Aldrich), ivermectin (Agromen Chemicals Co. Ltd., China), fipronil (Agromen Chemicals Co. Ltd., China) and formulated amitraz (12.5%, Schering Plough Saúde Animal Indústria e Comércio Ltd., Brazil) were used.

Larval Tarsal Test

The LTT was conducted at the IB and at the IPVDF following the protocol described previously in Lovis et al. (2011) with some modifications. Ready-to-use treated microtiter plates were prepared in advance in the CRA. Briefly, 20 µl of a coating solution (100% ethanol, olive oil (Sigma-Aldrich, Fluka), 400:1) was dispensed in the wells of flat bottom 96-well polystyrene plates (NUNC, Catalogue No. 260836, Denmark) and ethanol was allowed to evaporate overnight. Then 5 µl of acaricidal compounds diluted in DMSO to obtain 12 two-fold dilutions were distributed in the appropriate wells of the plates. The upper and lower rows as well as one of the inner rows contained DMSO only, and the inner row with DMSO was used as control. Plates were placed for 1 hour in an N_2 sampler concentrator (Techne DB-3 Dri-Block, Witec AG, Switzerland) or for 2 hours in a centrifugal vacuum concentrator (SC21017 SpeedVac® Plus, ThermoSavant) for complete DMSO evaporation. In order to avoid potential oxidation of the compounds, plates were placed in airproof plastic bags (ZU3605, Severin) and sealed (Folio bag sealer FS 3602, Severin) under N_2 atmosphere using an anaerobic chamber (Bactron anaerobic chamber model II, Shel Lab). In addition, the treated plates were kept with silica gel and were not exposed to direct light to optimise their preservation. Plates were shipped to the IB and IPVDF, kept at room temperature (20-28°C) and used for testing within 5 weeks after preparation.

Since eggs aggregate, they were separated by the use of glass beads in order to facilitate their distribution in the wells. In details, a portion (40 ml-volume) of 3 mm diameter glass beads was placed in a 100 ml glass bottle. A small amount (~30 mg) of talc (Fluka, Catalogue No. 86255) was added and mixed thoroughly with the beads to ensure that the surface of the beads was covered with talc. Egg clusters (300-1000 mg) were added to the beads and the bottle was closed. To

separate the eggs, the bottle was smoothly turned to mix the eggs and the beads and egg clusters were disrupted. At the beginning of the egg separation process, it helps to open the glass bottle to break the egg masses of big size with a spatula. If necessary, additional talc (~30 mg) was added to the beads to ensure that the eggs did not stick to the beads or to the walls of the glass bottle. When the separation was completed and in order to extract the eggs from the beads, the content of the bottle was poured in a sieve (mesh width: 0.9 mm) which allowed the eggs to pass through but not the beads. Eggs were collected in a glass Petri dish. Around 50 eggs (mean: 54.6; standard deviation: 4.6) were distributed per well by using a 2.5 mm-diameter spoon, corresponding to a 4 mm^3 volume (Meyerhoefer Chalazion Curette, Size 3, RUMEX, Catalogue No. 16066).

After distribution of the eggs, uncovered plates were kept for 24 hours at 28+/-2°C at ~95% RH. Then plates were sealed with a transparent sealing film (VIEWseal, Greiner bio-one, Catalogue No. 676070, Switzerland) and held at 28+/-2°C and 80-90% RH for 3 to 4 additional weeks. The sealing of the plates as well as egg distribution were performed on a static control mat (157 KIT, elme) to remove electrostatic charges. After incubation (i.e. 40-42 days after the collection of the females), plates were removed from the environmental chamber and larval mortality was evaluated by counting dead or surviving larvae using a stereomicroscope. Larval motility and general appearance were used as criteria to assess mortality.

The following concentrations were tested: flumethrin, fipronil, pyriprol: 0.003-6.25 mg/m^2; moxidectin: 0.05-100 mg/m^2; amitraz, cypermethrin, ivermectin: 0.1-200 mg/m^2; chlorpyrifos: 0.2-400 mg/m^2; coumaphos: 0.4-800 mg/m^2. Each dilution was tested in triplicates in separate plates.

Larval Packet Test

The LPT was carried out at the IB as previously described (FAO, 2004). Briefly, technical grade acaricides were dissolved in a mixture of trichloroethylene (Synth, Diadema-SP, Brazil) and olive oil (Sigma-Aldrich) (2:1) to prepare 1% active ingredient (AI) stock solutions which were subsequently diluted in trichloroethylene:olive oil to prepare 6-12 concentrations per compound. A volume of 670 µl of each dilution was used to impregnate 7.5 x 8.5 cm filter papers (Whatman No 1, Whatman International Ltd, Maidstone, United Kingdom) and trichloroethylene was allowed to evaporate at room conditions for a minimum of 24 hours. Each concentration was tested in triplicates and controls contained the diluent only. Treated papers were stored in a fridge and used within 3 weeks. For testing, they were folded in half and sealed on the sides with metal clips forming an open-ended

packet. Then, around 100 larvae were collected with a paintbrush from the glass vials and inserted in each treated packet. Packets were then sealed with a third clip and incubated at 28+/-2°C and 80-90% RH for 24 hours. Packets were removed from the incubator, opened and larval mortality assessed by counting dead and surviving larvae. Larvae that moved their legs but did not walk were counted as if dead.

For amitraz, the LPT protocol modified by Miller et al. (2002) was followed. Nylon fabric (Type 2320, Cerex Advanced Fabrics, Pensacola, FL, USA) was therefore used instead of filter papers and formulated amitraz was used instead of technical grade amitraz. In addition, impregnated nylon fabric was not stored in the fridge but used directly once the evaporation was completed.

Tested concentrations varied among strains and were included in the following ranges: amitraz: 0.0002-0.02% AI; fipronil: 0.0031-0.1% AI; ivermectin: 0.03-0.3% AI; chlorpyriphos: 0.0005-1% AI; cypermethrin: 0.13-5% AI.

Statistical analysis

Data were entered in Excel software (Microsoft Office 2003) and transferred to Intercooled STATA release 11.0 (StataCorp, College Station, TX, USA). Abbott's formula (Abbott, 1987) was used to normalize mortality values by the mortality of the control wells. Outer wells of the plates with increased mortality due to occasional edge effect in microplates were removed (Lovis et al., 2011). Statistical analysis was performed on the R software (version 2.12.0) using the drc package (version 2.0-1), specific for modelling dose-response curves (Ritz and Streibig, 2005). A five-parameter log-logistic function with the bottom and top limits fixed at 0 and 100 respectively was used to model the dose-mortality data (drm command). Lethal concentrations at 50% and 90% mortality (LC_{50}, LC_{90}, respectively) and their corresponding resistance ratios (RR) (RR50 and RR90) as well as their 95% CI were calculated with the ED and SI commands and the Delta options. Populations were considered to be susceptible to a specific compound when the RR was smaller or equal to 4, moderately resistant for $4<RR\leq10$ and highly resistant for RR greater than 10. Potential discriminating doses (DD) were calculated as 2 x the LC_{99} of the susceptible strains (Jonsson et al., 2007). The survival rates of the field strains at the DD were estimated with the PR command. Discriminating doses were not generated for amitraz as the use of a single DD is not recommended for this compound (FAO, 2004; Jonsson et al., 2007; Lovis et al., 2011).

Results

Lethal concentrations inducing 50% and 90% mortality and their respective 95% CI are presented for the 17 field strains in comparison with the reference strains (Tables 1 to 5). The RR50, RR90 and their respective 95% CI, as well as the survival rates of the field populations at the potential DD are presented as well.

The 95% CI of LC_{90} and of RR90 were wider than those calculated for LC_{50} and RR50. The resistance status was therefore based on RR50, while the RR90 was used for comparison reasons. Some discrepancy between RR50 and RR90 in the identification of resistance was observed in case of absence of parallelism between the dose-response curves of the field populations and the reference strain as illustrated in Fig. 1 for pyriprol and amitraz.

a) b)

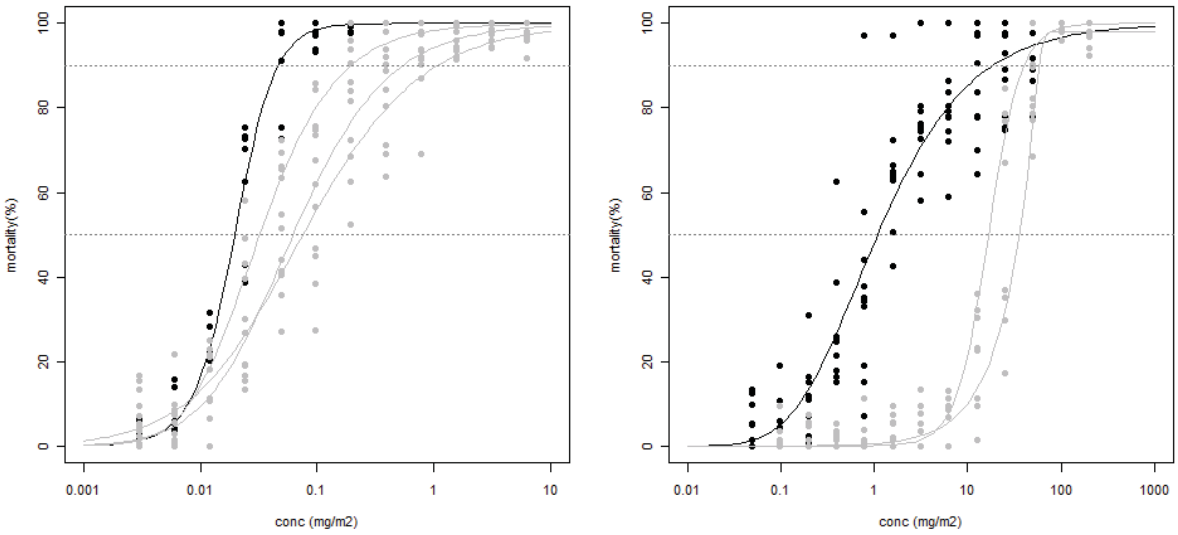

Figure 1. Dose-response curves obtained with the LTT a) when conducted with pyriprol: three field populations (ST48, ST49 and ST55, grey) in comparison to the susceptible reference Mozo strain (black) b) when conducted with amitraz: two field populations (ST41 and ST48, grey) in comparison to the susceptible reference Muñoz strain (black). The grey dotted horizontal lines indicate 50% and 90% mortalities.

Resistance status of the field populations based on RR50

Sixteen (94%) field populations showed evidence of resistance to SP with RR50 to cypermethrin ranging from 8.0 to 309.3 and RR50 to flumethrin ranging from 40.0 to 147.3. Three SP-resistant populations demonstrated resistance to cypermethrin only. Resistance ratios at 50% mortality appeared to be systematically higher when testing cypermethrin than when testing flumethrin.

Fourteen (82%) populations were found to be resistant to coumaphos, with RR50 between 4.9 and 72.9, and 11 (65%) populations were resistant to chlorpyriphos, with RR50 between 4.4 and 179.7. Five of these strains were considered as highly resistant to both OP compounds.

Amitraz resistance was detected in 88% (15/17) of the populations with RR50 ranging from 4.2 to 32.9. Six populations were considered as moderately resistant and 9 as highly resistant.

Eleven populations (65%) were resistant to fipronil, with RR50 ranging from 6.6 to 55.7, among which 8 populations were also resistant to pyriprol, with RR50 ranging from 4.3 to 43.9. Four populations were highly resistant to both PYZ compounds.

Finally, RR50 to ivermectin varied between 0.9 and 4.2. The population possessing the RR50 value of 4.2 (ST53) was considered as the single case of resistance to ML but two other populations (ST44 and ST55) had RR50 very close to the threshold value (3.6 and 4.0, respectively). No resistance at all to moxidectin was observed, with RR50 varying between 0.6 and 2.1.

Survival rates at potential DD

Survival at DD was calculated to see the ability of the use of DD to differentiate resistant from susceptible populations. Considering all compounds on resistant strains (based on RR50), survival rates at the DD ranged between 13.1 and 100% in 98% (90/92) of the tests. The other 2 cases were ST53 with ivermectin (0% survival at DD) and ST47 tested with fipronil (4% survival at DD). For susceptible strains (based on RR50), survival rates at DD were below 10% in all but 3 cases: ST55 tested with fipronil (14.8%), ST48 with pyriprol (20.4%) and ST55 tested with pyriprol (28.0%).

Table 1. Lethal concentrations 50 and LC_{90} and their 95% CI obtained for the 17 Brazilian field strains of *R. (B.) microplus* as well as their RR in comparison to the susceptible reference strain (Muñoz) and their survival rates at the DD when tested with cypermethrin and flumethrin. Concentrations are expressed in mg/m2.

		CYPERMETHRIN									FLUMETHRIN								
strain		LC50	(95% CI)	RR at LC50	(95% CI)	LC90	(95% CI)	RR at LC90	(95% CI)	PR**	LC50	(95% CI)	RR at LC50	(95% CI)	LC90	(95% CI)	RR at LC90	(95% CI)	PR**
Muñoz		0.5	(0.4-0.6)			2.2	(1.5-3)				0.01	(0.01-0.01)			0.03	(0.02-0.04)			
ST40	SãP	53.2	(47.9-58.4)	104.8	(83.2-127)	161.2	(119-204)	71.3	(36.6-106)	97.1	0.70	(0.5-0.9)	57.9	(41.7-74.1)	0.91	(0.1-1.7)	29.5	(-3-62.1)	99.9
ST41	SãP	71.9	(66.6-77.2)	141.0	(108-174)	211.7*	(164-260)	95.4	(32.8-158)	99.0	1.57	(1.4-1.7)	129.2	(102-156)	2.77	(1.9-3.7)	92.4	(42.1-143)	100.0
ST42	SãP	101.9	(75-129)	201.0	(144-258)	918.5*	(130-1707)	407.5	(59.1-756)	92.6	1.08	(0.9-1.2)	88.7	(63.2-114)	1.93	(1.5-2.3)	64.8	(31.3-98.4)	99.8
ST43	SãP	93.0	(65.5-121)	183.0	(133-233)	427.6*	(-20.2-875)	190.4	(24.2-356)	88.4	na	na	na	na	na	na	na	na	na
ST44	SãP	84.9	(65.2-105)	167.3	(127-207)	459.4*	(140-779)	203.4	(72.3-335)	95.7	1.80	(1.4-2.2)	147.3	(111-184)	3.10	(1.9-4.3)	105.1	(48.7-161)	99.9
ST45	MGS	4.0	(3.2-4.9)	8.0	(5.2-10.7)	25.9	(12.9-38.8)	11.5	(2.5-20.5)	13.4	0.01	(0.01-0.02)	1.1	(0.7-1.4)	0.25	(0.1-0.4)	8.2	(1.9-14.5)	17.8
ST46	ES	55.8	(46.5-65.1)	109.9	(82.5-137)	176.8	(106-248)	78.7	(33.9-124)	85.5	1.12	(0.8-1.5)	92.2	(67.4-117)	2.31	(0.9-3.7)	78.1	(33.7-123)	99.2
ST47	RGS	1.8	(1.6-2.1)	3.6	(2.8-4.5)	4.8	(3.5-6.2)	2.2	(1.1-3.3)	0.5	0.01	(0.01-0.01)	0.8	(0.6-0.9)	0.02	(0.02-0.02)	0.6	(0.3-0.9)	0.1
ST48	SãP	103.5	(84.2-123)	203.9	(154-254)	270.7*	(137-405)	120.4	(47.7-193)	95.4	0.77	(0.7-0.9)	63.3	(48.9-77.6)	1.65	(0.9-2.3)	55.5	(24.1-86.8)	100.0
ST49	MGS	79.1	(71.9-86.2)	155.7	(120-191)	165.3	(131-200)	73.6	(36.6-111)	98.2	0.91	(0.7-1.2)	74.8	(56.4-93.3)	1.40	(0.7-2.1)	46.9	(23.6-70.3)	99.9
ST50	MGS	36.2	(21.2-51.1)	71.3	(49.4-93.2)	192.7	(-32-417)	85.3	(16-155)	68.0	0.49	(0.4-0.6)	40.0	(30.1-50)	1.14	(0.8-1.5)	37.5	(6.8-68.2)	85.4
ST51	MGS	52.7	(45.1-60.3)	103.7	(77.9-130)	145.6	(98.7-193)	64.9	(29.9-99.9)	80.5	0.50	(0.4-0.6)	41.5	(31.4-51.7)	1.41	(1-1.8)	47.3	(29.9-64.7)	81.0
ST52	PA	112.8	(85.3-140)	221.5	(161-282)	757.2*	(158-1357)	339.4	(69-608)	95.7	1.42	(1.3-1.6)	116.1	(87.8-144)	2.66	(1.9-3.4)	91.1	(30.5-152)	100.0
ST53	RGS	62.4	(47.2-77.5)	122.8	(90.5-155)	307.4*	(70.7-544)	136.8	(34.7-239)	85.8	0.96	(0.8-1.1)	79.2	(62.4-96)	1.59	(1.4-1.8)	53.4	(35-71.8)	96.8
ST55	RGS	5.6	(3.7-7.4)	11.0	(7.8-14.1)	35.5	(3.9-67.1)	15.8	(4.5-27.2)	19.4	0.01	(0.01-0.01)	0.8	(0.6-1.1)	0.07	(0.01-0.13)	2.3	(0.8-3.9)	7.8
ST57	SãP	157.0	(107.9-206)	309.3	(200-419)	1651.0*	(-45.5-3348)	735.5	(-70.1-1541)	96.2	1.01	(0.9-1.1)	83.5	(68.1-98.9)	1.96	(1.5-2.4)	65.6	(37.3-94)	100.0
ST58	RGS	73.0	(64.2-81.8)	143.1	(113-173)	163.3	(114-213)	72.8	(37.3-108)	97.2	0.80	(0.7-0.8)	65.4	(52.5-78.3)	1.23	(1-1.5)	41.5	(20.9-62.1)	99.8

na: not available due to insufficient number of engorged females obtained

* estimates based on extrapolation (highest dose tested 200 mg/m2)

** predicted survival rates at the potential discriminating doses (2xLC_{99} of the susceptible reference strain)

Colour code: RR≤4.0 are represented on a light grey background; 4.0<RR≤10.0 are represented on a dark grey background; RR >10.0 are represented on a black background

Table 2. Lethal concentrations 50 and LC_{90} and their 95% CI obtained for the 17 Brazilian field strains of *R. (B.) microplus* as well as their RR in comparison to the susceptible reference strain (Mozo) and their survival rates at the DD when tested with chlorpyrifos and coumaphos. Concentrations are expressed in mg/m2.

		CHLORPYRIPHOS									COUMAPHOS								
strain	origin	LC50	(95% CI)	RR at LC50	(95% CI)	LC90	(95% CI)	RR at LC90	(95% CI)	PR**	LC50	(95% CI)	RR at LC50	(95% CI)	LC90	(95% CI)	RR at LC90	(95% CI)	PR**
Mozo		2.7	(2.6-2.9)			5.9	(5-6.8)				2.3	(2.2-2.4)			4.0	(3.6-4.3)			
ST40	SãP	53.9	(41-66.8)	19.8	(15.5-24.1)	188.0	(96.3-280)	32.3	(16.9-47.7)	65.6	28.6	(21.6-35.5)	12.7	(9.7-15.6)	74.8	(37.3-112)	18.8	(10.1-27.6)	79.3
ST41	SãP	20.6	(17.5-23.6)	7.6	(6.3-8.8)	103.0	(54.7-15)	17.6	(8.6-26.6)	35.6	35.3	(31.8-38.7)	15.5	(11.7-19.3)	120.6	(93.1-148)	30.8	(13.8-47.9)	79.2
ST42	SãP	13.7	(11.6-15.9)	5.1	(4.1-6)	70.3	(37.6-103)	11.9	(5.6-18.1)	24.5	30.4	(25.6-35.2)	13.4	(9.9-16.8)	65.0	(47.2-82.9)	16.6	(8.9-24.3)	75.1
ST43	SãP	na	na	na	na	na	na	na	na	na	67.6	(54.7-80.5)	29.9	(22.6-37.2)	231.4	(161-302)	58.0	(35.1-81)	75.3
ST44	SãP	77.8	(61.1-94.4)	28.7	(22.5-34.8)	1105.9*	(403-1809)	185.3	(65.6-305)	73.4	165.2	(130-201)	72.9	(53.7-92)	461.9	(220-704)	117.2	(45.2-189)	97.8
ST45	MGS	4.2	(2.6-5.7)	1.6	na	6.8	(3.9-9.7)	1.2	(1-1.3)	0.0	3.9	(3.5-4.4)	1.7	(1.3-2.2)	11.4	(6.9-15.9)	2.9	(0.4-5.4)	5.6
ST46	ES	442.3*	(281-603)	162.1	(114-210)	1187.7*	(-2014-4389)	210.3	(-158-578)	92.6	102.9	(78.9-127)	45.4	(32.7-58.1)	525.5	(251-801)	133.4	(51.4-216)	86.8
ST47	RGS	3.8	(3.4-4.2)	1.4	(1.2-1.6)	8.6	(5.9-11.3)	1.5	(0.9-2.1)	0.6	5.6	(4.9-6.3)	2.5	(2.1-2.9)	9.5	(5.2-13.8)	2.4	(1.1-3.7)	1.0
ST48	SãP	20.1	(18.3-21.8)	7.3	(6.1-8.6)	43.6	(32.9-54.3)	7.6	(4-11.1)	23.3	29.8	(21.7-37.9)	13.5	(8.7-18.3)	86.1	(49.5-123)	21.0	(8.6-33.5)	69.4
ST49	MGS	10.5	(9.8-11.2)	3.9	(3.4-4.4)	15.3	(11.9-18.6)	2.6	(1.6-3.6)	0.0	26.5	(19.8-33.2)	11.7	(8.8-14.7)	57.8	(36-79.7)	14.6	(8.6-20.6)	71.0
ST50	MGS	11.9	(10.2-13.6)	4.4	(3.7-5.1)	42.3	(27-57.5)	7.3	(4.2-10.3)	15.9	19.5	(13.1-25.9)	8.6	(5.8-11.4)	101.5	(41.3-162)	25.7	(10.5-40.9)	56.5
ST51	MGS	10.8	(9.2-12.4)	4.0	(3.4-4.5)	19.1	(9.5-28.6)	3.3	(1.9-4.7)	1.7	11.5	(9.1-14)	4.9	(3.3-6.5)	49.3	(28.1-70.5)	13.0	(5.3-20.7)	37.7
ST52	PA	484.6*	(272-697)	179.7	(97.3-262)	3763.8*	(-7330-14858)	790.4	(-1531-3112)	91.3	82.2	(65-99.5)	35.1	(26.4-43.8)	244.1	(96.6-392)	64.5	(30.3-98.7)	100.0
ST53	RGS	19.7	(15.9-23.6)	7.2	(5.7-8.8)	329.6	(139-521)	57.0	(21.9-92.1)	40.7	15.8	(10.9-20.7)	6.7	(4.5-9)	82.3	(28-137)	21.8	(7.4-36.2)	49.7
ST55	RGS	2.6	(2.3-2.9)	1.0	(0.8-1.1)	4.8	(3.5-6.2)	0.8	(0.5-1.1)	0.0	2.8	(2.4-3.2)	1.2	(0.9-1.5)	4.9	(3.1-6.7)	1.2	(-0.5-2.9)	0.0
ST57	SãP	> 400†	-	> 147	-	> 400†	-	> 26	-	98.9	107.6	(82.8-132)	45.7	(34.6-56.8)	278.0	(84.4-472)	75.1	(35.6-115)	100.0
ST58	RGS	23.2	(19.1-27.4)	8.5	(7-10.1)	144.9	(76.8-213)	24.8	(13-36.6)	41.0	20.7	(18.4-23)	8.8	(6.7-10.9)	49.4	(33.6-65.2)	13.0	(6.2-19.9)	70.2

na: not available due to insufficient number of engorged females obtained

* estimates based on extrapolation (highest dose tested 400 mg/m2)

† mortality of 13.6% at the highest dose tested (400 mg/m2). LC estimates not generated because of too much uncertainty

** predicted survival rates at the potential discriminating doses (2xLC_{99} of the susceptible reference strain)

Colour code: RR≤4.0 are represented on a light grey background; 4.0<RR≤10.0 are represented on a dark grey background; RR >10.0 are represented on a black background

Table 3. Lethal concentrations 50 and LC_{90} and their 95% CI obtained for the 17 Brazilian field strains of *R. (B.) microplus* as well as their RR in comparison to the susceptible reference strain (Muñoz) when tested with amitraz. Concentrations are expressed in mg/m2.

		AMITRAZ							
strain		LC50	(95% CI)	RR at LC50	(95% CI)	LC90	(95% CI)	RR at LC90	(95% CI)
Muñoz		1.1	(0.9-1.3)			18.5	(9-28)		
ST40	SãP	8.4	(6.5-10.3)	7.7	(5.4-10)	31.7	(14.6-48.7)	1.7	(0.5-3)
ST41	SãP	16.7	(15.2-18.1)	15.3	(12-18.5)	40.0	(30.6-49.4)	2.2	(0.9-3.5)
ST42	SãP	10.1	(8.3-12)	9.3	(6.7-11.9)	50.7	(28.1-73.3)	2.8	(0.8-4.8)
ST43	SãP	30.3	(23.2-37.3)	27.8	(19.3-36)	105.0	(45.7-164.3)	5.8	(1.4-10.1)
ST44	SãP	31.1	(25.4-36.8)	28.6	(21.7-35.2)	102.5	(53.7-151.2)	5.6	(2.1-9.2)
ST45	MGS	1.0	(0.6-1.4)	0.9	(0.6-1.2)	6.3	(1.1-11.6)	0.4	(0.1-0.6)
ST46	ES	9.4	(7.4-11.3)	8.6	(6.3-10.9)	30.2	(18.4-42)	1.7	(0.7-2.6)
ST47	RGS	3.7	(2-5.5)	3.4	(2.1-4.7)	13.7	(3.7-23.7)	0.8	(0.3-1.3)
ST48	SãP	35.2	(31.8-38.6)	32.3	(26.5-38.9)	56.8	(48.4-65.2)	3.2	(1.7-4.7)
ST49	MGS	6.0	(4.4-7.7)	5.5	(4.2-6.9)	19.0	(11.5-26.5)	1.0	(0.5-1.5)
ST50	MGS	12.6	(10.6-14.6)	11.6	(8.5-14.5)	37.3	(26.3-48.2)	2.0	(0.9-3.2)
ST51	MGS	4.6	(3.8-5.4)	4.2	(2.9-5.4)	16.8	(12.8-20.7)	0.9	(0.4-1.5)
ST52	PA	9.4	(7.5-11.4)	8.7	(6.1-11.1)	22.9	(14.5-31.3)	1.3	(0.5-2.1)
ST53	RGS	17.9	(12.4-23.5)	16.5	(10.8-22.1)	510.2*	(-13.2-1034)	27.3	(-2.8-57.5)
ST55	RGS	10.9	(9-12.9)	10.1	(7.6-12.4)	27.3	(14.9-39.6)	1.5	(0.6-2.5)
ST57	SãP	35.8	(26.5-45.1)	32.9	(23.3-42.1)	128.1	(47.9-208.4)	7.1	(2-12.2)
ST58	RGS	19.1	(15.1-23.1)	17.5	(12-22.8)	91.7	(45.4-138)	5.1	(1.1-9)

* estimates based on extrapolation (highest dose tested 200 mg/m2)

Colour code: RR≤4.0 are represented on a light grey background; 4.0<RR≤10.0 are represented on a dark grey background; RR >10.0 are represented on a black background

Table 4. Lethal concentrations 50 and LC_{90} and their 95% CI obtained for the 17 Brazilian field strains of R. (B.) microplus as well as their RR in comparison to the susceptible reference strain (Mozo) and their survival rates at the DD when tested with fipronil and pyriprol. Concentrations are expressed in mg/m2.

		FIPRONIL									PYRIPROL								
strain		LC50	(95% CI)	RR at LC50	(95% CI)	LC90	(95% CI)	RR at LC90	(95% CI)	PR**	LC50	(95% CI)	RR at LC50	(95% CI)	LC90	(95% CI)	RR at LC90	(95% CI)	PR**
Mozo		0.008	(0.01-0.01)			0.02	(0.01-0.03)				0.02	(0.02-0.02)			0.05	(0-0.1)			
ST40	SãP	0.026	(0.02-0.03)	3.1	(2.5-3.7)	0.07	(0.05-0.08)	3.2	(1.6-4.8)	2.8	0.04	(0.04-0.05)	2.1	(1.6-2.6)	0.10	(0.1-0.1)	2.2	(1-3.4)	1.0
ST41	SãP	0.165	(0.14-0.19)	20.1	(15.3-25)	2.10	(1.2-3)	100.8	(33.6-168)	57	0.16	(0.1-0.2)	8.0	(5.9-10)	1.59	(0.9-2.3)	33.9	(11.4-56.4)	39.8
ST42	SãP	0.028	(0.02-0.03)	3.4	(2.7-4)	0.08	(0.05-0.12)	4.0	(1.9-6.1)	5.2	0.03	(0.03-0.04)	1.6	(1.3-2)	0.10	(0.1-0.1)	2.1	(1-3.2)	2.5
ST43	SãP	0.077	(0.07-0.09)	9.4	(7.7-11.2)	0.14	(0.09-0.19)	6.8	(3.2-10.4)	15	na	na	na	na		na	na	na	na
ST44	SãP	0.095	(0.07-0.12)	11.8	(8.8-14.7)	0.21	(0.1-0.3)	9.7	(5.1-14.3)	35	0.10	(0.1-0.1)	5.1	(3.8-6.4)	0.54	(0.4-0.7)	11.8	(4.7-18.9)	24.2
ST45	MGS	0.029	(0.03-0.03)	3.6	(2.7-4.5)	0.05	(0.04-0.06)	2.4	(1.3-3.5)	0.1	0.03	(0.02-0.03)	1.4	(1-1.7)	0.13	(0.1-0.2)	2.8	(0.9-4.7)	5.0
ST46	ES	0.139	(0.11-0.17)	17.0	(12.6-21.5)	0.62	(0.3-1)	29.5	(10.3-48.8)	54	0.12	(0.1-0.1)	6.2	(4.6-7.7)	0.46	(0.2-0.7)	9.9	(3.1-16.7)	26.8
ST47	RGS	0.078	(0.07-0.08)	9.6	(8-11.2)	0.11	(0.1-0.13)	5.4	(2.8-8)	2	0.08	(0.1-0.1)	3.9	(2.8-5)	0.28	(0.2-0.4)	6.0	(2.4-9.5)	13.1
ST48	SãP	0.054	(0.04-0.07)	6.6	(5-8.2)	0.63	(0.3-1)	29.8	(10.7-49)	31	0.06	(0.05-0.08)	3.2	(2.3-4.1)	0.54	(0.2-0.9)	11.6	(3.5-19.8)	20.4
ST49	MGS	0.022	(0.02-0.03)	2.8	(2.1-3.4)	0.09	(0.06-0.12)	4.5	(1.7-7.3)	6.8	0.03	(0.03-0.04)	1.6	(1.2-2.1)	0.20	(0.1-0.3)	4.2	(1.5-6.9)	8.4
ST50	MGS	0.315	(0.23-0.41)	38.4	(27.3-49.6)	14.5*	(2.7-26.2)	702.9	(129-1276)	66	0.44	(0.3-0.6)	21.8	(14.6-29.1)	32.8*	(-5.8-71.3)	698.0	(32.7-1363)	59.4
ST51	MGS	0.067	(0.04-0.1)	8.2	(5.5-10.8)	0.68	(-0.4-1.8)	33.3	(-0.7-67.3)	35	0.09	(0.05-0.12)	4.3	(2.9-5.7)	1.69	(-0.6-4)	36.2	(2.1-70.2)	31.1
ST52	PA	0.459	(0.32-0.6)	55.7	(38.3-73.1)	5.30	(0.7-9.9)	258.4	(33.5-483)	80	0.87	(0.6-1.2)	43.9	(26.1-61.7)	6.03	(0.8-11.2)	129.5	(4.1-255)	73.1
ST53	RGS	0.033	(0.03-0.04)	4.0	(3.1-4.8)	0.05	(0.04-0.06)	2.5	(1.4-3.6)	0	0.03	(0.03-0.04)	1.7	(1.3-2.2)	0.14	(0.1-0.2)	3.0	(1-5.1)	5.3
ST55	RGS	0.019	(0.01-0.02)	2.3	(1.6-2.9)	0.21	(0.07-0.36)	10.5	(2.8-18.1)	15	0.08	(0.04-0.11)	3.9	nd	1.08	(-0.1-2.3)	22.9	nd	28.0
ST57	SãP	0.307	(0.26-0.35)	37.2	(28.9-45.6)	0.82	(0.3-1.3)	39.7	(10.2-69.3)	97	0.41	(0.3-0.5)	20.8	(15.7-26)	1.33	(0.6-2)	28.3	(8.5-48)	82.3
ST58	RGS	0.179	(0.14-0.22)	21.6	(15.6-27.6)	1.04	(0.4-1.7)	51.3	(10.9-91.7)	62	0.37	(0.2-0.5)	18.8	(10-27.7)	3.97	(0.5-7.5)	82.6	(-16.6-182)	61.0

na: not available due to insufficient number of engorged females obtained, nd: not defined

* estimates based on extrapolation (highest dose tested 6.25 mg/m2)

** predicted survival rates at the potential discriminating doses ($2xLC_{99}$ of the susceptible reference strain)

Colour code: RR≤4.0 are represented on a light grey background; 4.0<RR≤10.0 are represented on a dark grey background; RR >10.0 are represented on a black background

Table 5. Lethal concentrations 50 and LC_{90} and their 95% CI obtained for the 17 Brazilian field strains of R. (B.) microplus as well as their RR in comparison to the susceptible reference strain (Mozo) and their survival rates at the DD when tested with ivermectin and moxidectin. Concentrations are expressed in mg/m2.

		IVERMECTIN									MOXIDECTIN								
strain		LC50	(95% CI)	RR at LC50	(95% CI)	LC90	(95% CI)	RR at LC90	(95% CI)	PR**	LC50	(95% CI)	RR at LC50	(95% CI)	LC90	(95% CI)	RR at LC90	(95% CI)	PR**
Mozo		0.9	(0.7-1)			4.3	(1.8-6.7)				0.39	(0.3-0.4)			1.3	(1-1.7)			
ST40	SãP	1.7	(1.3-2.1)	2.0	(1.3-2.7)	6.7	(3.4-10.1)	1.6	(0.2-3)	0.3	0.43	(0.4-0.5)	1.1	(0.8-1.4)	1.3	(0.8-1.7)	1.0	(0.3-1.6)	0.2
ST41	SãP	1.4	(1.2-1.6)	1.7	(1.1-2.2)	8.6	(5.2-11.9)	2.0	(0.2-3.8)	1.3	0.64	(0.6-0.7)	1.7	(1.1-2.2)	2.2	(1.5-2.8)	1.7	(0.5-2.9)	0.6
ST42	SãP	1.4	(1.1-1.7)	1.6	(1-2.2)	5.1	(2.9-7.4)	1.2	(0.1-2.3)	0.2	0.62	(0.5-0.7)	1.6	(1.1-2.1)	2.7	(1.5-3.9)	2.0	(0.6-3.5)	1.4
ST43	SãP	na	na	na	na	na	na	na	na	na	0.35	(0.3-0.4)	0.9	(0.6-1.2)	1.7	(1-2.3)	1.3	(0.4-2.1)	1.0
ST44	SãP	3.1	(2.4-3.7)	3.6	(2.4-4.8)	11.6	(5.9-17.3)	2.6	(0.4-4.9)	0.8	0.66	(0.6-0.7)	1.7	(1.2-2.2)	2.3	(1.6-3)	1.7	(0.5-3)	0.8
ST45	MGS	0.8	(0.7-0.9)	0.9	(0.6-1.2)	3.0	(1.9-4.1)	0.7	(0.1-1.3)	0.1	0.26	(0.2-0.3)	0.7	(0.4-0.9)	0.6	(0.3-1)	0.5	(0.1-0.8)	0.0
ST46	ES	2.5	(2.3-2.8)	2.9	(2.1-3.8)	5.1	(3.6-6.6)	1.2	(0.2-2.1)	0.0	0.68	(0.5-0.8)	1.8	(1.2-2.3)	2.8	(1.5-4)	2.1	(0.5-3.7)	0.8
ST47	RGS	1.4	(1-1.7)	1.6	(1.1-2.1)	6.8	(1.1-12.5)	1.6	(0.1-3.1)	0.8	0.30	(0.3-0.3)	0.8	(0.6-1)	0.6	(0.4-0.9)	0.5	(0.2-0.8)	0.0
ST48	SãP	1.1	(0.9-1.3)	1.2	(0.8-1.7)	5.9	(3.2-8.6)	1.4	(0.1-2.7)	0.6	0.57	(0.4-0.8)	1.5	(0.9-2.1)	4.2	(1.4-7)	3.2	(0.6-5.8)	3.4
ST49	MGS	1.0	(0.9-1.1)	1.2	(0.8-1.6)	3.8	(2.7-5)	0.9	(0.1-1.7)	0.2	0.46	(0.4-0.5)	1.2	(0.8-1.6)	2.5	(1.5-3.5)	1.9	(0.4-3.4)	1.7
ST50	MGS	1.3	(1.1-1.5)	1.5	(1-2)	5.6	(2.8-8.3)	1.3	(0.2-2.5)	0.5	0.23	(0.2-0.3)	0.6	(0.4-0.8)	1.2	(0.4-2)	0.9	(0.3-1.6)	0.8
ST51	MGS	1.0	(0.9-1.1)	1.1	(0.7-1.5)	7.3	(5-9.6)	1.7	(0.2-3.3)	1.3	0.26	(0.2-0.3)	0.7	(0.4-0.9)	2.1	(1-3.2)	1.6	(0.3-2.8)	2.1
ST52	PA	1.4	(1.2-1.6)	1.6	(1-2.2)	7.4	(4.8-9.9)	1.8	(0.2-3.3)	0.7	0.64	(0.5-0.8)	1.7	(1.1-2.3)	2.1	(1.1-3.1)	1.6	(0.3-2.8)	0.4
ST53	RGS	3.6	(3-4.2)	4.2	(3-5.3)	6.6	(5.4-7.8)	1.6	(0.6-2.6)	0.0	0.80	(0.7-0.9)	2.1	(1.4-2.8)	2.4	(1.7-3.2)	1.8	(0.4-3.2)	0.5
ST55	RGS	3.4	(2.6-4.2)	4.0	(2.8-5.2)	5.8	(4.3-7.2)	1.4	(0.5-2.3)	0.0	0.83	(0.6-1)	2.1	(1.4-2.9)	1.6	(1.2-2)	1.2	(0.4-2)	0.0
ST57	SãP	2.3	(1.8-2.7)	2.6	(1.5-3.7)	8.5	(5.2-11.7)	2.0	(0.2-3.9)	0.2	0.56	(0.4-0.7)	1.5	(0.9-2)	2.9	(1.6-4.3)	2.2	(0.4-4)	1.7
ST58	RGS	1.8	(1.5-2.1)	2.1	(1.3-2.8)	8.8	(5.8-11.7)	2.1	(0.2-4)	1.0	0.80	(0.6-1)	2.1	(1.4-2.8)	1.8	(0.9-2.7)	1.3	(0.4-2.3)	0.0

na: not available due to insufficient number of engorged females obtained

** predicted survival rates at the potential discriminating doses (2xLC_{99} of the susceptible reference strain)

Colour code: RR≤4.0 are represented on a light grey background; 4.0<RR≤10.0 are represented on a dark grey background; RR >10.0 are represented on a black background

Larval Packet Test

Four field populations were tested with the LPT. Table 6 summarizes the RR50 and the resulting resistance-classification when using the LPT in comparison to the LTT. Resistance ratios obtained with the LTT were higher than those obtained with the LPT in 95% (18/19) of the tests. As a consequence, the LPT has failed to identify resistance in 6 cases where the LTT showed RR50 values clearly above 4.

Table 6. Resistance ratios based on the LC_{50} when assessed with the LTT and LPT for 4 Brazilian field populations

	Chlorpyriphos*		Cypermethrin**		Ivermectin*		Fipronil*		Amitraz**	
strain	LTT	LPT	LTT	LPT	LTT	LPT	LTT	LPT	LTT	LPT
ST40	19.8	3.1	104.8	65.6	2.0	1.3	3.1	1.5	7.7	2.4
ST41	7.6	1.9	141.0	69.2	1.7	1.5	20.1	2.1	15.3	7.9
ST42	5.1	na	201.0	106.6	1.6	1.2	3.4	0.8	9.3	27.8
ST44	28.7	3.4	167.3	45.5	3.6	1.4	11.8	1.4	28.6	12.8

na: not available because of insufficient data to generate the dose-reponse mortality curve
* Mozo is the reference strain
** Muñoz is the reference strain
Colour code: RR≤4.0 are represented on a light grey background; 4.0<RR≤10.0 are represented on a dark grey background; RR >10.0 are represented on a black background

Discussion

The LTT is a time-effective test which relies on the distribution of tick eggs in the wells of pre-treated 96-well plates, allowing testing 12 doses of 5 compounds in a single plate. It was shown previously to be equally sensitive and much more time effective than the LPT (Lovis et al., 2011). In this article, we present some additional information to facilitate the completion of the LTT and some alternatives to the equipment presented in Lovis et al. (2011). The use of glass beads and talc allows individualising eggs in an extremely effective way and with very basic material. In addition, the use of a curette to measure the quantity of eggs and to distribute the eggs instead of the seed counter avoids investing in cumbersome and costly equipment. Furthermore, static electricity can be removed during distribution and the sealing of the plates by using a static control mat which is a simple alternative to

other discharging systems. For the moment, DMSO evaporation after the coating of the plates with the acaricides still requires some particular equipment. We suggest here two possibilities (N_2 sampler concentrator, centrifugal vacuum concentrator) but a simplified system would be desirable. A possibility might be to consider using a different solvent which evaporates more easily while at the same time ensuring satisfactory dissolution of all tested compounds without damages to the polystyrene plates. In the setup of our study, plates treated with all compounds could be stored for at least 5 weeks without losing activity (data not shown). Finally, the incubation conditions of the plates should be kept as stable as possible to decrease the factors which could negatively impact the eggs between their distribution into the plates and their hatching.

The Mozo strain was meant to be used as susceptible reference strain for all compounds since it was tested in parallel to the field populations and in the same conditions. However, our Mozo isolate showed unexpectedly high resistance to both cypermethrin and flumethrin and moderate resistance to amitraz in comparison to the Muñoz strain (data not shown) and was for this reason replaced by the Muñoz strain for these compounds. The resistance of the Mozo strain is surprising since it has never been exposed to acaricides before and after its collection. Additionally, it has already been used as susceptible reference strain for SP (Mendes et al., 2011). Our isolate was established at the IPVDF in November 2010 and larvae used for testing were from the second generation. It is possible that the IPVDF isolate has been contaminated with ticks from a resistant isolate during that time.

The tested concentrations of the acaricidal compounds were suitable to calculate LC_{50} and LC_{90} of susceptible and resistant populations. Only in 5% and 10% of the tests an extrapolation from the dose-response curve was necessary to estimate the LC_{50} and LC_{90}, respectively. For studies aiming to evaluate the susceptibility of field populations without prior knowledge on their resistance status, we recommend testing the same concentration ranges (as described in the Material and Methods section), with the following two modifications: chlorpyriphos, 0.4-800 mg/m2 instead of 0.2-400 mg/m2; cypermethrin, if the populations are expected to be resistant, 0.4-800 mg/m2 instead of 0.1-200 mg/m2. These ranges should minimize the cases where LC_{90} have to be extrapolated from the model.

We observed particularly high resistance frequencies to OP, SP, amitraz and PYZ, most probably because farms were selected based on the observation of treatment failures. We reported 94% resistance to SP and 65% resistance to chlorpyriphos, which is comparable to the values determined

by Martins et al. (2008) with the AIT and by Mendes et al. (2011) with the LPT. In contrast, the coumaphos and amitraz resistance frequencies we observed (82% and 88%, respectively) are higher than those of previous studies (Campos Júnior and Oliveira, 2005; Farias et al., 2008; Martins et al., 2008). Resistance to fipronil was detected in 65% of the farms we surveyed, which is very high considering that resistance to this compound was reported only recently in Brazil (Martins et al., 2008; Castro-Janer et al., 2010). In 2008, Martins et al. reported an average of efficacy of fipronil of 88.5% among 723 populations tested with the AIT between 1997 and 2006. More recently, Castro-Janer et al. (2010) also reported some resistance to fipronil with the LIT. The value obtained in the present study is worrying and suggests that resistance to fipronil is spreading rapidly in Brazil. The presence of four populations demonstrating dose-response mortality curves typical of heterogeneous populations also reflects that fipronil resistance is in process of development in field populations. Similarly, resistance to pyriprol was also found in nearly half of the populations surveyed whereas this compound is not used to treat cattle against ticks. Finally, resistance to ivermectin has also already been reported several times in Brazil using the LIT (Klafke et al., 2006; Klafke et al., 2012).

In the present study, field populations were considered to be resistant when RR50 were greater than 4. This value allowed discriminating very well between SP susceptible and SP resistant populations since most of the RR50 estimates were greater than 40 for resistant, or around 1 for susceptible populations. In contrast, for OP, PYZ and amitraz, several populations possessed RR50 between 3 and 5, with some estimates smaller or equal to the threshold of 4.0, but having the upper 95%CI limit over 4.0. In these last cases susceptibility can be argued, but this is inherent to the use of cut-off values. Likewise two populations (ST44 and ST55) demonstrated RR50 estimates to ivermectin of 3.5 and 4.0, respectively and resistance could therefore be suspected. Finally, if populations had been considered resistant when RR50 was statistically significant and greater or equal to 2, instead of using our cut-off value, as it is has been done in previous studies (Castro-Janer et al., 2011; Klafke et al., 2012), many additional populations would have been considered resistant. Thus, all the populations would have been considered resistant to fipronil and 44% resistant to ivermectin.

The resistance status of the field populations was based on RR50 because they possess smaller 95%CI than RR90 and are therefore more reliable estimates. However, as it is essential not to miss resistance at its emergence, resistance statuses based on RR50 were also compared to those based on RR90. Considering RR90 instead of RR50 would have led to similar conclusions to distinguish resistant from susceptible field populations in 94% (124/132) of the cases, excluding amitraz. The

discrepancies were observed with flumethrin, fipronil and pyriprol, for which 1, 2 and 4 additional populations, respectively would have been considered resistant if based on RR90 instead of RR50 (Fig. 1a for pyriprol). This observation suggests that resistance is emerging in these populations. Inversely, the population considered as ivermectin-resistant based on RR50 would have been considered susceptible based on RR90, reflecting the steep dose-response curve of the field population. Amitraz situation (Fig. 1b) was particular since all the field populations demonstrated greater slopes than the reference strain. Thus, although a wide shift was observed between the response of the reference and the field populations at low concentrations, the curves intersect around 90-95% mortality. As a consequence, if the RR90 had been considered, only 5 out of the 15 amitraz resistant strains based on RR50 would have been diagnosed as resistant. To conclude, if the complete dose-response curve is obtained, we recommend generating both RR50 and RR90 estimates, considering RR50 as a priority and comparing them to RR90 to detect emerging resistance, with the exception of amitraz, for which the use of RR90 is clearly not indicated.

The use of DD to determine resistance has been recommended by the FAO (2004) but has been criticised (Jonsson et al., 2007). We therefore wanted to assess if DD would have been suitable in our study and found a wide agreement between the survival rates at the DD calculated as 2 x LC_{99} of the susceptible reference strain, and the RR50 or RR90. Survival rates at these DD exceeded 10% in all the populations diagnosed as resistant based on the RR50, with 3 exceptions. Additionally, the use of the DD would have allowed detecting 3 of the 7 cases of emerging resistance. However, since DD are particularly valuable to reduce the amount of work and of ticks needed to detect resistance, their interest is, to our opinion, limited in the case of the LTT and we would not recommend their use as a substitute of the full dose-response mortality curves.

The LPT and LTT have already been compared using the laboratory strains Muñoz and Ultimo and were shown to perform equally well in the detection of the resistance to diazinon, flumethrin, cypermethrin and amitraz (Lovis et al., 2011). In the present study, we repeated the comparison but limited it to four field populations due to the labour-intensive nature of the test. The LTT showed a higher sensitivity than the LPT to measure resistance, providing higher resistance ratios to all compounds. This was most visible for chlorpyriphos and fipronil, for which the LPT failed to detect resistance to chlorpyriphos and fipronil, while the LTT detected 4 and 2 resistant populations, respectively. Finally, since the LIT is getting increasingly used for the detection of resistance to ivermectin and fipronil (Klafke et al., 2006; Castro-Janer et al., 2011) and has been shown to perform

better than the LPT for the detection of fipronil resistance (Castro-Janer et al., 2009), it would be also relevant to compare the sensitivity of the LTT and the LIT to detect resistance to these compounds.

To conclude, the present study showed that the LTT is a reliable bioassay to diagnose acaricide resistance in *R. (B.) microplus* field populations of ticks. The original method as described by Lovis et al. (2011) was adapted to reduce the required lab-infrastructure for the test performance. A detailed protocol for the tick egg separation and the distribution into the microtiter plates is provided. With these modifications, the LTT can be carried out in laboratories without additional needs of expensive equipment and infrastructure. It allowed here to confirm the widespread resistance to OP, SP and amitraz, to identify a few cases of ivermectin resistance, but also to show the important on-going development of PYZ resistance in Brazil.

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4.5. Chapitre 5: Distribution patterns of three sodium channel mutations associated with pyrethroid resistance in *Rhipicephalus (Boophilus) microplus* populations from North and South America, South Africa and Australia

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Distribution patterns of three sodium channel mutations associated with pyrethroid resistance in *Rhipicephalus* (*Boophilus*) *microplus* populations from North and South America, South Africa and Australia

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Synthetic pyrethroid

ABSTRACT

Resistance to synthetic pyrethroids (SP) in the cattle tick *Rhipicephalus* (*Boophilus*) *microplus* is widespread throughout its distribution area. Three single nucleotide substitutions identified in Domains II and III of the sodium channel gene of *R.* (*B.*) *microplus* are known to be associated with target site pyrethroid resistance. We developed a multiplex PCR using allele-specific primers to amplify wild type or mutated genotypes of the three mutations simultaneously. This assay was used to screen tick samples originating from Brazil, Argentina, Mexico, South Africa and Australia whose phenotype to flumethrin and cypermethrin had been determined by the use of the Larval Tarsal test (LTT) or the Larval Packet Test (LPT). These mutations were found to have distinct geographical distributions and result in different resistance phenotypes. The L64I Domain II mutation conferring resistance to several SP compounds was found in all the Brazilian, Argentinean and Australian populations and in one South African population, with frequencies between 38% and 100% in flumethrin and cypermethrin resistant populations, respectively. In contrast, this mutation was not found in samples from Mexico, while the Domain III mutation was found exclusively in this country. The G72 V Domain II flumethrin-specific mutation was found in a single Australian population, at a very low frequency (6%). The homozygous resistant RR genotype of the L64I Domain II mutation correlated significantly with the survival rates at the discriminating doses of flumethrin and cypermethrin. This survey shows the widespread distribution of the L64I Domain II mutation and provides evidence of its geographic separation from the Domain III mutation.

1. Introduction

Resistance to synthetic pyrethroids (SP) in the one-host cattle tick *Rhipicephalus* (*Boophilus*) *microplus* is widespread throughout its area of distribution (Kemp et al., 1998; FAO, 2004; Graf et al., 2004; Rosario-Cruz et al., 2009a). Pyrethroid insecticides target the voltage-gated sodium channel, an integral transmembrane protein consisting of four homologous domains (I–IV) each containing six membrane spanning segments (S1–6) (Dong, 2007). Single nucleotide substitutions in the gene have been shown to cause SP resistance in numerous insect species (reviewed by Dong, 2007). In Australian *R.* (*B.*) *microplus*, increased metabolic detoxification was the first identified mechanism of SP resistance (Schnitzerling et al., 1983; Nolan et al., 1989). However, increased detoxification was not observed in some SP resistant populations in their studies, suggesting that target site insensitivity was present. Later, patterns of SP resistance found in some Mexican populations of *R.* (*B.*) *microplus* led to the hypothesis that both increased metabolic enzyme activity (esterase and cytochrome P450) and target site insensitivity (*kdr* mutation) were involved in SP resistance in these populations (Miller et al., 1999). Among the two phenomena, target site insensitivity is likely the major mechanism of economically significant resistance to SP in most populations of *R.* (*B.*) *microplus* (Guerrero et al., 2012).

Three point mutations associated with resistance to SP have been reported in the sodium channel gene of *R.* (*B.*) *microplus* (Table 1) (He et al., 1999; Morgan et al., 2009; Jonsson et al., 2010). The first mutation was identified in two tick populations from Mexico (He et al., 1999) in which target site-based resistance had been reported (Miller et al., 1999). This mutation is located in the S6 segment of the Domain III of the sodium channel gene and results in a phenylalanine to isoleucine amino acid substitution. A

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Table 1
Summary of the three point mutations in the sodium channel gene associated with resistance.

Domain	Segment	Nucleotide position	Nucleotide substitution	Amino acid substitution	Reference code	Identification	PCR assay
III	S6	2134	T → A	Phe → Ile	F1550I	He et al. (1999)	Guerrero et al. (2001)
II	S4–5 linker	190	C → A	Leu → Ile	L64I	Morgan et al. (2009)	Morgan et al. (2009)
II	S4–5 linker	215	G → T	Gly → Val	G72 V	Jonsson et al. (2010)	None

Accession number in GenBank: AF134216.

PCR diagnostic assay was developed to allow the detection of this mutation (Guerrero et al., 2001), and frequency of this mutation correlated with survival rates in permethrin dose-mortality bioassays. In addition, the allelic frequency of the mutation correlated with resistance ratios and survival rates at discriminating doses of SP assessed *in vitro* (Rosario-Cruz et al., 2005, 2009b; Li et al., 2007). Later, another mutation associated with SP resistance was identified in Australian populations of *R.* (*B.*) *microplus* (Morgan et al., 2009), and a PCR assay was developed to allow the detection of the mutation. More recently, an additional point mutation associated with resistance to flumethrin but not to cypermethrin was identified in Australian populations (Jonsson et al., 2010). These two mutations are located in the S4–5 linker of the Domain II of the sodium channel gene, leading to leucine to isoleucine (L64I) and glycine to valine (G72 V) amino acid substitutions, respectively.

The mutation identified by He et al. (1999) has been found throughout Mexico (Rosario-Cruz et al., 2005, 2009b) and in an outbreak strain in the United States (Miller et al., 2007) while it has not been reported in Brazilian SP resistant populations (Andreotti et al., 2011; Domingues et al., 2012) nor in Australia (Chen et al., 2009). Although the two Domain II mutations were initially identified in Australia, recent work showed that the L64I mutation reported by Morgan et al. (2009) was found in ticks outside Australia, however the G72 V mutation reported by Jonsson et al. (2010) appears to be isolated to Australia (Guerrero et al., 2012; Domingues et al., 2012).

Bioassays are complementary to molecular surveys and allow assessment of SP resistance without knowledge of the underlying mechanism. *In vitro* tests, such as the adult immersion test (Drummond et al., 1973), the larval packet test (LPT) (Stone and Haydock, 1962), both currently recommended by the FAO (FAO, 2004) and the recently developed larval tarsal test (LTT) (Lovis et al., 2011) can be used to identify resistant phenotypes. Ticks can be exposed to a range of doses of acaricides to establish a dose–response curve from which the doses inducing 50% or 90% mortality can be calculated and compared to a susceptible reference strain to determine the resistance ratios. Alternatively, ticks can be exposed to a single dose that has been pre-established based on data from bioassays conducted on a susceptible reference strain, and survival at this discriminating dose (DD) is considered as an indicator of resistance (FAO, 2004).

The objectives of this study were (1) to develop a multiplex diagnostic PCR assay to allow the simultaneous detection of the three known *R.* (*B.*) *microplus* sodium channel gene mutations that are associated with target site pyrethroid resistance, and (2) to investigate the presence of these three mutations in field and laboratory populations originating from Brazil, Argentina, South Africa, Mexico and Australia and, if present, to correlate their frequency to the phenotypic resistance to flumethrin and cypermethrin assessed *in vitro* with the LTT or LPT bioassays.

2. Materials and methods

2.1. Tick populations

Samples of tick populations were collected between February 2009 and November 2010 from Brazil, Argentina, South Africa, and Australia. Details about the origin of these populations are listed in Table 2. In addition, an Australian SP-resistant laboratory strain, Ultimo, was also included in the study. The Ultimo strain was originally collected in 1992 in central Queensland, Australia from SP and amitraz resistant ticks, maintained in colony at CSIRO and subsequently established at the Novartis Animal Health Research Centre (CRA), St-Aubin, Switzerland in 1999 where it was maintained without acaricide selection (Lovis et al., 2011). Ultimo ticks used for bioassays and molecular studies were from F31 to F33 generations.

Three Mexican strains (San Felipe, Coatzacoalcos, and Rio Bravo) and an additional Brazilian strain (Santa Luiza), established as laboratory strains at the Cattle Fever Tick Research Laboratory (CFTRL), Edinburg, Texas were also included in this study. The three Mexican strains were collected from pyrethroid resistant populations. The San Felipe and Rio Bravo strains have predominant pyrethroid resistance due to target site insensitivity, while the Coatzacoalcos strain is resistant through esterase-based metabolism. The San Felipe and Coatzacoalcos strains were reared under SP selection pressure and their origin, rearing conditions and characterization were described by Miller et al. (1999). The Rio Bravo strain originated from the city of the same name in Tamaulipas, Mexico and was collected at the importation inspection vats in Reynosa, Tamaulipas at the border with Texas. This strain was selected for pyrethroid resistance. The origin of the Brazilian Santa Luiza strain, which has a pyrethroid resistant phenotype, has been described by Li et al. (2007). San Felipe and Coatzacoalcos larvae used for molecular studies were from F16 to F26 generations, respectively, while LPT data for these two strains were from F8 to F16 generations. Rio Bravo ticks used for bioassays and molecular studies were from F1 to F3 generations, respectively. Santa Luiza larvae used for molecular studies were from the first generation established at the CFTRL in 2000 while it was tested later *in vitro* with F13 ticks.

2.2. Bioassays

The LTT was used to assess the susceptibility of tick populations to technical grade flumethrin (Sigma–Aldrich, Switzerland) and cypermethrin (Novartis, Switzerland). The LTT was performed at the CRA, following the protocol described by Lovis et al. (2011) for the Argentinean, South African and Australian populations. For the Brazilian field populations the LTT was conducted at the Instituto Biologico (IB), São Paulo and at the Instituto de Pesquisas Veterinárias Desidério Finamor (IPVDF) with the modifications described by Lovis et al. (submitted for publication-a).

The LPT (Stone and Haydock, 1962) was carried out at the CFTRL as previously described (Miller et al., 1999; Li et al., 2007) to determine permethrin (FMC, Philadelphia, PA), cypermethrin (Hoechst-Roussel, Mexico) and flumethrin (Bayer, Mexico) toxicity to the San Felipe and Coatzacoalcos strains and permethrin toxicity to the Rio Bravo and Santa Luiza strains.

2.3. DNA extraction

Genomic DNA was isolated from individual tick larvae of the Rio Bravo, San Felipe, Coatzacoalcos and Santa Luiza strains as described by Guerrero et al. (2001). Briefly, pooled larvae were stored frozen in plastic vials until DNA purification. Larvae were

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Table 2
Origin of the field populations and laboratory strains and information about their previous characterisation.

Strain	Country	State/Province	Origin	Year of collection	Bioassay used for characterization
ST40	Brazil	São Paulo	Field pop	2011	LTT
ST41	Brazil	São Paulo	Field pop	2011	LTT
ST42	Brazil	São Paulo	Field pop	2011	LTT
ST44	Brazil	São Paulo	Field pop	2011	LTT
ST46	Brazil	Espirito Santo	Field pop	2011	LTT
ST47	Brazil	Rio Grande do Sul	Field pop	2011	LTT
ST48	Brazil	São Paulo	Field pop	2011	LTT
ST49	Brazil	Mato Grosso do Sul	Field pop	2011	LTT
ST50	Brazil	Mato Grosso do Sul	Field pop	2011	LTT
ST51	Brazil	Mato Grosso do Sul	Field pop	2011	LTT
ST52	Brazil	Paranà	Field pop	2011	LTT
ST55	Brazil	Rio Grande do Sul	Field pop	2011	LTT
ST57	Brazil	São Paulo	Field pop	2011	LTT
ST58	Brazil	Rio Grande do Sul	Field pop	2011	LTT
Santa Luiza	Brazil	na	Lab strain	2000[a]	LPT
ST21	Argentina	Corrientes	Field pop	2010	LTT
ST22	Argentina	Corrientes	Field pop	2010	LTT
ST24	Argentina	Corrientes	Field pop	2010	LTT
ST25	Argentina	Corrientes	Field pop	2010	LTT
ST26	Argentina	Corrientes	Field pop	2010	LTT
ST27	Argentina	Corrientes	Field pop	2010	LTT
ST29	Argentina	Corrientes	Field pop	2010	LTT
ST30	Argentina	Corrientes	Field pop	2010	LTT
San Felipe	Mexico	Tamaulipas	Lab strain	1996	LPT
Coatzacoalcos	Mexico	Veracruz	Lab strain	1994[a]	LPT
Rio Bravo	Mexico	Tamaulipas	Lab strain	1998	LPT
ST11	South Africa	Western Cape	Field pop	2010	LTT
ST12	South Africa	Western Cape	Field pop	2010	LTT
ST15	South Africa	Mpumalanga	Field pop	2010	LTT
Ultimo	Australia	Queensland	Lab strain	1992	LTT
Urah	Australia	Queensland	Field pop	2010	LTT

na: not available.
[a] Year of establishment in the CFTRL.

placed on a Petri plate on dry ice, and individuals were transferred to pre-chilled 1.5 ml tubes (Kontes, Vineland, NJ). Liquid nitrogen-cooled disposable pellet pestles for 1.5 ml centrifuge tubes (Kontes, Vineland, NJ) were used to grind the larvae against the tube walls for 15 s. Twenty-five µl of GeneAmp 10X PCR buffer II (Applied Biosystems, Carlsbad, CA) were added, and larvae were ground an additional 15 s. The tubes were briefly centrifuged and boiled for 3 min. Samples were stored at −80 °C until PCR amplification.

Genomic DNA from the other tick populations was also extracted from individual larvae according to Guerrero et al. (2001) but with some modifications. Briefly, frozen larvae were transferred individually from plastic vials to pre-chilled 1.2 ml polypropylene tubes (Qiagen, Switzerland) kept on dry ice. Steel beads (5 mm diameter, Qiagen, Switzerland) were dispensed into the tubes using a 96-well dispenser (Qiagen, Switzerland) and 20 µl buffer (100 mM Tris, pH 8.3; 500 mM KCl) were added per tube. The tubes were grouped in a 96-well rack and placed in a mixer mill (Retsch, Haan, Switzerland, Type MM301) between two adaptors at an oscillation frequency of 25 Herz for a total of 4 min (2 x 2 min) to grind the larvae. The tubes were centrifuged for 1 min at 1500*g* and 4 °C, boiled for 5 min and centrifuged again for 1 min at 1500*g* at room temperature. The DNA extracts were pipetted from the polypropylene tubes to 96-well PCR plates (Thermo Scientific, Switzerland). Plates were sealed with adhesive foil sheets (Thermo Scientific, Switzerland) and samples stored at −80 °C until PCR amplification.

2.4. PCR Conditions

PCR amplification was carried out in two separate reactions to detect both the pyrethroid susceptible and pyrethroid resistant alleles. Twenty µl reactions were optimized and performed in thin-walled 0.2 ml microcentrifuge tubes (Rainin, Oakland, CA). During optimization, we designed and tested several oligonucleotide primers and varied their concentrations (0.5–4.0 µM) and annealing temperature (54–64 °C). The concentration of $MgCl_2$ was varied from 1.0 to 2.5 mM, and the concentration of each dNTP was varied from 0.05 to 0.2 mM. Final optimized reaction conditions used 1 µl of DNA from a single tick larva, 1 µl of 10X PCR buffer II, 1.75 mM $MgCl_2$, 0.1 mM of each dNTP, and 0.2 µl of a 1:1 vol:vol mix of Ampli*Taq* (Applied Biosystems, Carlsbad, CA) and TaqStart antibody (Clontech, Mountain View, CA). To detect the susceptible alleles, reactions included 4 µM of primers FG-221 and FG-424, 1 µM of primers DB-011 and FG-447, and 0.5 µM of primers LL-001 and FG-446 (Table 3). To detect the resistant alleles, reactions included 4 µM of primers FG-222 and FG-424, 1 µM of primers DB-012 and FG-447, and 0.5 µM of primers LL-005 and FG-446 (Table 3). Amplification was carried out using a DNA Engine (MJ Research, Watertown, MA) programmed for 2 min at 96 °C, followed by 37 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min. A final extension of 72 °C for 7 min was also included. PCR products were viewed on GelStar (Lonza, Rockland, ME) stained 4% NuSieve agarose and TBE gels (Lonza, Rockland, ME).

2.5. Data analyses

Statistical analysis of the LTT results was performed on the R software (version 2.12.0) using the drc package (version 2.0–1), specific for modelling dose–response curves (Ritz and Streibig, 2005). Concentrations inducing 50% (LC_{50}) and 90% (LC_{90}) mortality and their respective resistance ratios (RR50 and RR90) as well as the survival rates at potential discriminating doses (DD) were calculated as described previously (Lovis et al., 2011). Correlations between the frequency of the RR genotype or resistant allele frequency and the survival rates at the DD were estimated using the Pearson's correlation coefficients on the R software for flumethrin and cypermethrin. Due to non-linear correlations, correlations with

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Table 3
Sequences of primers selected for the multiplex PCR assay.

Primer ID	Sequence	Description	Annealing Site in AF134216 (nt#)
Domain III mutation			
FG-221[a]	5′-TTATCTTCGGCTCCTTCT-3′	Wild type-specific sense	2117–2134
FG-222[a]	5′-TTATCTTCGGCTCCTTCA-3′	Resistant-specific sense	2117–2134
FG-424	5′-TCATTGAAATTGTCGATAATAACAC-3′	Downstream non-diagnostic	2156–2180
L64I Domain II mutation			
FG-447	5′-GAACTTGTGTTTACTTTCTTCGTAGT-3′	Downstream non-diagnostic	266–291
DB-011[b]	5′-GGAAAACCATCGGTGCTC-3′	Wild type-specific sense	173–190
DB-012[b]	5′-GGAAAACCATCGGTGCTA-3′	Resistant-specific sense	173–190
G72 V Domain II mutation			
LL-001	5′-CTTGACCTTTGTCCTGGG-3′	Wild type-specific sense	198–215
LL-005	5′-CTTGACCTTTGTCCTGGT-3′	Resistant-specific sense	198–215
FG-446	5′-ACTTGTGTTTACTTTCTTCGTAGT-3′	Downstream non-diagnostic	266–289

[a] Primer sequences obtained from Guerrero et al. (2001).
[b] Primer sequences obtained from Morgan et al. (2009).

RR50 and RR90 were computed using the Kendall coefficient. These correlations were based on the populations which possessed PCR results for a minimum of 15 larvae. Statistical analysis of the LPT results was performed using Polo-PC (LeOra Software, 1987). Lethal doses inducing 50% and RR50 were calculated as described previously (Miller et al., 1999; Li et al., 2007). Tick population resistance phenotype was assigned based on three criteria: RR50, RR90 and survival at the DD. Populations were considered resistant when their RR50 or RR90 was greater than four or when the survival rate at the DD was over 10% (Lovis et al., submitted for publication-a,b).

3. Results

3.1. Multiplex PCR assay development

Guerrero et al. (2001) developed an allele-specific PCR assay to detect an A → T nucleotide substitution at the nucleotide #2134 of the tick sodium channel coding sequence (all numbering in this work is based upon the AF134216 GenBank nucleotide sequence fragment entry for *R* (*B.*) *microplus*) using primers FG-221 and FG-222 as diagnostic primers and FG-227 as the downstream non-diagnostic primer. The two diagnostic primers from this assay were used for the new multiplex assay, but a new non-diagnostic primer (FG-424) was designed leading to an amplified product of 64 bp (Fig. 1). FG-424 performed better than FG-227, because self-hybridization of FG-227 at its 3′-end led to primer-dimer formation during the amplification. Primer concentrations in the multiplex were adjusted from the 1.0 μM concentration used in the Guerrero et al. (2001) protocol as needed to optimize the performance of the multiplex.

Morgan et al. (2009) designed primers for an allele-specific PCR targeting a C → A nucleotide substitution at the nucleotide #173 of the tick sodium channel coding region (L64I Domain II mutation). Their two diagnostic primers were used for the new multiplex assay and renamed DB-011 (wild type) and DB-012 (resistant), but a new non-diagnostic primer (FG-447) was designed to lengthen the amplified fragment to 119 bp and hence allow its differentiation from other amplified fragments (Fig. 1). We initially designed primers to detect the mutation on the sense and antisense strands and observed the presence of an intron in the diagnostic assay based on the antisense strand, preventing the use of this version for the multiplex. To enhance specificity of the multiplex, the mismatch of a C to a T incorporated at the penultimate base of the diagnostic primers by Morgan et al. (2009) was also incorporated into our protocol. Primers were used at a concentration of 1.0 μM.

Jonsson et al. (2010) identified a G → T mutation at the nucleotide #215 of the tick sodium channel coding region (G72 V Domain II mutation) and we designed allele-specific primers LL-001 (wild type diagnostic), LL-005 (resistant diagnostic) and FG-446(non-diagnostic) to allow detection of this mutation in the multiplex reaction, producing a 92 bp amplification product. As noted previously, detection on the antisense strand would be hampered by the presence of an intron upstream of the mutation reported by Morgan et al. (2009). An additional intron was observed on the coding strand between the nucleotides #353 and #434. FG-446 was therefore used as the non-diagnostic primer to avoid this intron. The concentration of these primers was optimized at 0.5 μM and typical results from the multiplex PCR assay obtained for different genotypes are shown in Fig. 2.

3.2. Bioassays

Resistance ratios to flumethrin and cypermethrin at 50% and 90% mortality, survival rates at the DD obtained with the LTT, as well as the RR50 estimated with the LPT are shown in Table 4. Around 86% (24/28) of the populations evaluated with the LTT were found to be resistant to cypermethrin based on our criteria noted previously. Resistance to cypermethrin based on the LTT was observed in 13 out of 14 Brazilian, 7 out of 8 Argentinean, both of the Australian and one of the three South African populations. The four populations found to be susceptible to cypermethrin were also susceptible to flumethrin (ST11, ST12, ST21, ST47). One additional Brazilian population (ST55) was susceptible to flumethrin, by all the bioassay criteria, but was cypermethrin resistant. Resistance statuses to flumethrin based on RR50, differed from those based on RR90 and DD for four Argentinean populations (ST22, ST25, ST26, ST27) which would have been considered susceptible based on RR50 but resistant based on RR90 and DD. Finally, the three Mexican strains, tested with the LPT, were resistant to flumethrin, cypermethrin and permethrin, and the Brazilian Santa Luiza population was resistant to permethrin based on LPT.

3.3. Genotype frequencies

The frequency of larvae with specific genotypes to the three mutations is listed in Table 4 and correlation between RR genotype frequency and survival rate at the DD of flumethrin and cypermethrin represented in Fig. 3. The Domain III mutation was found in two of the three Mexican populations, San Felipe and Rio Bravo (Table 4). Both of these populations were phenotypically resistant to SP and had a resistant allele frequency of 94% and 100%, respectively. This Domain III mutation was not found in any samples from the other countries. The G72 V Domain II mutation was found in only a single larva of an Australian population as a heterozygote.

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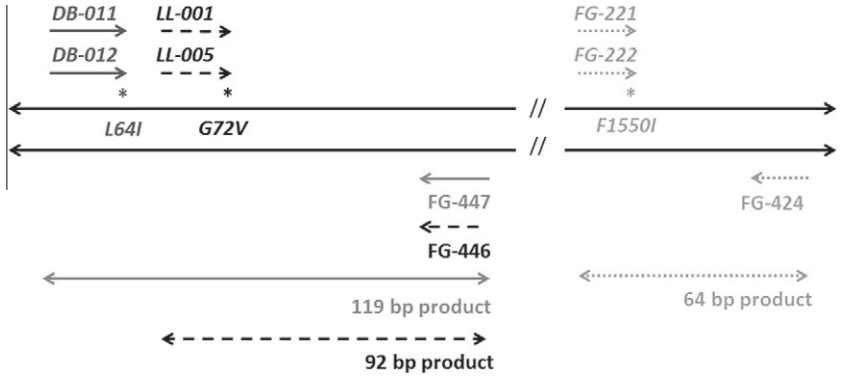

Fig. 1. Schematic representation of the locations of the primers selected for multiplex PCR assay. Asterisks indicate the location of the mutations. Italicised primers are the diagnostic primers.

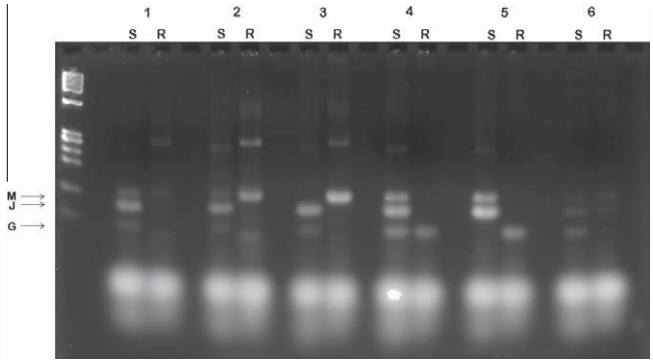

Fig. 2. Multiplex PCR assay of representative genotypes. Arrows point to the locations of the PCR amplification product that is diagnostic for each mutation: *G* = Domain III mutation, product size is 64 bp; *J* = G72 V Domain II mutation, product size is 92 bp; *M* = L64I Domain II mutation, product size is 119 bp. Two separate reactions were carried out to detect the susceptible alleles (S) and the resistant alleles (R). (1) larva homozygous susceptible to all three mutations; (2) larva homozygous susceptible to *G* and *J*, heterozygote to *M*; (3) larva homozygous susceptible to *G* and *J*, homozygous resistant to M; (4) larva heterozygote to *G*, homozygous susceptible to *J* and *M*; (5) larva homozygous resistant to *G*, homozygous susceptible to J and M; (6) larva homozygous susceptible to *G*, heterozygote to *J* and *M*.

It was not found in any other country. The L64I Domain II mutation was found in all the Brazilian, Argentinean and Australian populations and in one of the three South African populations. In contrast, the L64I Domain II mutation was not found in the samples that originated in Mexico, only the Domain III mutation was found in these populations. The frequency of the resistant allele was greater than 38% in all the non-Mexican populations resistant to cypermethrin, with the exception of ST55 where it was 8% (Table 4). Three of the four populations which were susceptible to both flumethrin and cypermethrin (ST11, ST12, and ST21) did not possess any RR genotype individuals and none or few RS individuals (0–17%) while the fourth population (ST47) had a resistant allele frequency of 28%.

4. Discussion

Target site insensitivity has been shown to be the major resistance mechanism in most of the SP resistant populations of *R.* (*B.*) *microplus* in Mexico (Rosario-Cruz et al., 2005, 2009b) and Australia (Morgan et al., 2009; Jonsson et al., 2010). Metabolic detoxification, through increased esterase activity (Jamroz et al., 2000; Pruett et al., 2002) or mutation in an esterase gene (Hernandez et al., 2000, 2002), is a complementary mechanism to *kdr* resistance. The three currently known mutations in the sodium channel gene of *R.* (*B.*) *microplus* have distinct geographical distributions and result in different resistance phenotypes. The Domain III mutation, widespread throughout Mexico but apparently limited to this country, confers very high resistance to flumethrin, cypermethrin and permethrin. For example, the Corrales and San Felipe Mexican strains were reported by Miller et al. (1999) as possessing RR > 1000 to permethrin and RR > 2300 to cypermethrin following laboratory selection with SP. Guerrero et al. (2001) showed the Corrales and San Felipe strains had 99% and 86% resistant allele frequency, respectively. The two Domain II mutations seem to provide lower levels of resistance than the Domain III mutation as assayed in our study. The L64I Domain II mutation identified by Morgan et al. (2009) in Australian populations has also been found in Brazil by other researchers (Domingues et al., 2012) and provides resistance to the same spectrum of acaricides as the Domain III mutation, but with low to moderate resistance ratios. For example, the Parkhurst strain was reported by Nolan et al. (1989) to have RR to SP between 100 and 450 and Brazilian populations possessing very high frequency of this mutation had RR to cypermethrin between 16 and 25 (Domingues et al., 2012). The G72 V Domain II mutation reported by Jonsson et al. (2010) confers low levels of resistance to flumethrin but offers no resistance to cypermethrin (Jonsson et al., 2010).

Allele specific PCR assays allow the identification of wild type or mutated alleles at the individual level. They offer several advantages in comparison to bioassays, requiring low numbers of larvae and allowing results to be obtained in less time than the larval *in vitro* tests, such as the LPT or LTT, which usually require 6 weeks for completion. In addition, PCR assays can detect resistance early in its emergence because resistant alleles can be detected at low frequency in populations that may still demonstrate a susceptible phenotype in bioassays (Rosario-Cruz et al., 2005). However, PCR assays require specific infrastructure, well-trained technicians and cold-chain sample maintenance to preserve nucleic acid quality. Furthermore, PCR assays can only identify the genotype of the mutation for which it was designed. Yet-to be discovered mutations or metabolic detoxification mechanisms cannot be detected. Hence, PCR assays should not substitute for bioassays but the two should be carried out in conjunction when possible. The use of the present multiplex PCR assay allowed the successful detection of the three currently known target site resistance-associated mutations in *R.* (*B.*) *microplus* in one assay, increasing significantly the efficiency of the detection of these mutations.

In this paper, we report the widespread distribution of the L64I Domain II mutation outside Australia. This mutation was found in all the phenotypically SP resistant bioassayed populations of Brazil, Argentina, South Africa and Australia, with resistant allele fre-

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Table 4
Resistance ratios at 50% and 90% mortality to flumethrin and cypermethrin or permethrin and kdr genotypes to the three mutations, in populations of *R.* (*B.*) *microplus* ticks from Brazil, Argentina, Mexico, South Africa and Australia.

Strain	Country	Flumethrin			Cypermethrin			% of larvae larvae with specific kdr genotype												
								L64I Domain II					Domain III				G72 V Domain II			
		RR50[a]	RR90[b]	Surv[c] (%)	RR50[a]	RR90[b]	Surv[c] (%)	SA[d]	RR[e]	SR[e]	SS[e]	R[f]	SA[d]	RR[e]	SR[e]	SS[e]	SA[d]	RR[e]	SR[e]	SS[e]
ST40	Brazil	57.9	29.5	99.9	104.8	71.3	97.1	7	86	14	0	93	8	0	0	100	9	0	0	100
ST41	Brazil	129.2	92.4	100	141	95.4	99	8	62	38	0	81	7	0	0	100	9	0	0	100
ST42	Brazil	88.7	64.8	99.8	201.1	407.5	92.6	8	100	0	0	100	9	0	0	100	9	0	0	100
ST44	Brazil	147.3	105.1	99.9	167.3	203.4	95.7	6	100	0	0	100	6	0	0	100	9	0	0	100
ST46	Brazil	92.2	78.1	99.2	109.9	78.7	85.5	9	89	11	0	94	0	No amplif.			9	0	0	100
ST47	Brazil	0.8	0.6	0.1	3.6	2.2	0.5	16	25	6	69	28	22	0	0	100	9	0	0	100
ST48	Brazil	63.3	55.5	100	203.9	120.4	95.4	20	100	0	0	100	29	0	0	100	9	0	0	100
ST49	Brazil	74.8	46.9	99.9	155.7	73.6	98.2	5	100	0	0	100	7	0	0	100	9	0	0	100
ST50	Brazil	40	37.5	85.4	71.3	85.3	68	5	40	60	0	70	6	0	0	100	7	0	0	100
ST51	Brazil	41.5	47.3	81	103.7	64.9	80.5	7	86	14	0	93	8	0	0	100	9	0	0	100
ST52	Brazil	116.1	91.1	100	221.5	339.4	95.7	8	50	12	38	56	16	0	0	100	15	0	0	100
ST55	Brazil	0.8	2.3	7.8	11	15.8	19.4	13	0	15	85	8	14	0	0	100	16	0	0	100
ST57	Brazil	83.5	65.6	100	309.3	735.5	96.2	10	80	10	10	85	17	0	0	100	17	0	0	100
ST58	Brazil	65.4	41.5	99.8	143.1	72.8	97.2	5	100	0	0	100	11	0	0	100	14	0	0	100
Santa Luiza	Brazil	na	na	na	na	na	90.7*	18	78	22	0	89	18	0	0	100	18	0	0	100
ST21	Argentina	<1.0	0.2	0	1.7	2.1	0	18	0	17	83	8	16	0	0	100	17	0	0	100
ST22	Argentina	1.2	36.5	34.5	6.8	21.7	20.5	17	24	29	47	38	11	0	0	100	18	0	0	100
ST24	Argentina	21.8	46.3	67.2	27.2	30.2	40.5	18	22	39	39	42	16	0	0	100	18	0	0	100
ST25	Argentina	2.5	31.3	31.9	6.6	16.4	16.7	15	47	47	7	70	11	0	0	100	17	0	0	100
ST26	Argentina	1.3	27.8	26.1	5.6	23.2	18.2	16	81	19	0	91	16	0	0	100	16	0	0	100
ST27	Argentina	0.9	32.2	33.4	4.2	19.4	16.1	16	19	75	6	56	18	0	0	100	13	0	0	100
ST29	Argentina	23	24	83.1	57	30.8	67.4	18	100	0	0	100	18	0	0	100	18	0	0	100
ST30	Argentina	na	na	na	21.5	116.2	41.2	17	88	12	0	94	17	0	0	100	17	0	0	100
San Felipe	Mexico	>24,300	na	na	>2300	na	>1000*	17	0	0	100	0	17	88	12	0	17	0	0	100
Coatzacoalcos	Mexico	15.7	na	na	57.2	na	166*	0	No amplification				17	0	0	100	17	0	0	100
Rio Bravo	Mexico	na	na	na	na	na	99.6**	17	0	0	100	0	17	100	0	0	17	0	0	100
ST11	South Africa	<4.0	<1.6	0	2.3	2.1	0.3	17	0	0	100	0	17	0	0	100	17	0	0	100
ST12	South Africa	<4.0	<1.6	0	1.7	1.4	0.1	18	0	0	100	0	18	0	0	100	18	0	0	100
ST15	South Africa	51.9	46.1	87.1	101.5	43.7	79.4	16	100	0	0	100	18	0	0	100	18	0	0	100
Ultimo	Australia	157.8	113.7	99.7	113.1	67.8	78.3	6	100	0	0	100	6	0	0	100	6	0	0	100
Urah	Australia	43.4	58.3	78	33.9	48.8	47.9	18	94	6	0	97	18	0	0	100	18	0	6	94

na: not available.
[a] Resistance ratios at 50% mortality.
[b] Resistance ratios at 90% mortality.
[c] Survival rates at DD.
[d] No. of larvae with successful amplification.
[e] RR = homozygous resistant genotype; RS = heterozygous genotype; SS = homozygous susceptible genotype.
[f] Percentage of resistant alleles: R = No. of resistant alleles divided by the total number of successful amplified alleles (= RR% + 0.5*RS%).
* Resistance ratios at 50% mortality of permethrin.
** Surviving rate at a DD of 0.2% AI of permethrin.

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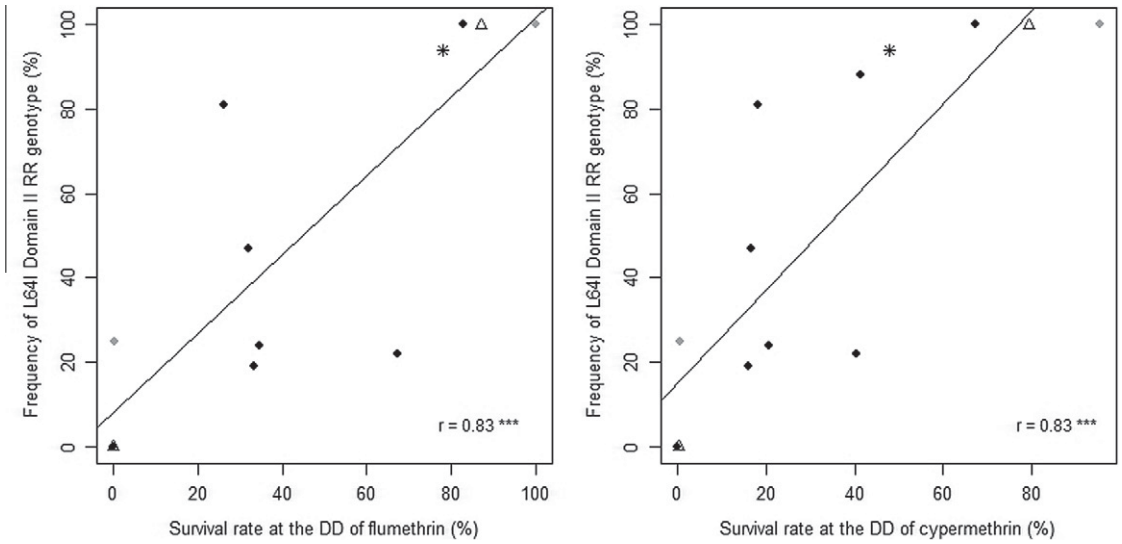

Fig. 3. Correlation between RR genotype frequency of the L64I Domain II mutation and survival rate at the DD of (a) flumethrin and (b) cypermethrin, measured with the LTT for the Brazilian (grey diamonds), Argentinean (black diamonds), South African (triangles) and Australian (stars) populations. The *r* value is the Pearson correlation coefficient; ***indicates that $p < 0.001$. Correlations were based on the populations which possessed PCR results for a minimum of 15 larvae.

quency between 38% and 100% in all of them, except in one population from Brazil (ST55, 8%) (Table 4). Interestingly, the L64I Domain II mutation was not found in any of the three Mexican strains whereas the Domain III mutation was present in two of these strains, with resistant allele frequency over 95% (Table 4). Thus, the Domain III mutation was limited to Mexico, further supporting the absence of this mutation outside North America as previously reported (Chen et al., 2009; Andreotti et al., 2011; Domingues et al., 2012). In addition this study provides evidence that the L64I Domain II mutation, whose major importance has been demonstrated in Australia (Morgan et al., 2009; Jonsson et al., 2010) and very recently in Brazil (Domingues et al., 2012), is probably also a major mechanism conferring resistance to SP in Argentina, and is also present in South Africa. Our results suggest that this mutation may be the mechanism responsible for SP resistance in some of the phenotypically resistant Brazilian populations studied by Andreotti et al. (2011) that failed to show the Domain III mutation in this group's PCR assays. The low incidence of the G72 V Domain II mutation, being limited to a single copy in an Australian population, is consistent with the phenotypes we observed and the one reported to be associated to the G72 V Domain II mutation. Indeed, Jonsson et al. (2010) showed that this mutation provides resistance to flumethrin but not to cypermethrin while none of the populations included in the present study demonstrated a similar phenotype. The geographical boundary between the Domain III mutation found in Mexico and the Domain II mutation found in South America is not known yet. Further sampling in Central America and north and south of the Amazon would provide very interesting information on the region where the distribution areas of these two mutations meet. Most of the tick populations included in this study were collected in farms in which some lack of treatment efficacy had been reported by the owners. Therefore, our data do not allow extrapolating SP resistance prevalence. Furthermore, it would be interesting to test additional field tick populations from Mexico to confirm the absence of the L64I Domain II mutation since the three Mexican samples used in the present study are all laboratory strains which have been occasionally exposed to acaricidal pressure during their maintenance.

When excluding the Mexican populations, the frequency of the RR genotype of the L64I Domain II mutation was found to be linearly correlated with survival rates at DD of both flumethrin and cypermethrin (Fig. 3; $r = 0.83$ ($r^2 = 0.69$), $p < 0.001$, for both compounds). The correlation coefficients between the resistant allele frequency and the *in vitro* results were very close to those values. RR genotype and resistant allele frequencies were also found to be associated with RR50 and RR90, but with a nonlinear correlation whose coefficients were lower than those for DD (data not shown).

As we see from the correlation results (r^2 values), 69% of the variation in the survival at the flumethrin and cypermethrin DD can be explained by the variation in the frequency of the L64I Domain II mutation RR genotype. Hence there is still an important part of the variation in the phenotypic results which is not explained by the L64I Domain II mutation genotype. Thus, for example, a 50% frequency of the L64I Domain II mutation RR genotype was sufficient to lead to 100% survival at the flumethrin DD in ST52, whereas for ST29 a RR genotype frequency of 100% led to only 67.4% survival at the cypermethrin DD (Table 4). Variability was also observed when comparing the L64I Domain II mutation genotype frequency with RR50 or RR90. Hence, RR genotype frequency of the L64I Domain II mutation of 100% resulted in RR50 ranging from 23 for flumethrin up to >200 for cypermethrin. These differences may be due to the presence of additional mechanisms of resistance such as metabolic detoxification or a yet undiscovered mutation, which may increase the resistance conferred by the L64I Domain II mutation in some of the tick populations. Furthermore, increased metabolic activity is probably the major mechanism of SP resistance in the ST55 population. Indeed, this Brazilian field population was shown to be cypermethrin resistant and flumethrin susceptible while it possessed a resistant allele frequency of the L64I Domain II mutation of only 8%. Inversely, another Brazilian population (ST47) possessed a resistant allele frequency of 28% while it demonstrated a susceptible phenotype to both flumethrin and cypermethrin. Lack of efficacy of cypermethrin treatment was reported by the farmer who provided this strain, supporting the molecular diagnosis. Interestingly, the four Argentinean populations (ST22, ST25, ST26, ST27) which appeared susceptible to flumethrin based on RR50 but resistant based on RR90, indicating the emergence of SP resistance in these populations, possessed resistant allele frequency between 38% and 91%

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(Table 4), confirming their resistance status and the importance of considering RR90 in addition to RR50 to allow the detection of resistance in development with bioassays.

The Brazilian Santa Luiza strain has previously been widely studied. In 2007, Li et al. reported a 100% susceptible genotype of the Domain III mutation in the Santa Luiza strain while its RR50 to permethrin was 90.7. Increased metabolic detoxification was first thought to be the cause of the resistance but later studies made detoxification less likely to be a dominant cause of SP resistance (Li et al., 2008). In fact, these authors noted the possibility that another mutation besides the Domain III mutation might exist in Santa Luiza (Li et al., 2008). Our report of the L64I Domain II mutation in the Santa Luiza strain therefore confirms their hypothesis. In addition, Li et al. (2008) presumed that the permethrin resistance in the Santa Luiza strain was inherited as an incomplete recessive trait, which is compatible with the hypothesis that the L64I Domain II mutation is a recessive trait (Morgan et al., 2009).

The three Mexican strains included in the present study have also been widely studied and our results corroborate the previous findings. The San Felipe strain is one of the two populations in which the Domain III mutation was originally discovered (He et al., 1999). The frequency of the resistant allele was shown to be between 80% and 86% (Guerrero et al., 2001; Li et al., 2007) while a lack of significant metabolic resistance had been shown (Miller et al., 1999). The resistant allelic frequency observed in the present study (94%, Table 4) is in line with the previous results. The Rio Bravo strain was selected in 1998–1999 for permethrin resistance for two generations (personal communication, Robert Miller) and the resistant allelic frequency we found (100%, Table 4) is therefore consistent. In contrast, the SP resistance in the Mexican Coatzacoalcos strain is known to be driven by increased metabolic activity. Indeed, metabolic resistance was first shown using synergists (Miller et al., 1999) and later confirmed with measures of esterase activity (Jamroz et al., 2000) while only very few individuals carrying the Domain III mutated allele (resistant allele frequency of 4%) were identified in this strain (Guerrero et al., 2001). In the present study, we did not find any of the three investigated mutations in Coatzacoalcos, supporting the view that metabolic resistance is likely a major mechanism of resistance in the Coatzacoalcos strain. The presence of an additional mutation not yet identified cannot be excluded.

The mutation found in the Mexican strains is located in the Domain III of the sodium channel gene of *R.* (*B.*) *microplus* and seems to provide higher resistance to SP than the two Domain II mutations. Single nucleotide substitutions in the sodium channel gene are either found as single mutations in resistant populations or in combination with another mutation, which can provide an additive or synergistic increase of resistance (Soderlund and Knipple, 2003; O'Reilly et al., 2006). Hence, very high resistance levels to SP are generally observed when there is the simultaneous presence of two mutations (Williamson et al., 1996; Guerrero et al., 1997; Liu et al., 2000). Mutations occurring in the Domain III are less common than those in the Domain II (O'Reilly et al., 2006), but have also been reported in insects (Pittendrigh et al., 1997). Pittendrigh et al. (1997) identified three mutations in the Domain III of *Drosophila melanogaster*, among which two occur at positions that are similar to the positions of mutations identified by Williamson et al. (1996) in Domain II in super-kdr house flies. Strains carrying both mutations possessed higher resistance levels to deltamethrin than the additive effects of the two single mutations. However, in the case of *R.* (*B.*) *microplus*, the Phenylalanine (Phe) to Isoleucine (Ile) Domain III S6 mutation provides very high levels of resistance (>1000) while it was not found to be combined with another mutation. Tan et al. (2005) demonstrated that the Domain III S6 mutation identified in *R.* (*B.*) *microplus* provides knockdown resistance in cockroaches, abolishing the sensitivity of the cockroach sodium channel expressed in *Xenopus laevis* oocytes to type I and type II pyrethroids, by reducing the pyrethroid binding to the sodium channel. The authors also observed that an aromatic residue at the position of the Phe to Ile Domain III S6 mutation is essential for the action of pyrethroids. O'Reilly et al. (2006) proposed a model of the housefly sodium channel where the pyrethroid binding site is located in a hydrophobic cavity delimited by the domain II S4-S5 linker and the Domain II S5 and III S6 helices. Their model suggests that the Phe to Ile Domain III S6 mutation could disrupt the interactions between the Domain III S6 helix and the Domain II S4-S5 linker and alter their relative positions. The crucial role of the aromatic amino acid at the pyrethroid binding site of the sodium channel may explain why this mutation confers such high levels of resistance to *R.* (*B.*) *microplus* and why the Leu to Ile and Gly to Val mutations of the Domain II S4-S5 linker confer such lower levels of resistance.

To conclude, this study provides evidence of the geographic separation of the three previously identified mutations. We provide data on the extent of the L64I Domain II mutation outside Australia, and show that the Domain III mutation is present only in North America. The L64I mutation is located in the Domain II of the sodium channel, the region that usually contains the *kdr* and *superkdr* mutations in insects (Soderlund and Knipple, 2003). Perhaps this Domain is somehow more amenable to mutation compared to Domain III which may explain the widespread occurrence of the Domain II mutation throughout arthropods. In addition, the widespread distribution of the L64I Domain II mutation and its association to SP resistance is consistent with the hypothesis that only a few mutations in the sodium channel gene conferring SP-resistance may exist in *R.* (*B.*) *microplus* (Guerrero et al., 2001). Increased detoxification, such as hydrolytic esterase activity, is likely to complement the target site insensitivity observed in the present study. However, other metabolic resistance mechanisms in *R.* (*B.*) *microplus* such as detoxification through cytochrome P450 and glutathione-S transferase cannot be ruled out.

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4.6. Chapitre 6 : Acaricide Resistance Mechanisms in *Rhipicephalus (Boophilus) microplus*

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Acaricide resistance mechanisms in *Rhipicephalus* (*Boophilus*) *microplus*

Mecanismos de resistência aos acaricidas em *Rhipicephalus (Boophilus) microplus*

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Abstract

Acaricide resistance has become widespread in countries where cattle ticks, *Rhipicephalus* (*Boophilus*) *microplus*, are a problem. Resistance arises through genetic changes in a cattle tick population that causes modifications to the target site, increased metabolism or sequestration of the acaricide, or reduced ability of the acaricide to penetrate through the outer protective layers of the tick's body. We review the molecular and biochemical mechanisms of acaricide resistance that have been shown to be functional in *R. (B.) microplus*. From a mechanistic point of view, resistance to pyrethroids has been characterized to a greater degree than any other acaricide class. Although a great deal of research has gone into discovery of the mechanisms that cause organophosphate resistance, very little is defined at the molecular level and organophosphate resistance seems to be maintained through a complex and multifactorial process. The resistance mechanisms for other acaricides are less well understood. The target sites of fipronil and the macrocyclic lactones are known and resistance mechanism studies are in the early stages. The target site of amitraz has not been definitively identified and this is hampering mechanistic studies on this acaricide.

Keywords: Cattle tick, resistance mechanisms, target site mutation, metabolism.

Resumo

A resistência aos acaricidas tornou-se amplamente difundida nos países onde os carrapatos bovinos, *Rhipicephalus* (*Boophilus*) *microplus,* são um problema. A resistência surge por meio de alterações genéticas em uma população de carrapatos que causam modificações no local de ação, aumento do metabolismo ou sequestro do acaricida, ou ainda redução na capacidade do acaricida em penetrar através das camadas protetoras do corpo do carrapato. Neste artigo, foram revisados os mecanismos moleculares e bioquímicos da resistência aos acaricidas que ocorrem em *R. (B.) microplus*. A partir de um ponto de vista dos mecanismos envolvidos, a resistência aos piretróides tem sido caracterizada em maior grau do que em qualquer outra classe de acaricida. Embora uma grande quantidade de pesquisas têm sido direcionada para a descoberta de mecanismos que causam resistência aos organofosforados, muito pouco é conhecido ao nível molecular, e essa resistência parece ser mantida por intermédio de um processo multifatorial e complexo. Os mecanismos de resistência para os demais acaricidas são bem menos compreendidos. Os alvos de ação do fipronil e das lactonas macrocíclicas são conhecidos, e os estudos dos mecanismos de ação envolvidos estão ainda em estágios iniciais. O alvo de ação do amitraz ainda não foi definitivamente identificado, e isso é limitante aos estudos dos mecanismos envolvidos na resistência a esse acaricida.

Palavras-chave: Carrapato bovino, mecanismos de resistência, mutação no local de ação, metabolismo.

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Introduction

McKosker (1979) estimated the global costs of ticks and tick-borne diseases to agriculture was over $ 7 billion. Although this was a crude estimate using broad assumptions, there is no doubt that ticks have a great impact on agricultural productivity and tick control is a necessary part of cattle production. The identification of chemicals with acaricidal properties quickly led to the adoption of chemical acaricides as the predominant method of tick control throughout the world. The cattle tick, *Rhipicephalus (Boophilus) microplus*, presents a challenge to cattle producers, as its life cycle and broad distribution through the tropical and subtropical regions of the world present conditions optimal for the rapid development of acaricide resistance.

In general terms, resistance can arise through several mechanisms in individual cattle ticks. Generally these mechanisms are broadly classified as target site, metabolic, or reduced penetration. Penetration resistance in ticks could arise through alterations in the ability of an acaricide to penetrate or otherwise enter an individual that is treated with acaricide. Although this resistance mechanism has been identified in a few arthropods (NOPPUN et al., 1989), including *R. (B.) microplus* (SCHNITZERLING et al., 1983), investigations into this mechanism in the cattle tick have not been reported recently. Target site resistance exists when an allele of the gene coding for the target molecule attacked by the acaricide has an amino acid mutation that confers resistance to the acaricide. This resistance mechanism is common, particularly well-studied in the case of pyrethroid class of acaricides, and will be discussed extensively below. Metabolic resistance to acaricides occurs through changes in the ability of an individual to detoxify or sequester an acaricide. The enzyme families known as cytochrome P450s, esterases, and glutathione S-transferases are generally involved in metabolic resistance and this type of resistance has been studied in *R. (B.) microplus* and will be discussed below. Often chemicals known as synergists are utilized to help discern resistance mechanisms in cattle ticks through bioassays. Synergist studies are especially helpful to detect metabolic resistance and common synergists are piperonyl butoxide (PBO), triphenyl phosphate (TPP) and diethylmaleate (DEM), which are generally believed to be specific for the cytochrome P450s, carboxylesterases, and glutathione S-transferases, respectively. However, the effects of synergists are not gene family-specific, as demonstrated by Young et al. (2005) who reported PBO effected pyrethroid resistance-associated esterases, and these studies must be interpreted with care.

Pyrethroid Resistance

The voltage-gated sodium channel is the target site for pyrethroid activity and target site resistance to pyrethroids has been studied in many arthropod species. An early report of target site-based resistance in *R. (B.) microplus* was Miller et al. (1999) working with pyrethroid resistant tick populations from Mexico. Their work was verified by He et al. (1999) who used gene sequencing to discover a specific amino acid substitution in Domain III (phenylalanine to isoleucine) of the *R. (B.) microplus* sodium channel in those Mexican tick populations. A PCR diagnostic assay (GUERRERO et al., 2001) was developed that allowed the rapid detection of this amino acid substitution in individual ticks, larvae or eggs. Large numbers of ticks were assayed by this method and this target site mechanism was found to be widespread throughout Mexico (ROSARIO-CRUZ et al., 2005, 2009) and in an outbreak strain in the United States (MILLER et al., 2007). The location of this sodium channel mutation is a bit out of the ordinary, as more arthropod sodium channel pyrethroid resistance-causing mutations are located in Domain II than the other three domains (SODERLUND; KNIPPLE, 2003). Interestingly, a survey of three pyrethroid resistant *R. (B.) microplus* populations from Mato Grosso do Sul, Brazil did not find this sodium channel mutation (ANDREOTTI et al., 2011). Our own survey, although limited in scope, also has not found the Domain III sodium channel mutation in either Brazilian or Australian tick populations (Table 1). However, recently Morgan et al. (2009) and Jonnson et al. (2010) have reported nucleotide differences in the Domain II region of the *R. (B.) microplus* sodium channel from pyrethroid resistant populations in Australia. These nucleotide differences in the Australian cattle tick sodium channel gene led to amino acid changes that correlated with pyrethroid resistance. In the case of the Morgan et al. (2009) report, the amino acid change is from leucine to isoleucine, while the change in the Jonsson et al. (2010) report is a glycine to valine.

The phenotypic effect of each of the three mutations differs significantly. The Domain III phenylalanine to isoleucine change confers a very high level of resistance to permethrin, cypermethrin, and flumethrin in the homozygous state as seen in the highly acaricide-selected Mexican Corrales strain (>1000-fold, MILLER et al., 1999). The Domain II mutations are reported to convey lesser levels of resistance. The leucine to isoleucine change reported by Morgan et al. (2009) conveys moderate levels (100-400-fold estimates) of resistance to permethrin, cypermethrin, and flumethrin, while the glycine to valine change was flumethrin resistance-specific and seemed to provide lower levels of resistance (JONSSON et al., 2010). Thus there are at least three target site mechanisms for pyrethroid resistance in *R. (B.) microplus*. The Domain III mutation seems to be localized to North America, the Morgan et al. (2009) mutation was discovered in Australia but has been seen outside of Australia (Table 1) while the Jonsson et al. (2010) mutation is only reported in Australia. The discovery of the Morgan et al. (2009) mutation allowed a successful conclusion to mechanistic studies of the multiply-resistant Brazilian Santa Luiza strain of *R. (B.) microplus*. Li et al. (2008) had reported genetic and synergist studies on the 93-fold permethrin resistance of Santa Luiza. Metabolic resistance was ruled out by the lack of significant synergism of pyrethroid resistance by PBO, DEM, or TPP. Molecular studies did not find the Domain III sodium channel mutation of Guerrero et al. (2001), thus suggesting a novel sodium channel mutation was present in the Santa Luiza strain. We have now confirmed that Santa Luiza contains the Morgan et al. (2009) Domain II mutation and this likely leads to the permethrin resistance phenotype of this Brazilian strain (F. GUERRERO, unpublished data).

The molecular aspects of metabolic resistance are not yet well-defined in *R. (B.) microplus*. While metabolic resistance has been generally attributed to the cytochrome P450s, esterases, and

Table 1. Pyrethroid resistance sodium channel mutation diagnostic assay results[1].

Country	Populations number	Larvae number	Percentage of mutant larvae[2]		
			Morgan et al. (2009)	Jonsson et al. (2010)	Guerrero et al. (2001)
Brazil	2	27	96	0	0
Argentina	8	133	76	0	0
Mexico	2	36	0	0	100
South Africa	4	69	26	0	0
Australia	1	17	100	0	0

[1]Data from PhD research of Léonore Lovis. [2]Percentage calculation includes larvae that were either heterozygous or homozygous for the indicated mutation.

glutathione S-transferases, each of these are large gene families in the cattle tick with 115, 81, and 39 individual members, respectively (BELLGARD et al., 2012). As knowledge of genetics and genomics of arthropods and the cattle tick advances, it will become possible to specify a gene-mediated metabolic resistance mechanism as has been done for glutathione S-transferase-based DDT resistance in *Anopheles gambiae* (RANSON et al., 2001) and cytochrome P450-based pyrethroid resistance in *Musca domestica* (TOMITA et al., 1995). Biochemical synergist studies are often used to determine if metabolic resistance mechanisms are present in resistant populations of *R. (B.) microplus* and these are useful. As noted previously, biochemical synergists can only serve as a guide for determination of the mechanism of metabolic resistance. Nevertheless, several *R. (B.) microplus* populations have been examined by synergist studies and metabolic resistance identified. Generally, where target site- and metabolism-based pyrethroid resistance coexists, the target site resistance plays the major role in product failure. However, in some cases metabolic resistance is a major mechanism. For example, studies with PBO and TPP showed the Mexican Coatzacoalcos population of *R. (B.) microplus* had a significant metabolic resistance component within the population's overall 166-, 57-, and 16-fold resistance to permethrin, cypermethrin, and flumethrin, respectively (MILLER et al., 1999). Protein and molecular studies showed only a low percentage of individuals from Coatzacoalcos (8%) had a single copy of the Domain III sodium channel target site mutation (GUERRERO et al., 2001). However, Coatzacoalcos overproduced a specific esterase, designated CzEst9, that hydrolyzed permethrin (JAMROZ et al., 2000; PRUETT et al., 2002). Baffi et al. (2007, 2008) later reported this esterase played a major role in pyrethroid resistant *R. (B.) microplus* ticks from Mato Grosso, Brazil. Synergist studies with PBO have indicated that cytochrome P450s play a role in pyrethroid resistance mechanisms in *R. (B.) microplus* from Mexico (MILLER et al., 1999), but molecular studies have not been reported, and, with PBO's lack of complete specificity towards the cytochrome P450 family, a mechanism cannot be attributed to a specific P450. Finally, although a number of glutathione S-transferases have been identified in *R. (B.) microplus* (BELLGARD et al., 2012), a significant involvement with pyrethroid resistance mechanisms has yet to be reported.

Organophosphate Resistance

Organophosphates and carbamates target the acetylcholinesterase protein. Although bioassay and synergist studies have been used to provide evidence regarding resistance mechanisms in various *R. (B.) microplus* populations, specific mechanisms have not been identified. There is uncertainty about the identity of the transcript encoding the acetylcholinesterase that is functionally relevant for acaricide resistance in *R. (B.) microplus*. In fact, more than one acetylcholinesterase might be involved in acaricide responses (BAFFI et al., 2008; TEMEYER et al., 2010). Seven contigs with significant sequence similarity to acetylcholinesterase were reported in the most recent *R. (B.) microplus* transcriptome (BELLGARD et al., 2012). Temeyer et al. (2010) expressed three acetylcholinesterase-like transcripts isolated from two organophosphate resistant and one organophosphate susceptible strain of *R. (B.) microplus* and showed that variant alleles existed among individuals in the strain that showed differential response to organophosphate. These authors concluded that "phenotypic resistance to OPs may be complex and multigenic in character". Unfortunately, no specific mutations in acetylcholinesterase have been correlated to organophosphate resistance in field populations, despite considerable effort to find mutations (F. GUERRERO, unpublished data). The concept of multiple forms of acetylcholinesterase in *R. (B.) microplus* was noted as far back as 1972 (NOLAN et al., 1972) where five forms of this enzyme were purified by electrophoretic and verified by biochemical methods. The uncertainty about the specific acetylcholinesterase targeted by organophosphates or mutations that affect the acaricide-target site interaction has prevented the identification of specific target site-mediated resistance mechanisms in *R. (B.) microplus*. Earlier, biochemical kinetic and inhibition studies of acetylcholinesterase in *R. (B.) microplus* had attributed organophosphate resistance in the Mexican Tuxpan, Tuxtla, San Roman, and Caporal strains (WRIGHT; AHRENS, 1988; PRUETT, 2002) and the Argentinian G Goya strain (REICH et al., 1978) to target site insensitivity. More recently, the attribution of resistance to a target site mechanism has sometimes been through the absence of significant effects on toxicity in the presence of synergists such as PBO, TPP, or DEM (LI et al., 2003). In this case, the absence of synergism indicated a lack of metabolic resistance, leading to an inference that target site resistance must be present. In an interesting study, Baffi et al. (2008) reported that a malathion resistant Brazilian strain of *R. (B.) microplus* seemed to have increased amounts of acetylcholinesterase compared to malathion susceptible strains. Thus a target site gene amplification or mutations within the gene promoter region might be the specific resistance mechanism.

Metabolic mechanisms play a role in *R. (B.) microplus* organophosphate resistance, though generally reported in the

presence of target site resistance. Bull and Ahrens (1988) reported coumaphos metabolism, likely through an esterase-based hydrolysis, was a component of the Tuxpan and Tuxtla Mexican *R. (B.) microplus* populations which were also shown to possess a target site-mediated resistance to organophosphates (WRIGHT; AHRENS, 1988). Jamroz et al. (2000) used biochemical analyses to quantify the esterase-based metabolic mechanisms of organophosphate resistance in the Mexican Tuxpan, Coatzacoalcos, and Corrales strains of *R. (B.) microplus*. A carboxylesterase, termed Est10, was found to be more abundant in the coumaphos resistant Tuxpan strain, so perhaps this esterase plays a significant role in this strain's metabolic resistance. Villarino et al. (2003) detected esterase-based metabolic resistance to organophosphates in the integument of adult female *R. (B.) microplus*. Li et al. (2003) reported evidence that a cytochrome P450-mediated coumaphos resistance was a significant mechanism in four coumaphos resistant Mexican populations of *R. (B.) microplus*. Interestingly, their PBO synergist studies did not show a P450-mediated resistance mechanism for diazinon in the diazinon resistant Tuxpan and San Roman strains. The coumaphos resistant Mexican San Roman strain showed increased expression of a cytochrome P450-like transcript following treatment with low doses of coumaphos, perhaps playing a role in the metabolic resistance mechanism of that strain (GUERRERO et al., 2007). A coumaphos susceptible strain also showed induction of the cytochrome P450-like transcript upon treatment with coumaphos, but the response of the resistant strain was more robust indicating a greater capacity to respond to the acaricide treatment. As is the case for target site organophosphate resistance, the metabolic mechanisms are not simple to define at the molecular level, although the advent of the *R. (B.) microplus* transcriptome database (BELLGARD et al., 2012) brings this closer to feasibility. The molecular analysis by Saldivar et al. (2008) reported the first evidence for the involvement of a specific glutathione S-transferase in *R. (B.) microplus* acaricide resistance (although see below for resistance in Pesqueria strain). The Mexican multiply-resistant San Alfonso strain showed 2-, 91-, and 600-fold resistance to coumaphos, permethrin, and amitraz, respectively. When treated with a low level dose of coumaphos (lethal to approximately 8% of the individuals), the expression of a specific transcript, TC9004, was increased over 5-fold. When translated, the protein encoded by transcript TC9004, which is from the BmiGI Version 2 gene index of *R. (B.) microplus* (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=b_microplus), showed high amino acid sequence similarity to glutathione S-transferase.

Resistance Mechanisms to Other Acaricides

The target site of amitraz has not been definitively identified, although candidates such as monoamine oxidase, octopamine receptor, and alpha-2-adrenceptors have been proposed (JONSSON; HOPE, 2007). The lack of information hampers the development of assays for and identification of target site-based resistance and no resistance-associated mutations in any of the proposed targets of amitraz activity have been reported. Synergist studies with PBO, TPP, and DEM, showed metabolic resistance played a role in amitraz resistance of Mexican strains of *R. (B.) microplus* (LI et al., 2004). Fragozo-Sanchez et al. (2011) suggested that resistance to amitraz is controlled by a recessive inheritance, also it seems that more than one gene are involved in this process. Target site resistance was proposed as the major resistance mechanism in the Brazilian Santa Luiza *R. (B.) microplus* strain by these researchers, although no direct evidence for target site resistance was presented. The Mexican Pesqueria strain was found to be resistant to both diazinon and amitraz, and DEM synergized the toxicities of both chemicals, indicating possible involvement of glutathione S-transferases in metabolic resistance to both amitraz and diazinon in this strain.

Fipronil acts on both the 4-aminobutyric acid (GABA)-gated chloride channel and the glutamate-gated chloride channel (ZHAO et al., 2004). This activity on dual targets probably plays a role in delaying or preventing the buildup of high levels of resistance. However, one of these targets is shared with the cyclodiene class of pesticides and low levels of fipronil resistance can be associated with resistance to dieldrin in *Drosophila melanogaster* (BLOOMQUIST, 1994). Fipronil resistance has been documented in field populations of *R. (B.) microplus* in Uruguay (CUORE et al., 2007; CASTRO-JANER et al., 2010a, 2011) and Brazil (CASTRO-JANER et al., 2010b), although mechanistic studies were not reported.

Macrocyclic lactones are increasingly being used for cattle tick control and the target site for this class of molecules are also believed to be the GABA- and glutamate-gated chloride channels. Resistance has been reported in Brazil (MARTINS; FURLONG, 2001; KLAFKE et al., 2006, 2012) and Mexico (PEREZ-COGOLLO et al., 2010), but studies of resistance mechanisms are just beginning. Because fipronil and the macrocyclic lactones both are believed to act on the glutamate-gated chloride channel and the 4-aminobutyric acid (GABA)-gated chloride channel, the possibility of cross resistance must be considered. Castro-Janer et al. (2011) observed that *R. (B.) microplus* populations that were treated with ivermectin but never treated with fipronil were susceptible to fipronil and tick populations that were resistant to fipronil were susceptible to ivermectin. This indicates the molecular site of action of fipronil and ivermectin are not identical and cross resistance was not detected in Uruguay.

As discussed above, the molecular target sites of a number of acaricidal compounds have yet to be elucidated and characterized. The use of molecular biology and genomics to understand acaricide resistance in *R. (B.) microplus* will play a major role in the comprehension of the molecular mechanisms of resistance. In addition, there is currently very limited molecular information on detoxification genes associated with metabolic resistance, although their importance has been highlighted by numerous biochemical studies in mites and ticks (VAN LEEUWEN, et al., 2010).

Conclusion

The resistance mechanisms for pyrethroids that act in the cattle tick are beginning to be understood at the molecular level. There is a basic understanding of metabolic mechanisms supporting organophosphate resistance in cattle ticks, however, target site resistance appears complex. Very little is known about

the resistance mechanisms that are active in amitraz, fipronil, and macrocyclic lactone resistant cattle ticks. Further studies are needed to address these deficiencies in the basic knowledge of cattle tick acaricide resistance. There is a general consensus that advances in tick genomics will accelerate the development of new molecular targets and new diagnostic tools for acaricide resistance detection, which in turn can improve strategies for tick control.

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5. GENERAL DISCUSSION

Rhipicephalus (B.) microplus is an ectoparasite which has a major economic impact on cattle industry by its direct and indirect negative effects. Cattle tick control mainly relies on acaricidal treatments and livestock production could not be imagined without the use of acaricides in countries where *R. (B.) microplus* is present (Ghosh et al. 2007). However, the heavy use of acaricides has led to the emergence and spread of drug and multi-drug resistance through its area of distribution. It is therefore very important for farmers facing lack of treatment efficacy to obtain information on the acaricidal resistance pattern of the tick populations established on their property in order to choose the most suitable replacement compound. To do so, ticks need to be sent to laboratories for *in vitro* testing of the resistance pattern.

Thus, the primary objective of this thesis was to develop a new bioassay to evaluate acaricidal resistance in *R. (B.) microplus* and to apply it to field populations from various countries. Finally, the phenotypic approach was completed by a genotypic approach of the resistance to synthetic pyrethroids.

The **first part** of this general discussion summarises the characteristics of bioassays, with a particular focus on the LTT, its advantages and utility. In the **second part** of this chapter, some selected cases of resistance observed in the field with the LTT are highlighted. Then, in the **third part**, the advantages and the limitations of the molecular tools as well as their complementarity to bioassays is developed with regard to the data obtained in this thesis. In the **fourth part**, the need for standardised criteria to interpret bioassay results is discussed, followed by some thoughts in the **fifth part** on the additional work which would be useful to improve the LTT and to have a more complete picture of the test. Finally, in the **last part**, the importance of a prudent use of acaricides is emphasised as well as the importance of integrated pest management to slow down the development of acaricide resistance.

5.1. Characteristics of the LTT and other bioassays

Bioassays are very useful tools to detect resistance in field populations and usually do not require expensive laboratory equipment. Each bioassay has its own advantages and disadvantages. Hence, while adult tests such as the AIT may be completed in two weeks, which rapidly provides farmers with results, allowing them to adapt rapidly their treatment strategy, larval tests (LPT, LIT, and LTT) require 6 weeks before the results are available. On the other hand, the AIT requires a high number of engorged females, limiting the number of tests which can be carried out, whereas larval tests and the LTT particularly,

require low numbers of engorged females to produce sufficient larvae for testing. In addition, when comparing larval tests, the time-consuming or inversely the time-effective nature of the tests is an important point which needs to be considered. Thus, although they all require the same time before the results are available, the time of active work to obtain the results differ drastically from one test to the other. Indeed, we estimated that with the LTT the distribution of the eggs into the plates and the evaluation of the tests requires approximately ten-fold less time than the loading of the larvae in the packets and the evaluation of the tests with the LPT (Chapter 1). The time required with the LIT is probably close to the time required with the LPT, although this was not investigated precisely in the present work. This last characteristic, as well as the number of engorged females required for testing, have a considerable impact on the data which can be obtained with the different bioassays. Hence, to reduce the need of ticks for the AIT a single dose is generally tested. This dose is usually the DD (FAO 2004) but if not available, the dose recommended for treatments on cattle can also be used (Chapter 2). The survival rate to this DD is considered as the percentage of the population resistant to the tested compound. However, the use of such DD has been criticised (Jonsson et al. 2007). The low requirement of ticks for the larval tests should allow testing several doses per compound, however, due to the time-consuming nature of the LPT, DD are also sometimes used to reduce the amount of work (FAO 2004). The LTT combines the two advantages of requiring very few engorged female ticks for testing and to be time-effective. These characteristics allow testing several doses per compound on a routine base aiming to obtain a full dose-response mortality curve from which LC_{50} or LC_{90} values are generated and compared to a susceptible strain to calculate resistance ratios providing more detailed results and probably more reliable results than the use of a single DD. In addition, it allows testing more compounds, increasing the knowledge about the resistance pattern of the tested populations. Testing more than one compound per class is valuable, because susceptibility to compounds of the same class may differ. Thus, for example, differential response to diazinon and coumaphos has been observed (Miller et al. 2008) while some strains demonstrate resistance to some SP compounds, but not to other ones (Jonsson et al. 2010a).

In addition, full dose-response mortality curves offer an additional advantage which is the information contained in its slope. Thus, as stated in FAO 2004, the slope is an indicator of the establishment of the resistance in a population. Hence, a homogeneously susceptible or a homogeneously resistant population should possess a steep dose-response curve, with a high slope, while a heterogeneous population is characterized by a flatter curve and therefore a smaller slope. A smaller slope leads to greater resistance ratios based on the LC_{90} (RR90) than those based on LC_{50} (RR50). The value of the slope, or the comparison of the RR50 and RR90 therefore allows determining if the resistance is emerging or in process of establishment in a field population. This is very important to consider, because

RR50 may be small, not allowing the suspicion of emerging resistance and if considered alone, may lead to counterproductive advice given to the farmers.

Finally, testing *in vitro* several compounds of the same classes can sometimes provide information on the resistance mechanisms and lead for example, to the suspicion of the presence of the domain II mutation reported by Jonsson et al. (2010) if resistance to flumethrin is observed but not to cypermethrin. *In vitro* data could also lead to the suspicion of enzymatic degradation through esterase hydrolysis if an isolate is resistant to permethrin but not to flumethrin and cypermethrin (Robert Miller, personal communications). In addition, if SP target-site resistance is suspected, resistance ratios can also provide an indication about the mutation which provides the resistance, leading to the suspicion of the Domain III Mexican mutation if resistance ratios are very high, or if they are lower, to the suspicion of the Domain II Morgan mutation.

In addition to these favourable characteristics, the LTT demonstrated an equal or even higher sensitivity in measuring resistance than the LPT. The comparison of the two tests using laboratory strains (Chapter 1) showed that resistance ratios obtained with both tests were in the same range, with the exception of coumaphos for which the resistance ratio was 20-fold higher when estimated using the LPT than with the LTT. Later, the comparison of the two tests carried out in Brazil with field populations demonstrated nearly systematically higher sensitivity of the LTT to detect resistance, although resistance ratios obtained with both tests were usually again in the same range (Chapter 4).

5.2. New information about the emergence or extend of resistance observed in the field

The present work further supports the previous reports of the widespread resistance to OP, SP and amitraz in Brazil and Australia. Although few data are available for Argentina and South Africa, the resistance to OP and SP we reported, as well as amitraz resistance in South Africa, are not new. The most interesting results are probably the observation of: (1) resistance to amitraz in Argentina, as this is the first report of amitraz resistance in this country (Chapters 2 and 3); (2) the large extend of the resistance to phenylpyrazol compounds in Brazil (Chapter 4), and (3) the high frequency of emerging resistance to flumethrin in Argentina (Chapter 3).

Amitraz resistance in Argentina is worrying because this compound currently plays a major role in the eradication program of ticks in this country. This tick control program has been based for 70 years on treatments in dipping vats at a 21-day interval (SENASA 1999) leading to the development of resistance to most of the existing compounds used in dipping vats. As a consequence, amitraz is currently the main

active ingredient used for treatment, hence the important impact of the development of amitraz resistance.

Fipronil resistance was reported for the first time in Brazil in 2010 in the states of São Paulo and Rio Grande do Sul (Castro-Janer et al. 2010a). The present work shows that fipronil resistance is also present in the states of Mato Grosso do Sul, Paraná and Espírito Santo, and that it was present in a unexpectedly and worrying large proportion of the farms included in this study. The use of fipronil was not reported in the farms where resistance to this compound was observed and furthermore, this compound seems to be only rarely used in Brazil (Mendes 2011). Since fipronil acts on the same receptors than ML, cross-resistance between the two classes may be suspected, however, resistance to ML or its suspicion was observed in only very few Brazilian strains refuting the hypothesis of cross-resistance between ML and phenylpyrazol compounds in the present situation. In contrast, this hypothesis remains valid for the South African strain (ST15) for which pyriprol resistance and suspicion of fipronil resistance was observed as well as emerging resistance to ivermectin. In addition to fipronil resistance, a high frequency of pyriprol resistance was also observed in Brazil, while this compound is not used for cattle tick treatments. Therefore, one can hypothesize potential cross-resistance between these two phenylpyrazol compounds.

As to Argentina, SP resistance was reported for the first time in 1996 (Caracostantógolo et al. 1996) and this study shows the on-going spread of resistance to this class of compounds, with half of the populations demonstrating emerging resistance to flumethrin, and all the other populations but one with established resistance to flumethrin and cypermethrin. The diagnosis of emerging SP resistance based on the bioassays was supported by the results of the molecular survey. Indeed, the observation of mixed genotypes of the Morgan et al. (2009) mutation, with simultaneous presence of RR, RS and SS genotypes confirms that these populations are heterogeneous and supports the hypothesis that the resistant allele is probably in process of establishment in these populations. As we can see with this example, phenotype and genotype data are complementary.

5.3. Complementarity of bioassays and molecular tools

Bioassays and molecular tests are complementary and molecular tools, when available, should be used in conjunction with bioassays, rather than substituting them.

In comparison to bioassays, molecular assays have the advantage to provide results within a day, to require very few ticks and to provide confirmatory information about resistance mechanisms. Furthermore, molecular tools have the advantage that they can be carried out using material (larvae or

DNA) which has been stored for years. In the present study for example, the multiplex was performed using DNA extracted in 1999 for the three Mexican strains. In addition, studies on the Brazilian Santa Luiza strain had demonstrated the absence of the domain III mutation (Li et al. 2007) and led to the hypothesis of the presence of another mutation (Li et al. 2008). Four years later, we could confirm this hypothesis demonstrating the presence of the domain II Morgan mutation in this strain, using stored frozen larvae. In contrast, molecular assays have the disadvantages to require specific laboratory equipment, cold-chain sample maintenance and well-trained staff.

Bioassays allow measuring resistance to compounds whose resistance mechanisms are unknown and irrespective to the underlying resistance mechanisms. Hence, resistance to OP, ML, fipronil and amitraz can be assessed with bioassays while no molecular tools are available for these compounds. In contrast, molecular tools allow detecting only what is known and what it has been designed for. *R.* (*B.*) *microplus* SP resistance is an example to illustrate this. Efforts have been made to identify the domain III mutation in ticks from Brazil and Australia (Chen et al. 2009, Andreotti et al. 2011), but no mutation were found. It was not until the recent identification of the domain II mutation by Morgan et al (2009), 10 years later after the identification of the domain III mutation, that the mutation providing target-site resistance in Brazil and Australia could be identified. Furthermore, resistance does not always results from a single mechanism (Miller et al. 1999). Indeed, target-site resistance and metabolic resistance can be combined and their effects add up. Therefore, investigation of one of them does not always provide a complete picture of the resistance. This is probably the case for some of the Brazilian and Argentinean field populations investigated in the present work. Thus, although the frequency of homozygous resistant genotypes of the Morgan et al. (2009) mutation explained about 70% of the variability in the phenotypic resistance to cypermethrin and flumethrin, it remains 30% of unexplained variation. Hence, in some populations, the presence of metabolic resistance can be suspected and it would be valuable to investigate detoxification activity of the CzEst9 esterase (Guerrero et al. 2002, Pruett et al. 2002) to complement our data.

5.4. Need for a standardisation of the interpretation of the bioassay data

Interpretation of bioassay data is not standardised. The criteria used to consider an isolate as resistant differ between studies. Some authors consider a strain to be resistant as soon as the resistance ratio is statistically significant based on their 95% confidence intervals (Perez-Cogollo et al. 2010, Mendes et al. 2011), others combine this criteria with the fact that the RR must be greater than 2 (Castro-Janer et al. 2011, Klafke et al. 2012), some authors use an interval (3-5) in which RR corresponds to tolerance (Bianchi et al. 2003), other authors use a threshold value above which RR are considered to indicate

resistance (Chevillon et al. 2007) while, some authors finally classify resistance in different levels, whose classes differ between compounds (Bianchi et al. 2003, Mendes et al. 2007). Interpretation of the same data may therefore differ drastically for populations with low resistance ratios, and standardization of the criteria to be used would be desired. However, standardization of such a criterion is a very difficult task, because the optimal criterion probably depends on the compound tested and on the bioassay used. Furthermore, defining such a criterion would require stall tests to establish correlations between *in vivo* lack of treatment efficacy and *in vitro* results.

In the present study, a threshold value of 4 was used for all the compounds. This value is pretty conservative and should avoid over-diagnosing resistance while it may miss resistant isolates, decreasing the sensitivity of the test. However, establishing the most discriminative value for a resistance ratio is very difficult and would require a lot of additional work. Ideally, the susceptibility of the field isolates tested *in vitro* in the present work should have also been tested *in vivo* for each compound in order to provide a basis for comparison with *in vitro* data. However, this is logistically and economically not feasible. The tick populations which would have provided the most valuable information to establish a threshold value are those with resistance ratios close to the potential threshold value. In addition, as mentioned previously, these values would probably vary between tests, because the exposition of the ticks to the acaricides differs between bioassays. For example, although resistance ratios obtained with the LTT and the LPT were in the same range (Chapter 1), those obtained with the LTT were shown to be generally higher than with the LPT (Chapter 4), which may impact on the threshold value to be use.

In the present study, despite the absence of *in vivo* comparative results for the field strains, the molecular results obtained for the SP provide valuable information to verify the suitability of our threshold value of 4. The populations with resistance ratios to cypermethrin lower than 10 are probably those which provide most information. If the RR50 was greater than 4 and the Morgan mutation well-established, or inversely, if the RR50 was smaller than 4 and the Morgan mutation low, then the molecular results support our phenotypic interpretation. In contrast, if the RR50 was was smaller than 4 and the Morgan mutation well-established, this would lead to suspect that our threshold value was too high. Thus, the four Argentinean strains (ST22, ST25-ST27) which demonstrated RR50 slightly higher than 4 (4.2-6.8) possessed Morgan resistant allele frequencies between 38 and 91%, supporting the diagnosis of cypermethrin resistance based on the LTT. The two South African strains (ST11 and ST12) which had RR50 of 2.3 and 1.7 did not possess any copy of any of the three known mutation, which also corroborates the phenotypic diagnosis of cypermethrin susceptibility. In contrast, the situation of the Brazilian ST47 strain is different. A RR50 to cypermethrin of 3.6 was obtained in this strain, which was therefore considered as susceptible. However, a Morgan resistant allele frequency of 28% was found in the population, leading to the hypothesis that using our threshold value of 4, we missed this resistance.

In addition, lack of efficacy of cypermethrin treatment was reported by the farmer who provided us with this strain, supporting the molecular diagnosis. Additional data of this type would be necessary to determine if our threshold value of 4 was too high and should have been decreased to another value, such as 3 potentially. However, decreasing the threshold value to 3 would not have had a major global impact on the diagnosis of resistance in the present work since it would have lead to a different interpretation of the RR50 or RR90 in less than 5% of the total tests (25/516). The question of the threshold value can also be raised for the molecular results: from which value of resistant allele frequency onwards should a population be considered as resistant?

Finally, another aspect to which we need to be very careful when assessing resistance is the quality of the data of the susceptible reference strain on which the comparison with the tick isolates is based. Thus, it is crucial to obtain reliable baseline data. For this, the data of the susceptible reference strain should be obtained in the same laboratory as the one where the field isolates are analysed, to ensure they are tested in the same conditions. Furthermore, the reference strain data should be based on repeated testing aiming to include natural variation between replicates and batch of ticks is taken into account.

5.5. Potential future improvements of the LTT

We have discussed above the strengths of the LTT, however, some further investigations and improvements would be desired. First, although the LTT was adapted to be performed using minimal equipment, there is still one step which requires specific lab-infrastructure. Indeed, the mode of evaporation of the pre-treated plates currently requires equipment which may represent a considerable financial investment for some laboratories and hamper its adoption. In the current protocol, the acaricidal compounds are dissolved in dimethyl sulfoxide (DMSO), which requires an N_2 sampler evaporator or a vacuum centrifuge to be evaporated. For the purpose of this study, the plates were prepared in Switzerland and then shipped to destination for testing, but this is a short term solution only. Therefore, it would be relevant choosing a different solvent which evaporates more easily while ensuring a satisfactory dissolution of all the chemicals to be tested without damage to the polystyrene plates. Secondly, another important piece of information to be obtained to facilitate the use of the LTT in routine is the time of storage of the plates under normal atmospheric conditions before degradation of the chemicals occurs. Indeed, it would be interesting for a laboratory to prepare a stock of plates which could be used to test field populations as they arrive to the laboratory for evaluation. Thus, to gain this knowledge, it would be necessary to test the plates at different time points after their preparation, keeping them at different potential room temperatures. Since a low relative humidity rate

is easy to obtain by the use of silica gel, it is recommended to always keep plates in a dry environment to limit the effect of humidity. As we can expect from other bioassays (LPT, AIT) and from our own observations with the LTT, amitraz will probably be the compound which degrades most rapidly over time. Finally, it would be worth investigating the potential effect of the talc and the separation of the eggs on their hatchability. Both factors may increase egg desiccation or may mechanically affect the eggs. We have observed a very high variability in the hatching rates of the eggs in the plates, ranging from 60% to 90%. To clarify if this variability results from the egg separation procedure or is inherent to batch of eggs, eggs should be mixed carefully and thoroughly prior to separation, keeping a sample of un-separated eggs in the same conditions as the plates, and whose hatching rate could be estimated visually and compared to the ones observed in the plates. Maintenance of the plates in an environment with a very high and stable humidity after the egg distribution seemed to limit egg desiccation. However, despite this, a very high variation in hatching rates was observed between and within strains. Interestingly, hatching rates varied a lot between strains, even for strains which were tested at the same time and whose plates were kept together in the same conditions. In addition, while hatching rates were always very close among the three replicates of each test, which were always performed together but on separate plates, high variations of hatchability were sometimes observed if the same strain was tested repeatedly and independently using a different batch of ticks. Hence, while we could observe that a careful separation of the eggs combined with stable humidity conditions for the maintenance of the plates allowed obtaining very high hatching rates, we observed that for some isolates, the same conditions led to much lower hatching rates. Hence, even though we cannot exclude a potential effect of talc and of the individualisation of the eggs, it should occur systematically, and not only in some cases. Therefore, it can be hypothesiszed that the hatching rates observed in the plates reflect the hatchability of the non-individualised eggs. Indeed, this can vary naturally due to the fitness of the engorged females, to the conditions in which the tick samples were sent to the laboratory or to the potential contact of the females with acaricidal compounds prior to tick collection. However, if a negative effect of talc egg separation procedure was observed, it would be interesting to investigate if this effect can be reduced by distributing eggs just before they hatch, reducing their exposure to factors and risks which may affect hatching.

Finally, the evaluation of larval mortality is a critical step. Since we have to evaluated the survival or mortality of 50 larvae in a very small space (1 well of a 96-well plate), this step can be difficult and must be done with care. In the present work, we used and recommend to base larval mortality assessment on the observation of the motility and general appearance. Within a laboratory, plates should be evaluated by the same person to ensure that comparison with the reference strain is consistent.

5.6. Conclusion

To conclude, with the LTT a new sensitive bioassay has become available to assess acaricidal resistance in *R. (B.) microplus*. The LTT presents several advantages. First, as a larval test, it requires very low number of engorged females. The main advantage of he LTT is clearly its time-effective nature, which is due to the handling of eggs instead of larvae (for the LPT) and its realisation in 96-well plates, which allow testing a high number of compounds and doses in a short amount of time. Hence, with the same number of engorged females obtained from a farm, more information on the pattern and level of resistance can be obtained than with the LPT. When presented at the World Association for the Advancement of Veterinary Parasitology (WAAVP) in Buenos Aires in 2011, this new bioassay has received much attention from people from Latin America who expressed their interest in learning about it. In the meantime, the method has been established in the Instituto Biológico in São Paulo, Brazil, while other labs in the USA (the Cattle Fever Tick Research Laboratory (CFTRL) in Texas) and in Argentina (the Instituto Nacional de Tecnología Agropecuaria in Buenos Aires (INTA Castelar)) are interested in implementing it. The future will show, if this new test will be accepted as an adequate tool to diagnose acaricide resistance in cattle ticks.

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8. APPENDICES

8.1. Appendice 1: Distribution of *R. (B.) microplus* according to Pal and Wharton (1974)

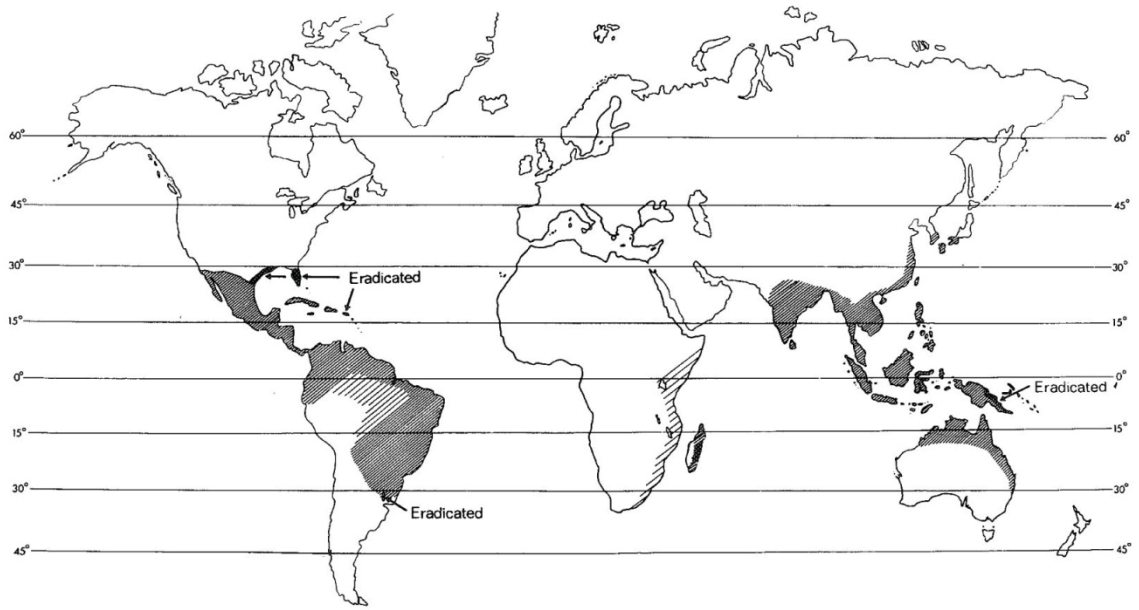
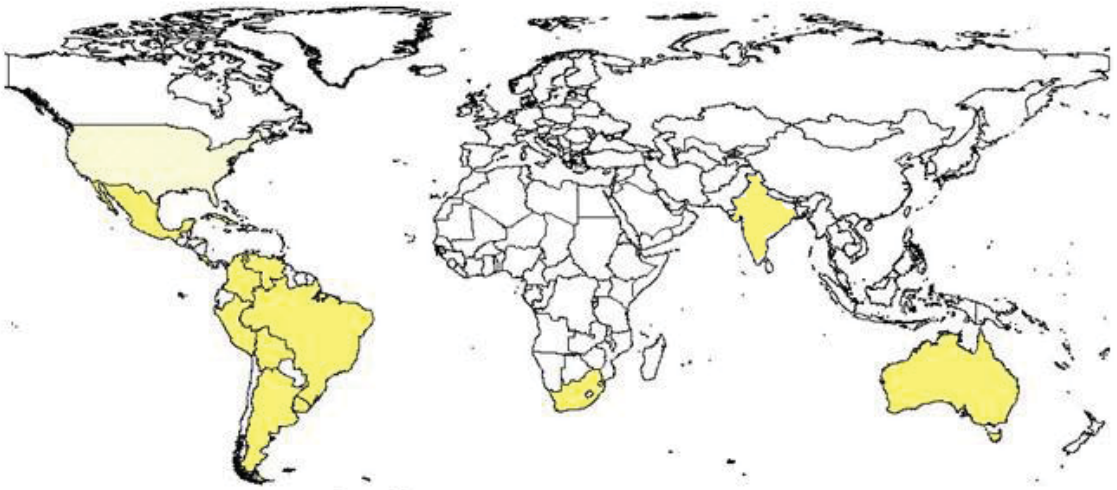


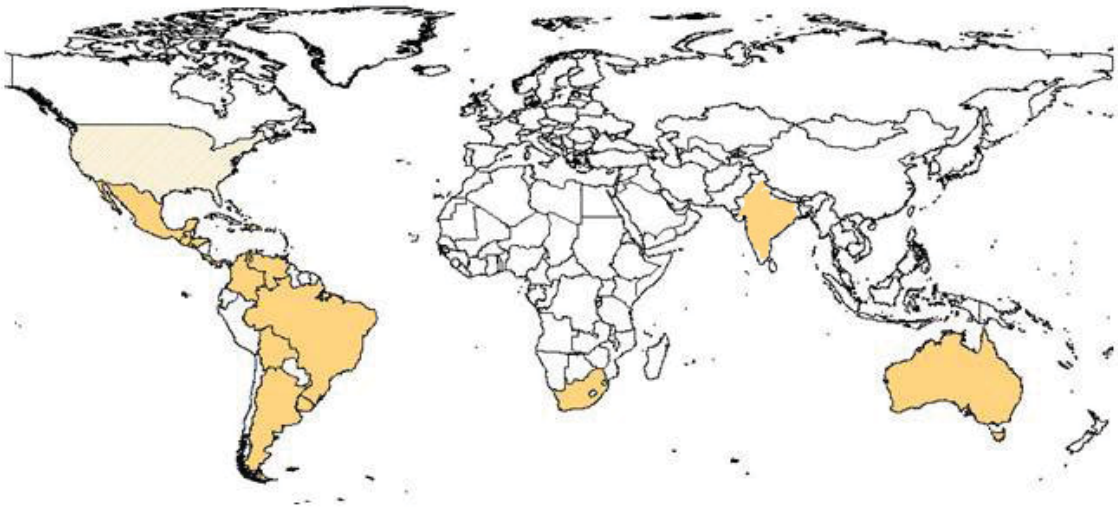
Figure 1. Distribution of *Boophilus Microplus*

8.2. Appendice 2: Distribution of the resistance to OP, SP, amitraz, ML and fipronil (June 2012)

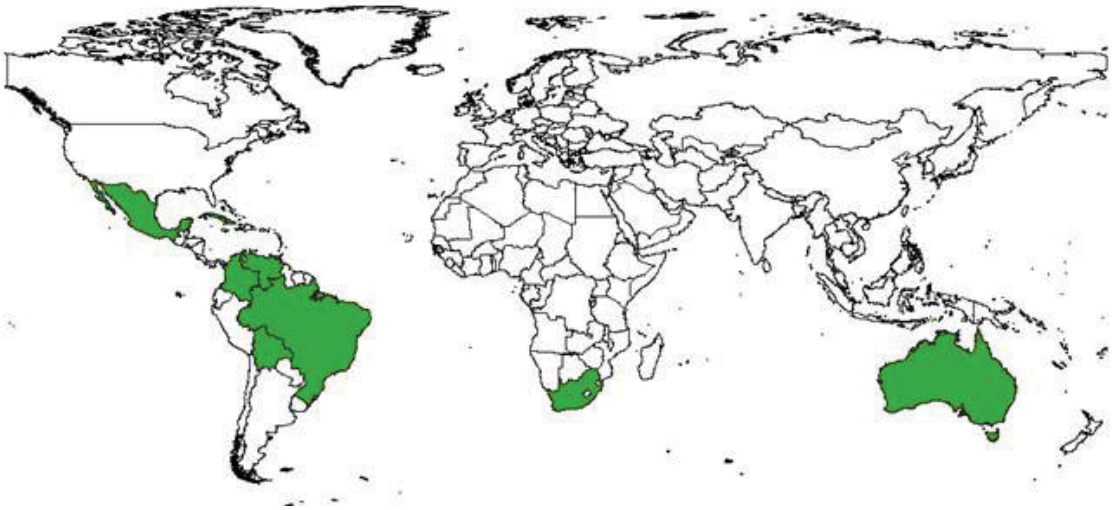
a) Resistance to organophosphates (OP)



b) Resistance to synthetic pyrethroids (SP)



c) Resistance to amitraz



d) Resistance to macrocyclic lactones (ML)

e) Resistance to fipronil

8.3. Appendice 3: Reports of resistance to organophosphates

This list is incomplete

Continent	Country	Chemical	International journals	National journals	Proceedings, FAO reports, other reports
North America	Mexico	Coumaphos	(Villarino et al. 2002); (Li et al. 2003); (Rosario-Cruz et al. 1997)*		*(FAO 2004) ; (Li 2004)*
		Diazinon	(Li et al. 2003)		*(FAO 2004)*
		Chlorpyriphos			*(FAO 2004) ; (Li 2004)*
		Ethion			*(FAO 2004) ; (Li 2004)*
		Dioxathion, dimethoate			*(FAO 2004)*
		OP	(Rodriguez-Vivas et al. 2007)		(Ortiz 1995)* ; *(Santamaria Vargas et al. 1999)*
	United States	Coumaphos	(Miller et al. 2005)		
Central America	Costa Rica	Coumaphos	(Rosario-Cruz et al. 1997)*		(Alvarez-Calderon et al. 2000)*
		Chlorpyrifos			(Alvarez-Calderon et al. 2000)*
		OP			(Alvarez-Calderon et al. 1999)* ; *(Pérez and Alvarez 1995) ; (Alvarez-Calderon 1999)*
	Cuba	Chlorfenvinphos			*(Rodriguez et al. 1999)*
	Jamaica	Dimethoate	(Rawlins and Mansingh 1978)*		
		Chlorfenvinphos	(Rawlins and Mansingh 1978)*		
South America	Argentina	Coumaphos & other OP		(Grillo Torrado and Gutiérrez 1970) ; (Grillo Torrado and Pérez Arrieta 1977) ; (Perez Arrieta et al. 1980)	
	Bolivia	Coumaphos			*(Kemp et al. 1998)*[a]

	Brazil	Coumaphos	(Patarroyo and Costa 1980); (Amaral et al. 1974)		(Farias 1999); (Furlong 1999); (Saueressig 1999)
		Chlorpyrifos	(Patarroyo and Costa 1980); (Mendes et al. 2011)	(Mendes et al. 2007)	
		Ethion, diaxothion	(Amaral et al. 1974)		
		OP		(Arteche et al. 1975);	(Martins et al. 2008); *(Kemp et al. 1998)*
	Colombia	Coumaphos		(Benavides et al. 2000); (Romero et al. 1997)	*(FAO 2004)*
		Diazinon		(Benavides et al. 2000); (Romero et al. 1997)	
		Chlorfenvinphos		(Benavides et al. 2000)	
		OP			(Benavides 1995)*; (Benavides and Romero 2002)
		Diazinon			*(FAO 2004)*
		Cylophos, trichlorfon, ethion			*(FAO 2004)*
	Peru	OP			*(Kemp et al. 1998)*
	Uruguay	Diazinon			*(Kemp et al. 1998)*
		OP		(Cuore et al. 2007)	
	Venezuela	Coumaphos		(Bravo et al. 2008)	(Coronado 1995)
		Chlorfenvinphos			(Coronado 1995)
		OP			(Coronado 1999); (Kemp et al. 1998); *(Santamaria Vargas et al. 1999)*
Africa	South Africa	Chlorfenvinphos			(Strydom and Peter 1999)[b]
		Diazinon		(Baker et al. 1979)	
		Carbophenothion, dicrotophos, dioxathion, ethion, fenitrothion, quintiofos		(Baker et al. 1979)	
		OP			(Kemp et al. 1998)[b]

Australia	Australia	Coumaphos	(Roulston et al. 1981) ; (Shaw 1966, Roulston et al. 1968)		
		Diazinon	(Shaw and Malcolm 1964) ; (Roulston et al. 1968)		
		Chlorpyrifos	(Roulston et al. 1981) ; (Shaw 1966)		
		Dioxathion	(Roulston et al. 1981) ; (Shaw 1966); (Shaw and Malcolm 1964)		
		Dimethoate, cyanophos	(Roulston et al. 1981)		
		Carbophenothion	(Shaw 1966); (Shaw and Malcolm 1964)		
		Ethion	(Roulston et al. 1968)		
		Parathion	(Shaw 1966)		

Italicized characters indicate that the reference does not correspond to the original articles, but contains a reference to the original source

* abstract available only

[a] resistance not confirmed, suspicion only

[b] *Boophilus* spp.

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8.4. Appendice 4 : Reports of resistance to macrocyclic lactones

Continent	Country	Chemical	International journals	Proceedings, FAO reports, other reports
North America	Mexico	ivermectin	(Perez-Cogollo et al. 2010a) ; (Perez-Cogollo et al. 2010b); (Fernandez-Salas et al. 2011)	
South America	Brazil	ivermectin	(Klafke et al. 2006, Klafke et al. 2012) (Martins and Furlong 2001)	
		doramectin	(Martins and Furlong 2001)	
		moxidectin	(Martins and Furlong 2001)	
	Colombia	ivermectin		(Benavides and Romero 2002)*[a]
	Uruguay	ivermectin	(Castro-Janer et al. 2011)[b]	

* abstract available only

[a] resistance not confirmed: strong indication of being resistant

[b] emerging resistance

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8.5. Appendice 5: Reports of resistance to fipronil

Continent	Country	Chemical	International journals	National journals	Proceedings, FAO reports, other reports
South America	Brazil	fipronil	(Castro-Janer et al. 2010a);		(Martins et al. 2008)
	Uruguay	fipronil	(Castro-Janer et al. 2010b) ; (Castro-Janer et al. 2011);	(Cuore et al. 2007)*	

* Abstract only

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Castro-Janer, E., L. Rifran, P. Gonzalez, J. Piaggio, A. Gil, and T. T. Schumaker. 2010b. *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) resistance to fipronil in Uruguay evaluated by in vitro bioassays. Vet Parasitol. 169:172-177.

Cuore, U., A. Trelles, J. Sanchis, V. Gayo, and M. A. Solari. 2007. Primer diagnostico de resistencia al Fipronil en la garrapata comun del ganado *Boophilus microplus*. Veterinaria 42:35-41.

Martins, J. R., Furlong, J., Prata, M. C. A., and Doyle, R. L.2008. Acaricide resistance in Brazil and the use of mixture as chemical alternative for tick control, VI Seminario Internacional de Parasitologia Animal, Boca del Río, Veracruz, Mexico.

8.6. Appendice 6: Dose-response mortality obtained with the LTT for the Argentinean populations

Figure 1: ST21 & ST22

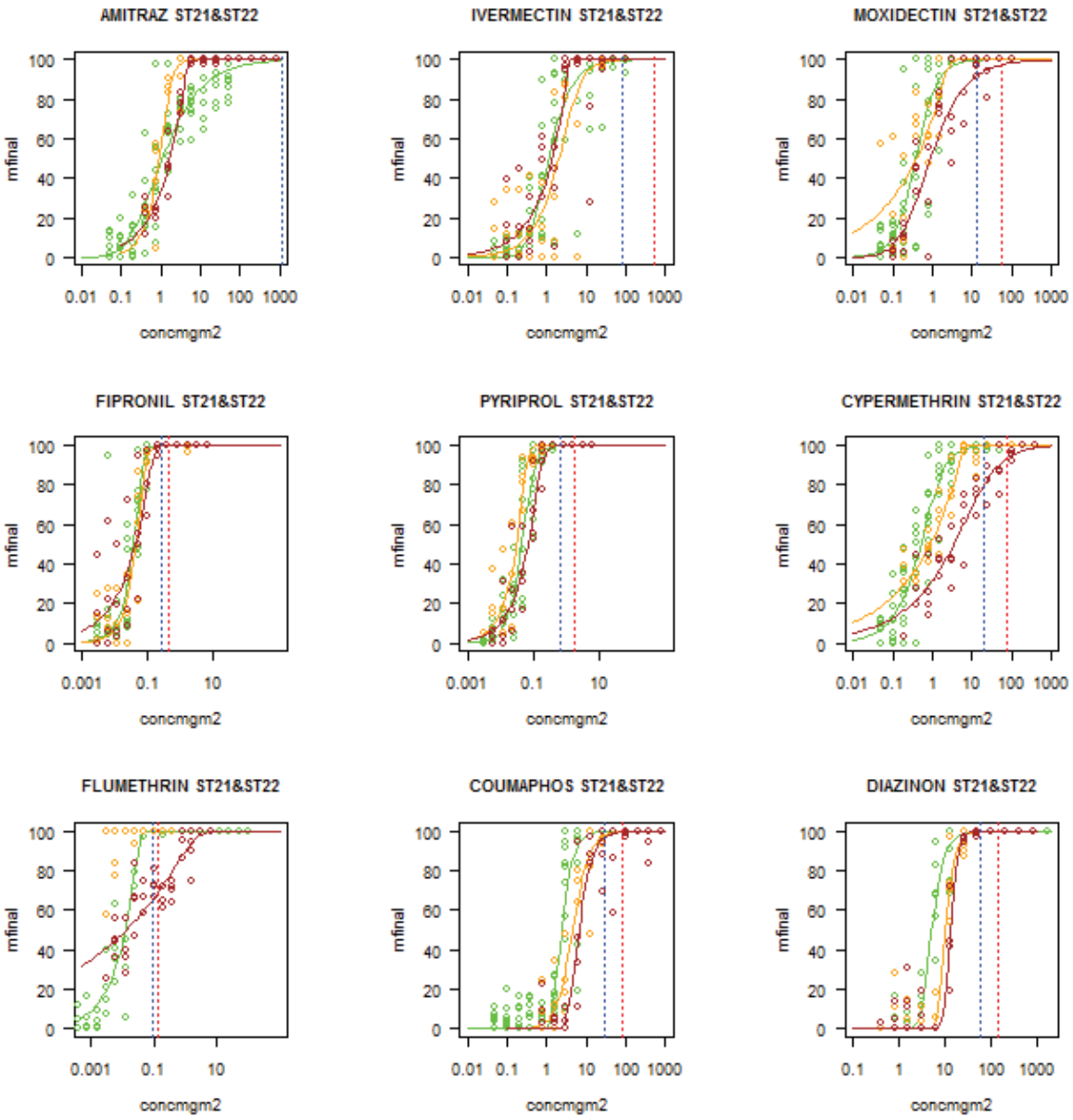


Fig. 1. Dose-response mortality obtained with the LTT for the Argentinean field populations ST21 (orange) and ST22 (brown) and for the susceptible Muñoz strain (green). Dashed lines indicate the concentrations corresponding to 2x LD_{99} (blue) and 2x $LD_{99.9}$ (red) of the susceptible Muñoz strain.

Figure 2: ST23 & ST24

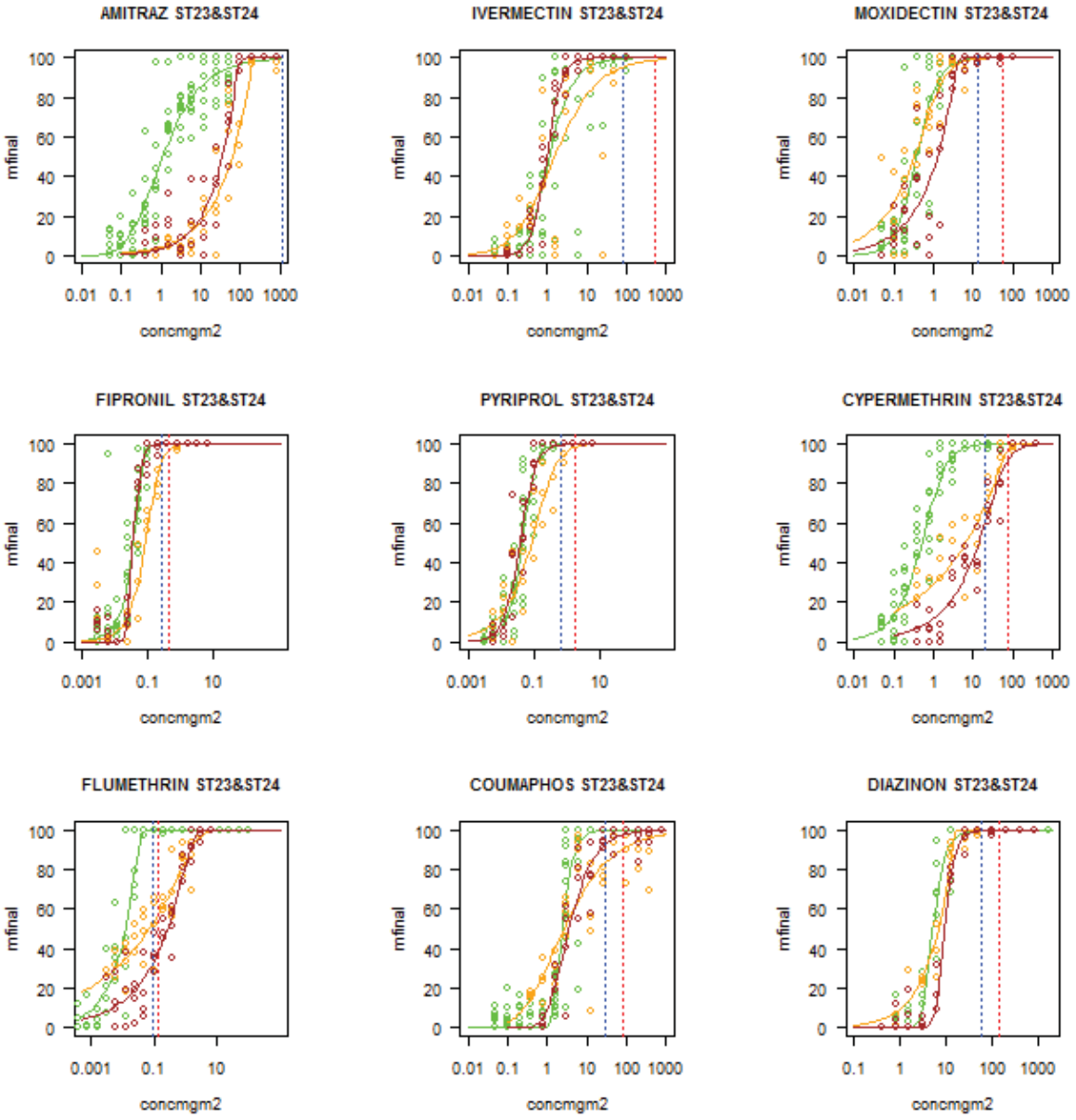


Fig. 2. Dose-response mortality obtained with the LTT for the Argentinean field populations ST23 (orange) and ST24 (brown) and for the susceptible Muñoz strain (green). Dashed lines indicate the concentrations corresponding to 2x LD_{99} (blue) and 2x $LD_{99.9}$ (red) of the susceptible Muñoz strain.

Figure 3: ST25

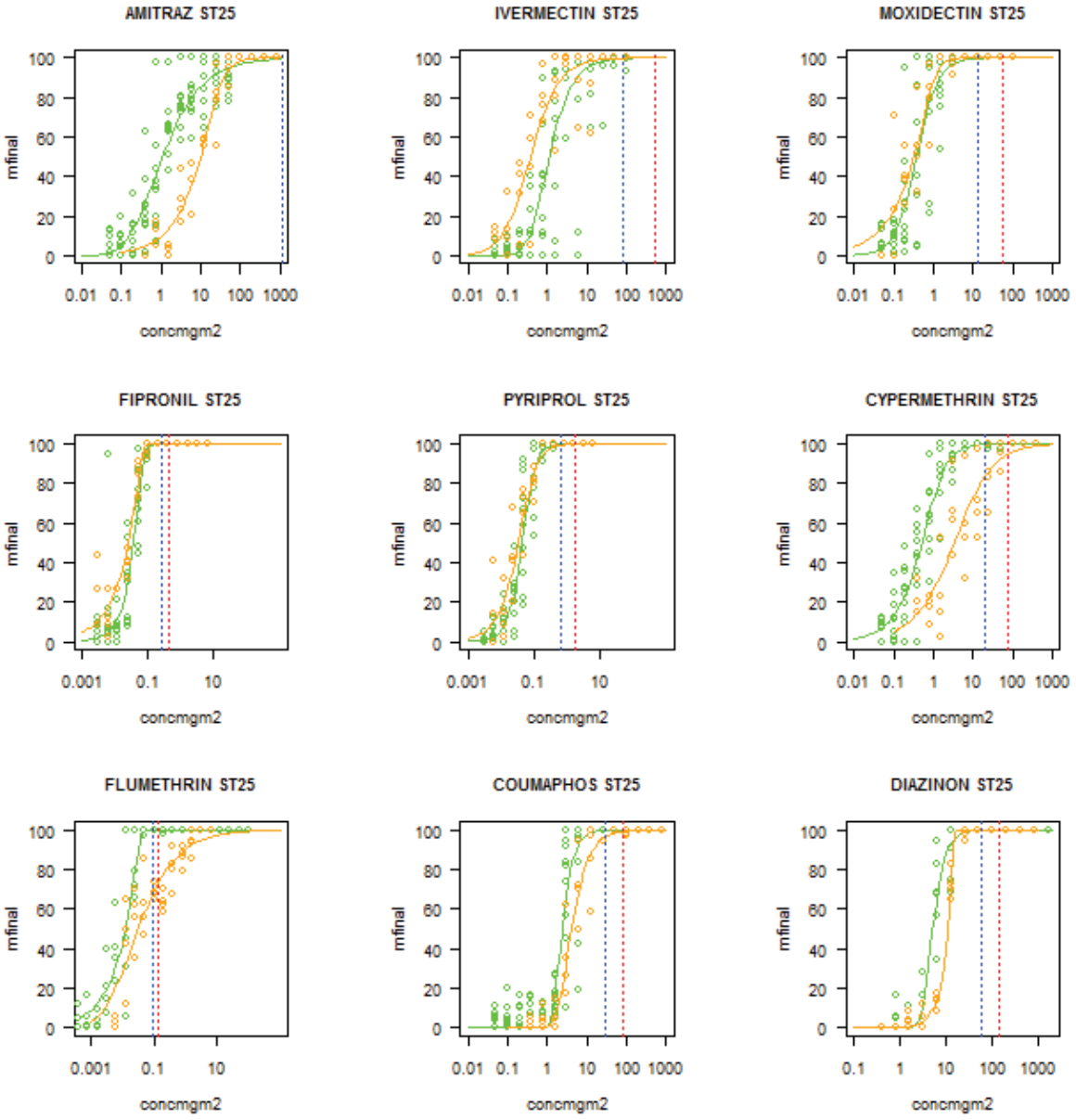


Fig. 3. Dose-response mortality obtained with the LTT for the Argentinean field population ST25 (orange) and for the susceptible Muñoz strain (green). Dashed lines indicate the concentrations corresponding to 2x LD_{99} (blue) and 2x $LD_{99.9}$ (red) of the susceptible Muñoz strain.

Figure 4: ST26

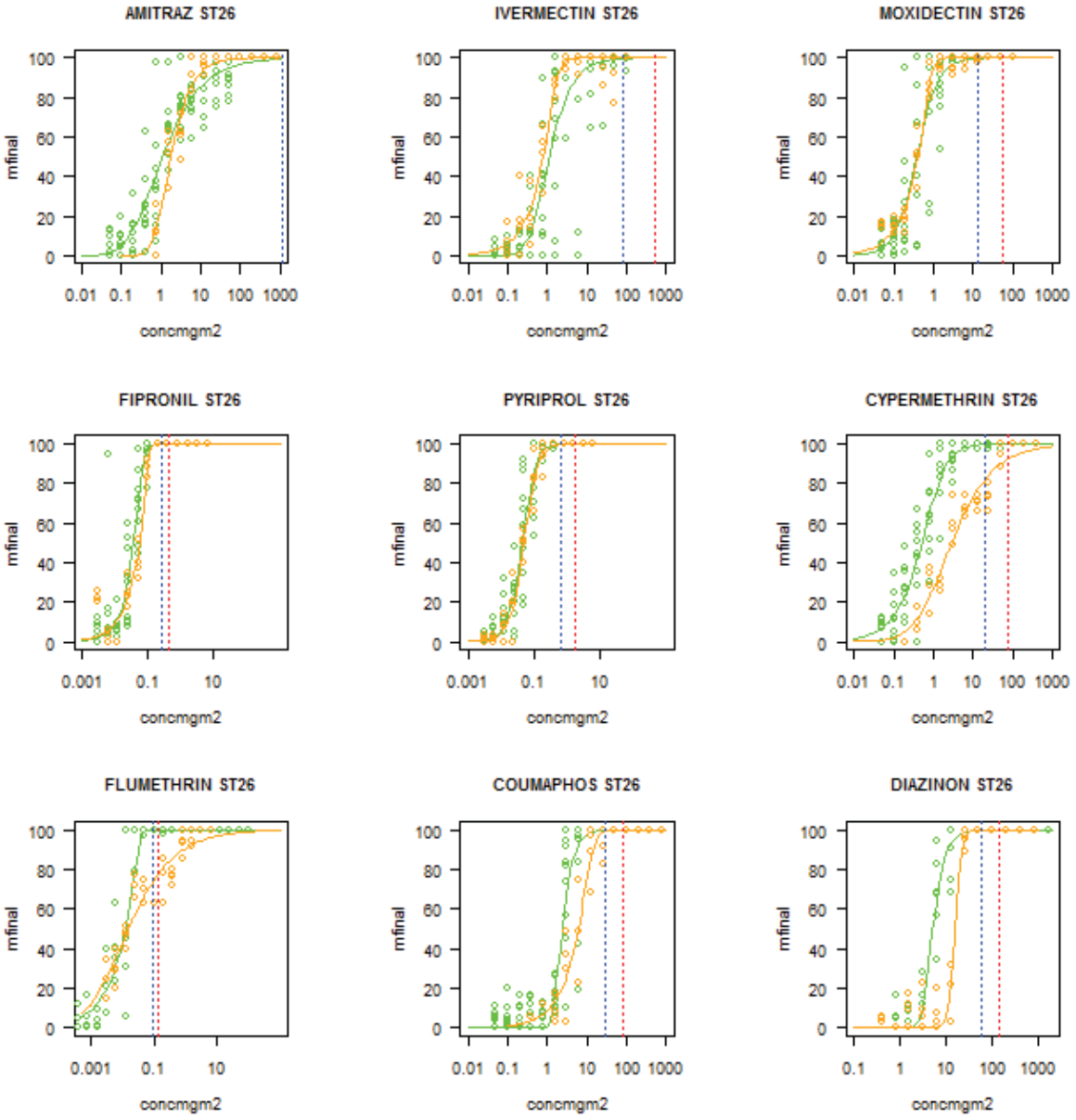


Fig. 4. Dose-response mortality obtained with the LTT for the Argentinean field population ST26 (orange) and for the susceptible Muñoz strain (green). Dashed lines indicate the concentrations corresponding to 2x LD_{99} (blue) and 2x $LD_{99.9}$ (red) of the susceptible Muñoz strain.

Figure 5: ST27

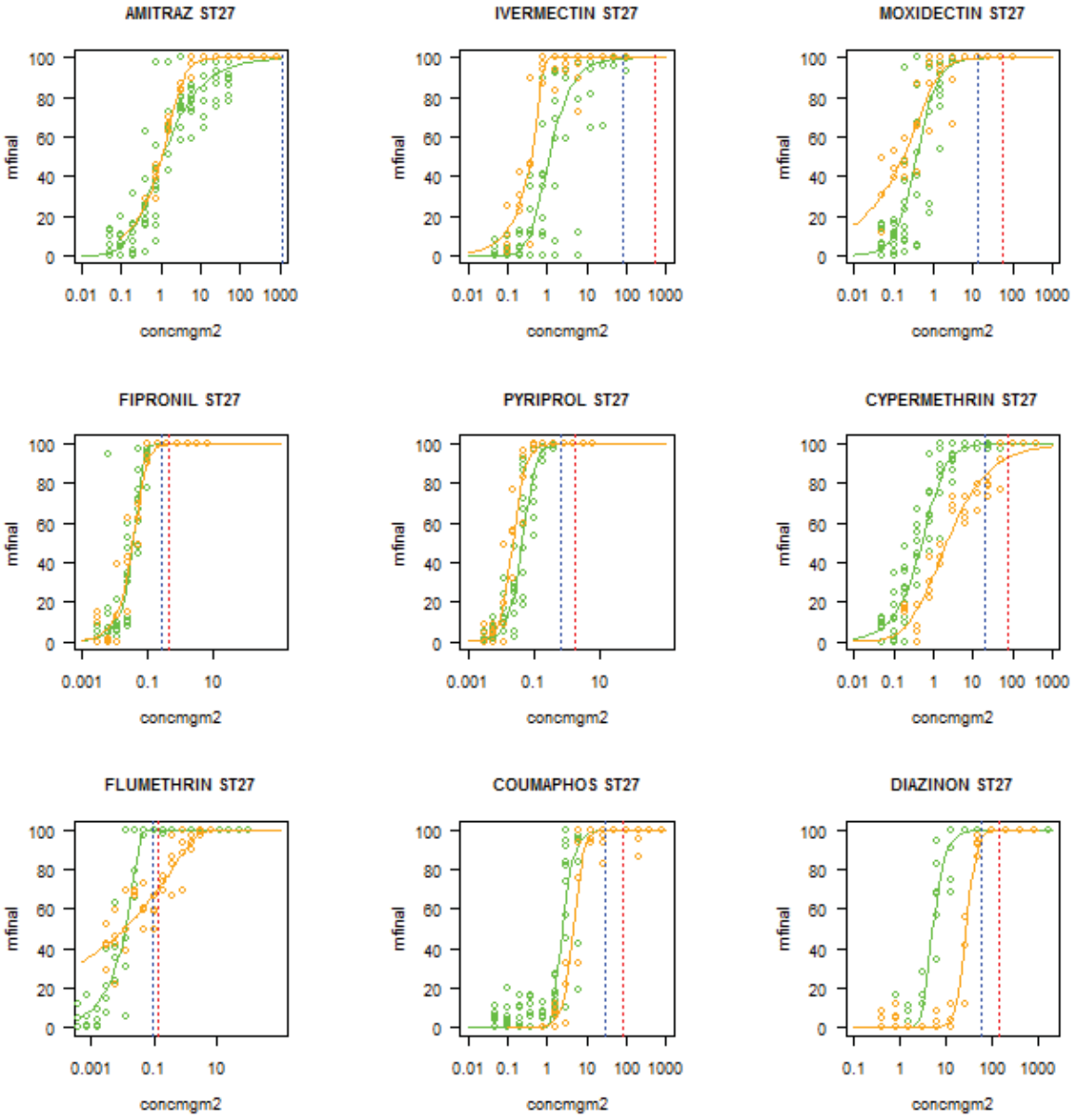


Fig. 5. Dose-response mortality obtained with the LTT for the Argentinean field population ST27 (orange) and for the susceptible Muñoz strain (green). Dashed lines indicate the concentrations corresponding to 2x LD_{99} (blue) and 2x $LD_{99.9}$ (red) of the susceptible Muñoz strain.

Figure 6: ST29

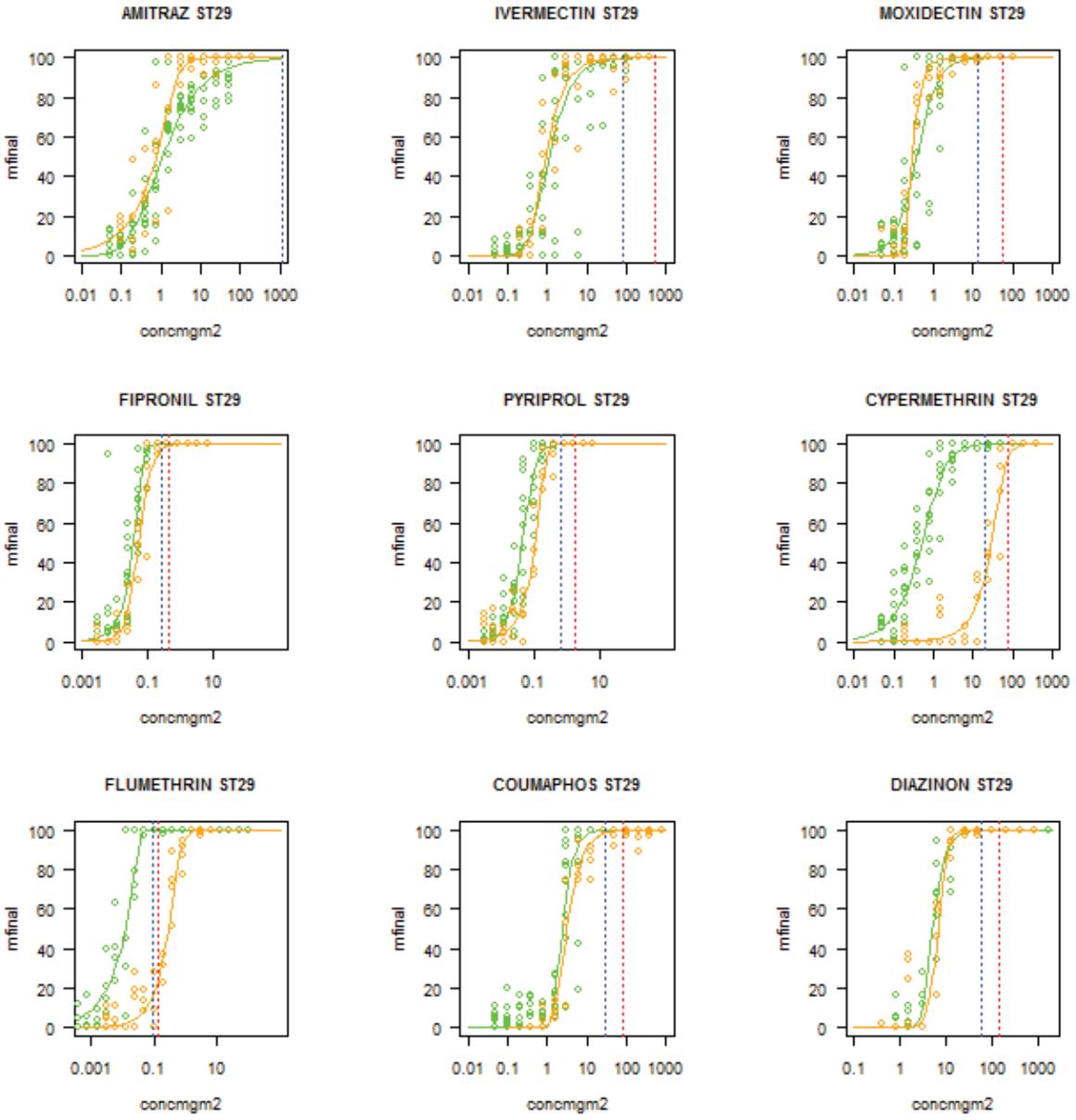


Fig. 6. Dose-response mortality obtained with the LTT for the Argentinean field population ST29 (orange) and for the susceptible Muñoz strain (green). Dashed lines indicate the concentrations corresponding to 2x LD_{99} (blue) and 2x $LD_{99.9}$ (red) of the susceptible Muñoz strain.

Figure 7: ST30

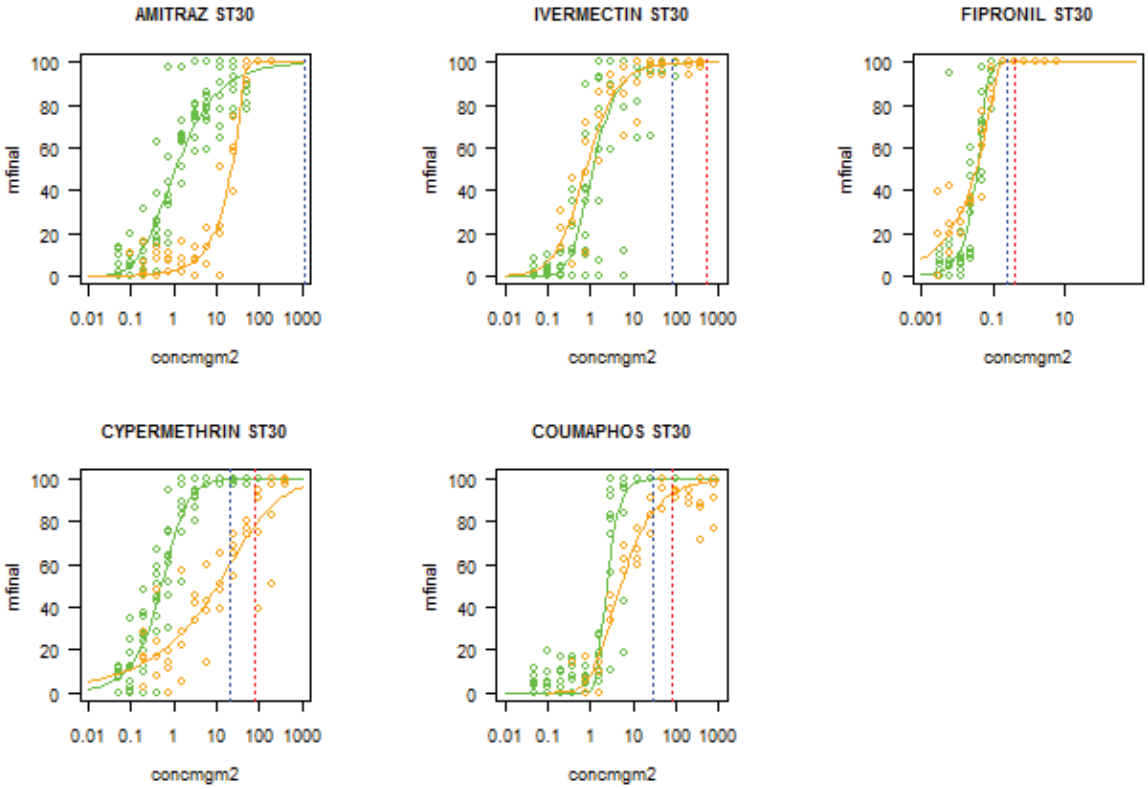


Fig. 7. Dose-response mortality obtained with the LTT for the Argentinean field population ST30 (orange) and for the susceptible Muñoz strain (green). Dashed lines indicate the concentrations corresponding to 2x LD_{99} (blue) and 2x $LD_{99.9}$ (red) of the susceptible Muñoz strain.

8.7. Appendice 7: Dose-response mortality obtained with the LTT for the South African populations

Figure 1: ST11

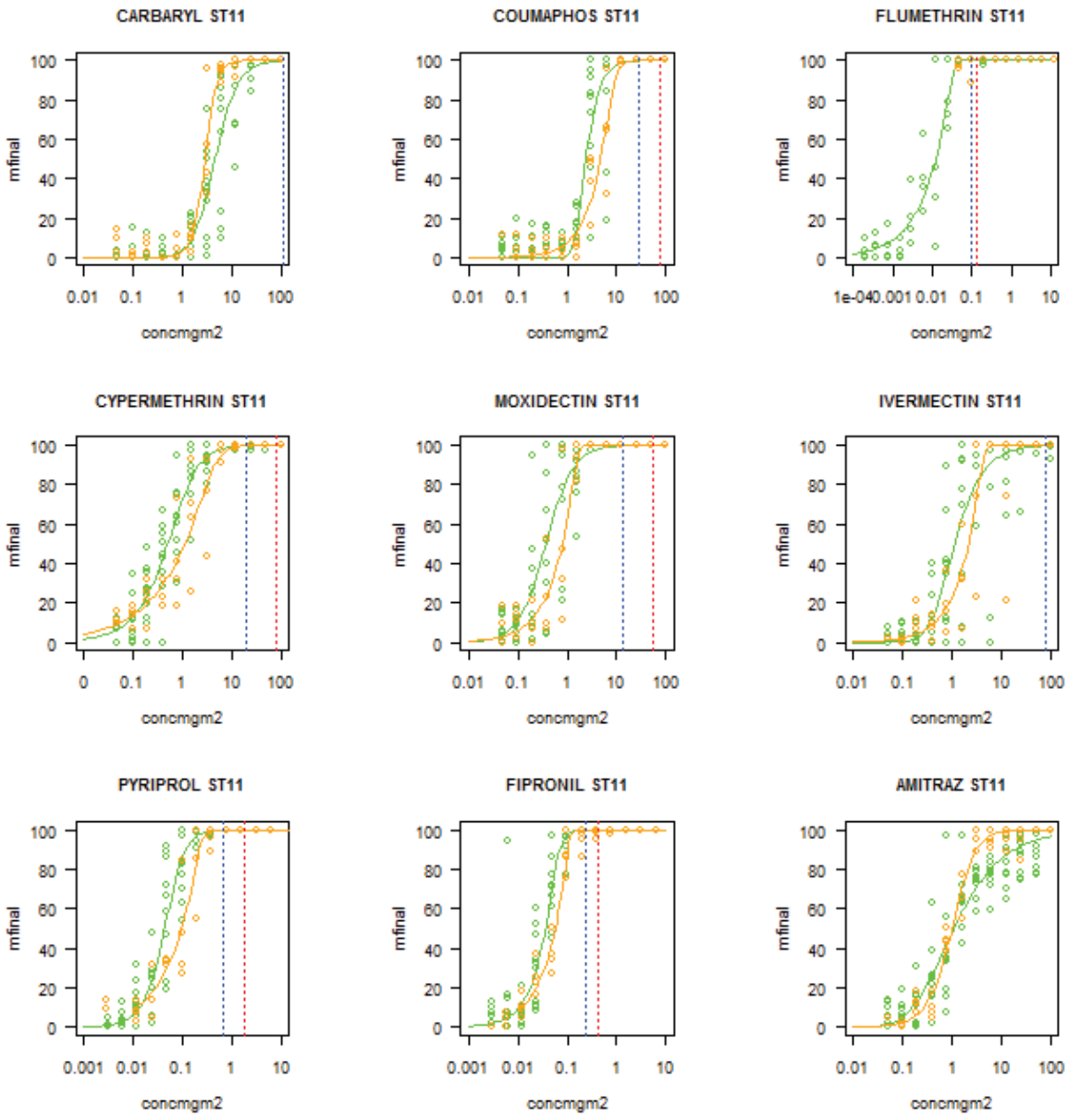


Fig. 1. Dose-response mortality obtained with the LTT for the South African field population ST11 (orange) and for the susceptible Muñoz strain (green). Dashed lines indicate the concentrations corresponding to 2x LD_{99} (blue) and 2x $LD_{99.9}$ (red) of the susceptible Muñoz strain.

Figure 2: ST12

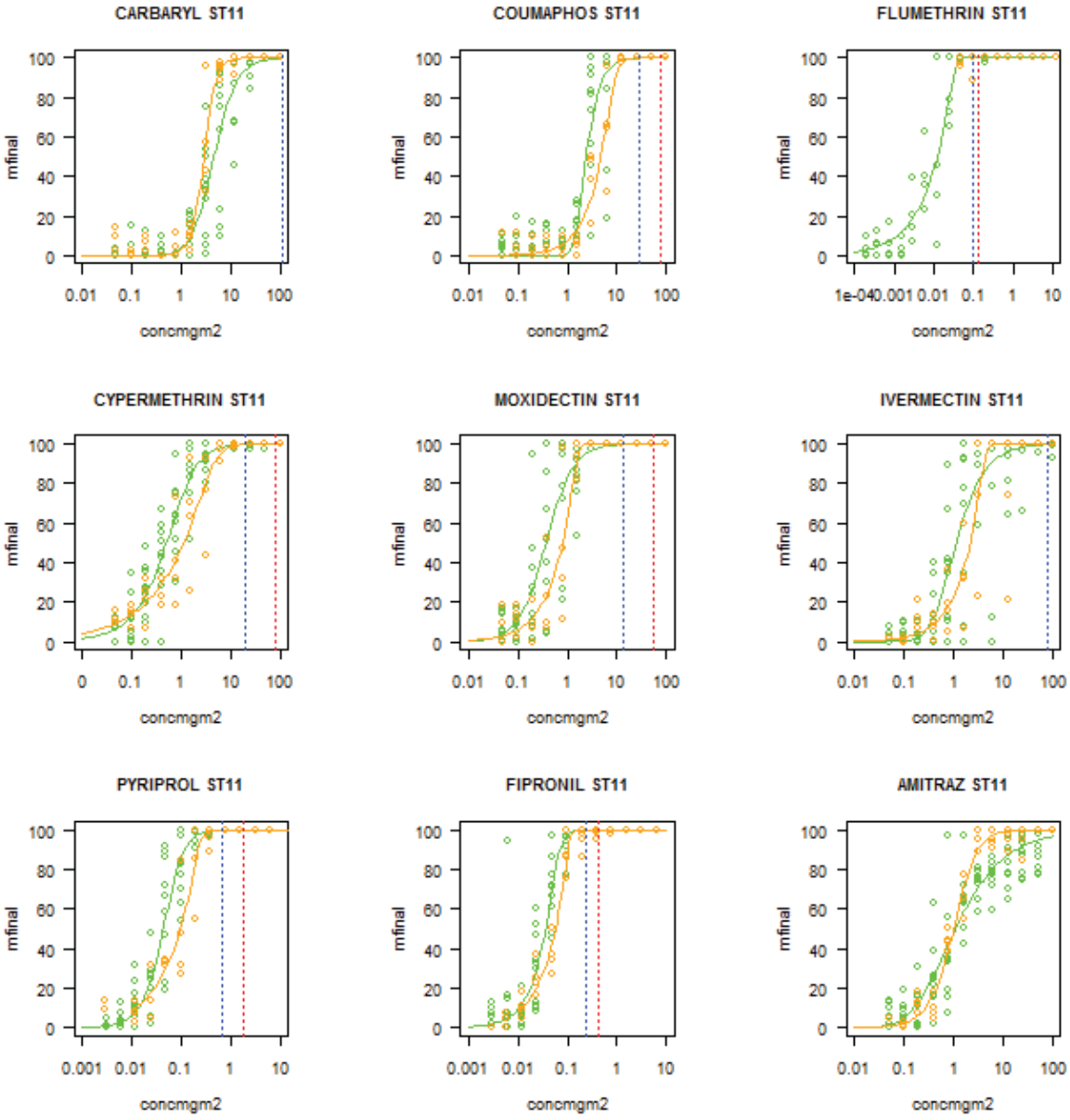


Fig. 2. Dose-response mortality obtained with the LTT for the South African field population ST12 (orange) and for the susceptible Muñoz strain (green). Dashed lines indicate the concentrations corresponding to 2x LD_{99} (blue) and 2x $LD_{99.9}$ (red) of the susceptible Muñoz strain.

Figure 3: ST15

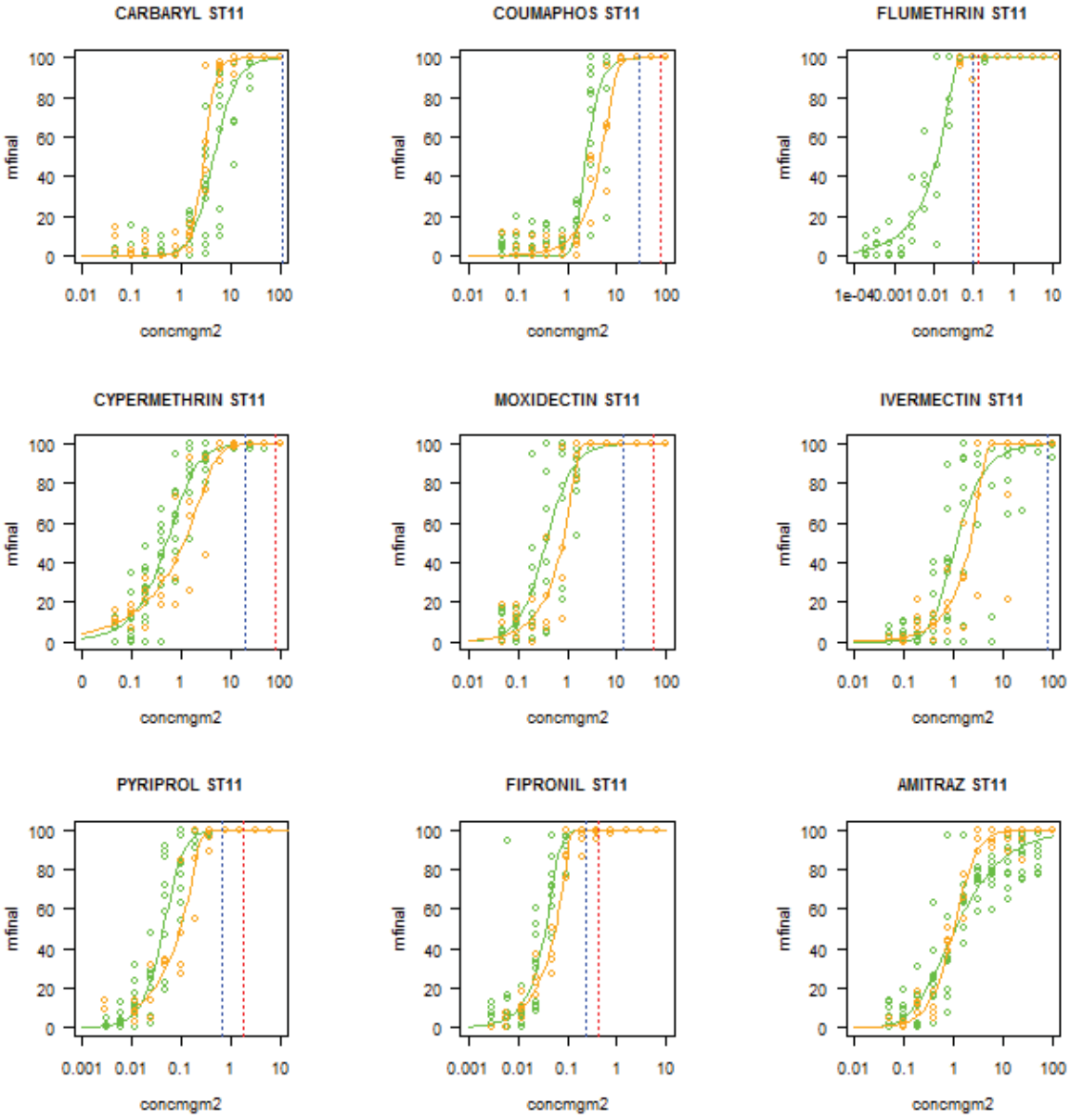


Fig. 3. Dose-response mortality obtained with the LTT for the South African field population ST15 (orange) and for the susceptible Muñoz strain (green). Dashed lines indicate the concentrations corresponding to 2x LD_{99} (blue) and 2x $LD_{99.9}$ (red) of the susceptible Muñoz strain.

8.8. Appendice 8: Dose-response mortality obtained with the LTT for the Australian populations

Figure 1: Urah

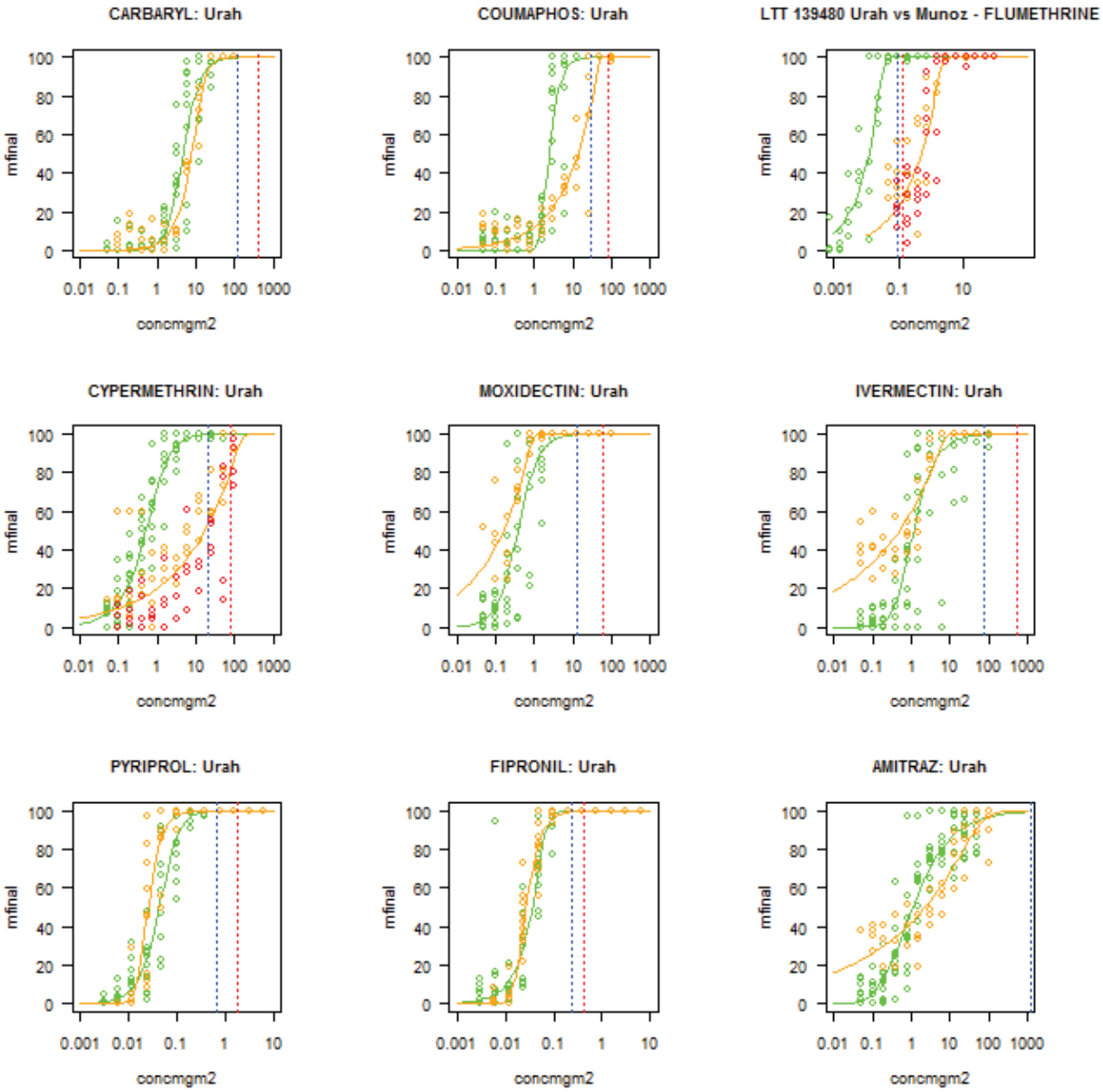


Fig. 1. Dose-response mortality obtained with the LTT for the Australian field population Urah (orange) and for the susceptible Muñoz strain (green). Dashed lines indicate the concentrations corresponding to 2x LD_{99} (blue) and 2x $LD_{99.9}$ (red) of the susceptible Muñoz strain.

Figure 2: Curra

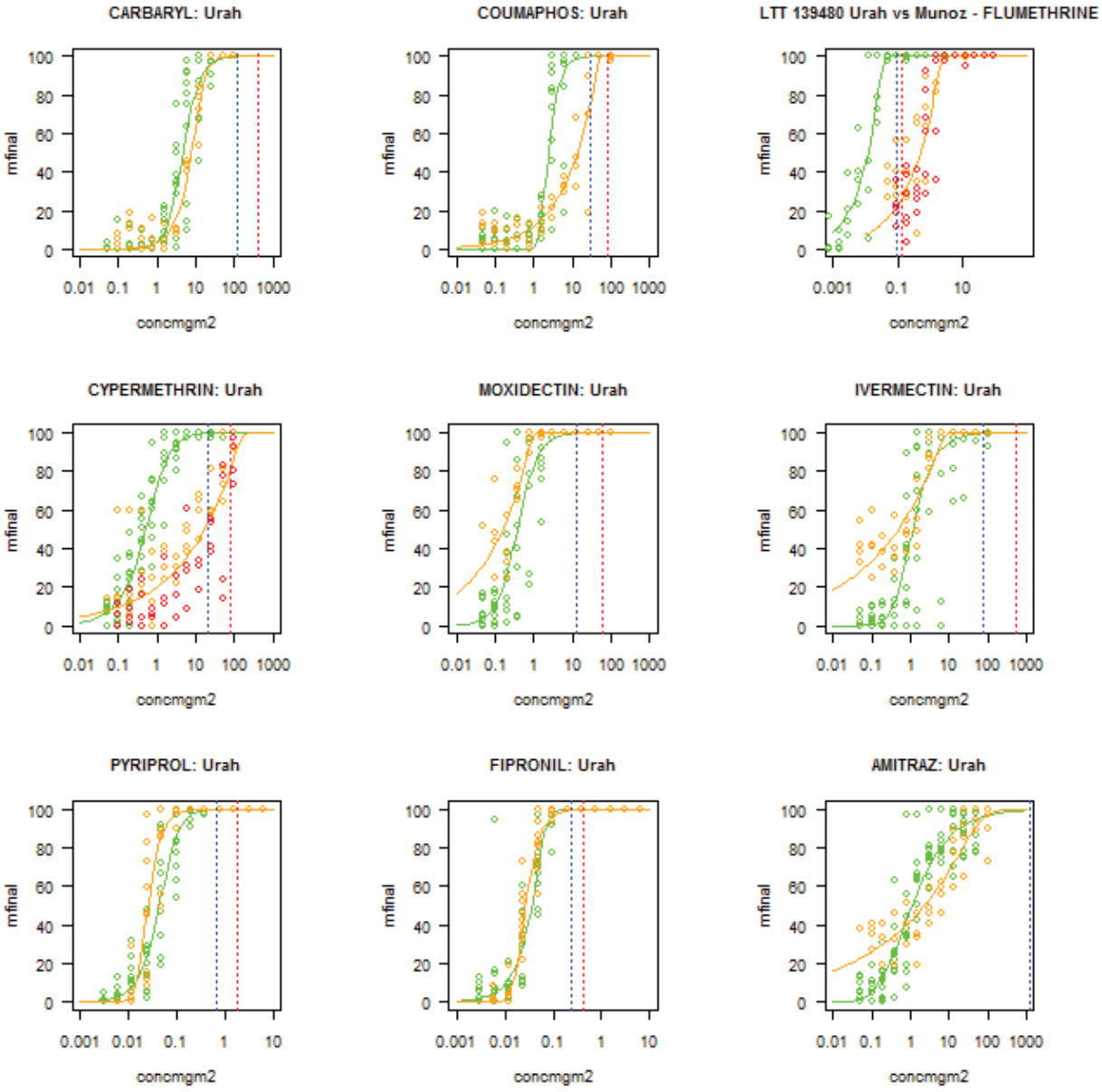


Fig. 2. Dose-response mortality obtained with the LTT for the Australian field population Curra (orange) and for the susceptible Muñoz strain (green). Dashed lines indicate the concentrations corresponding to 2x LD_{99} (blue) and 2x $LD_{99.9}$ (red) of the susceptible Muñoz strain.

8.9. Appendice 9: Dose-response mortality obtained with the LTT for the Brazilian populations

Figure 1: ST40

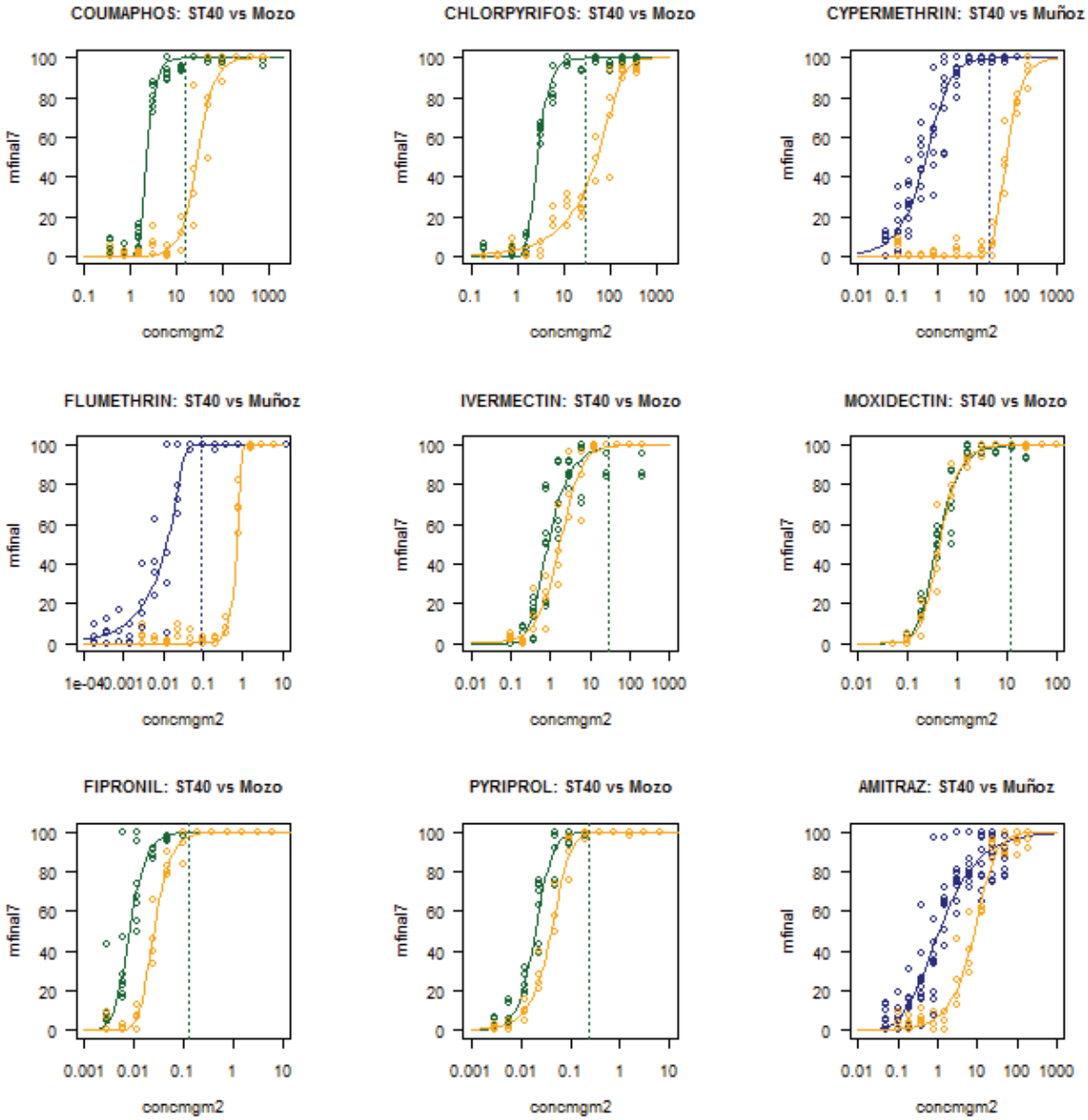


Fig. 1. Dose-response mortality obtained with the LTT for the Brazilian field population ST40 (orange) and for the susceptible Mozo strain (green) or Muñoz strain (blue). Dashed lines indicate the concentrations corresponding to 2x LD_{99} of the susceptible strain

Figure 2: ST41

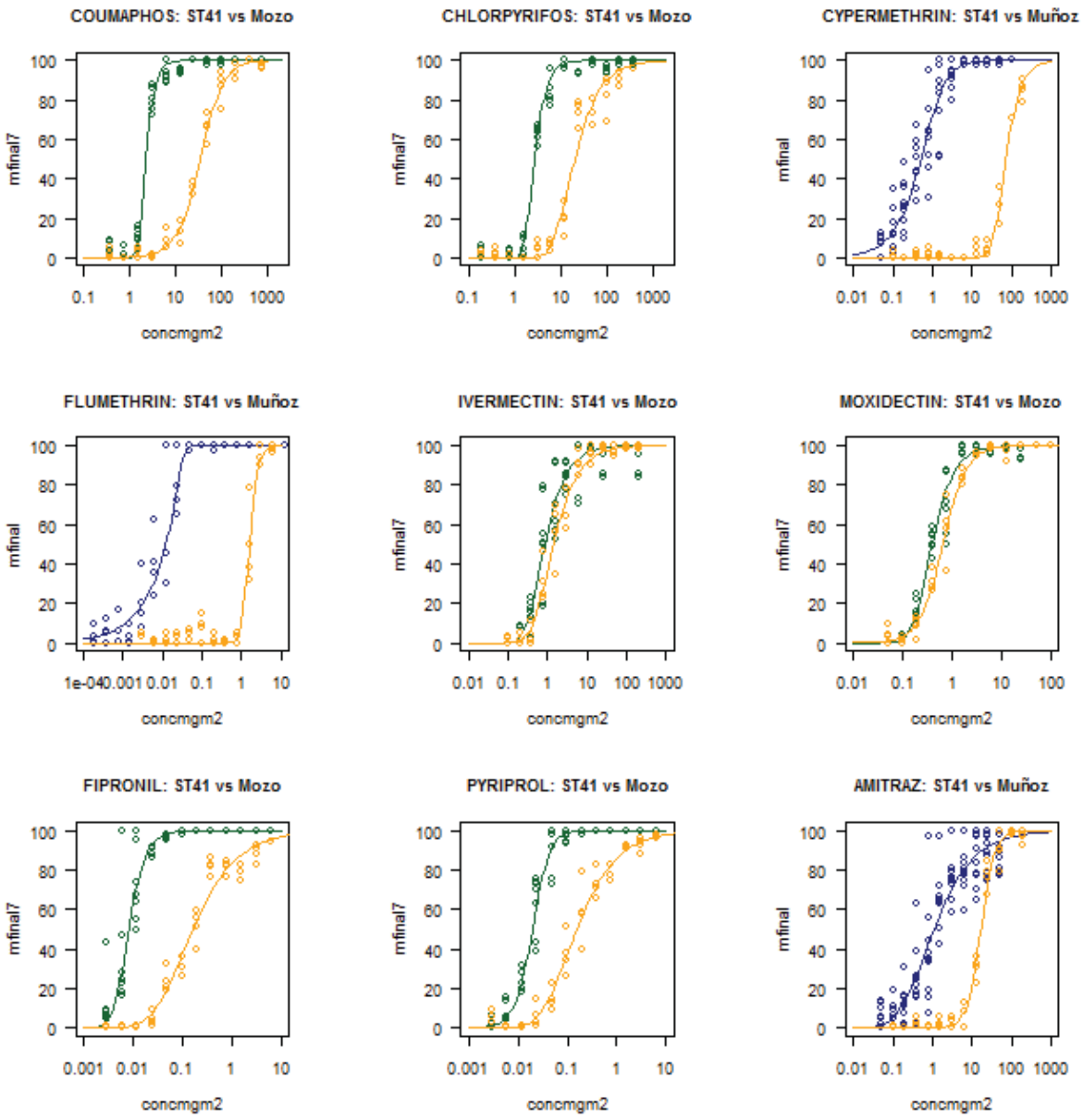


Fig. 2. Dose-response mortality obtained with the LTT for the Brazilian field population ST41 (orange) and for the susceptible Mozo strain (green) or Muñoz strain (blue).

Figure 3: ST42

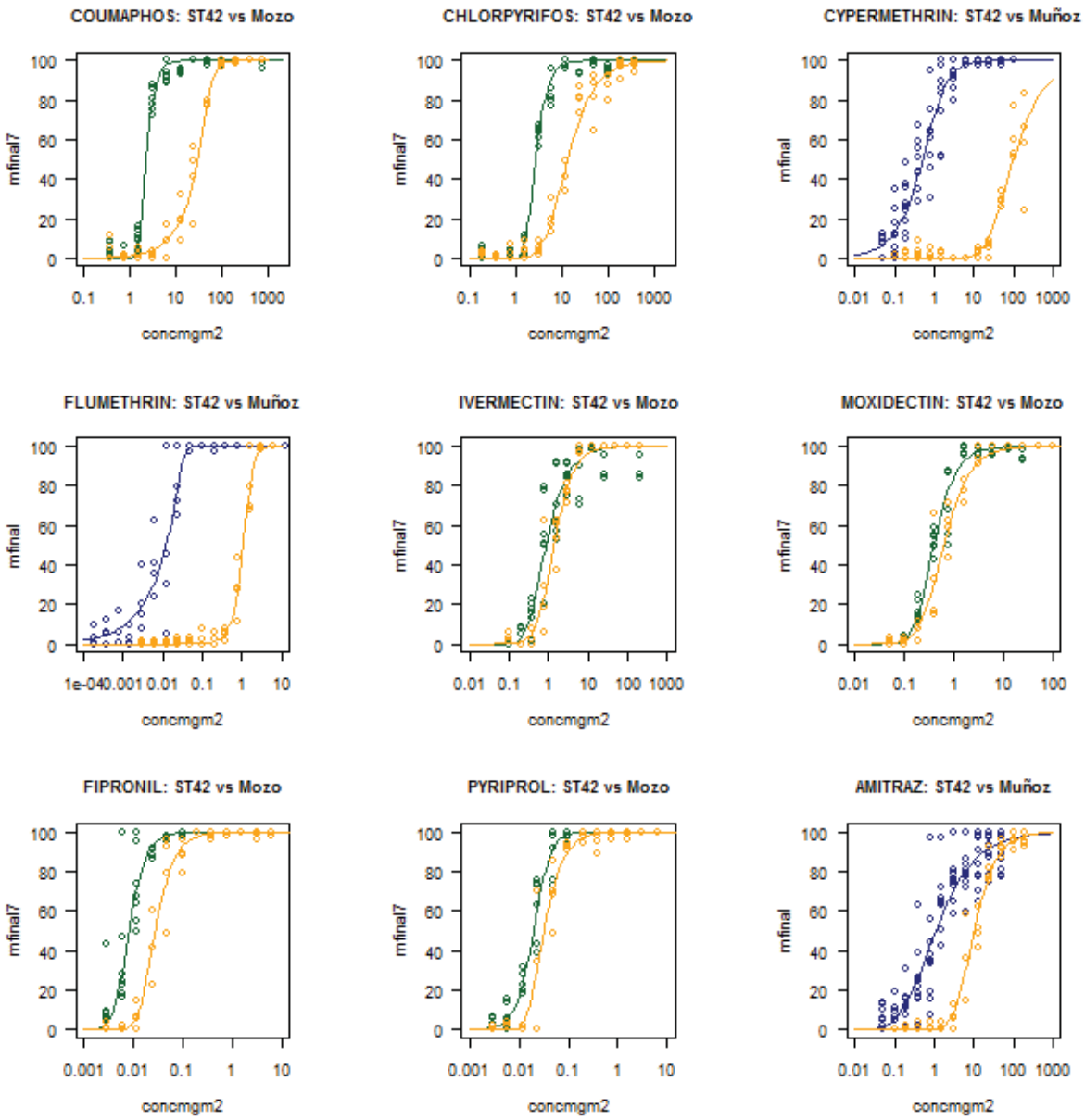


Fig. 3. Dose-response mortality obtained with the LTT for the Brazilian field population ST42 (orange) and for the susceptible Mozo strain (green) or Muñoz strain (blue).

Figure 4: ST44

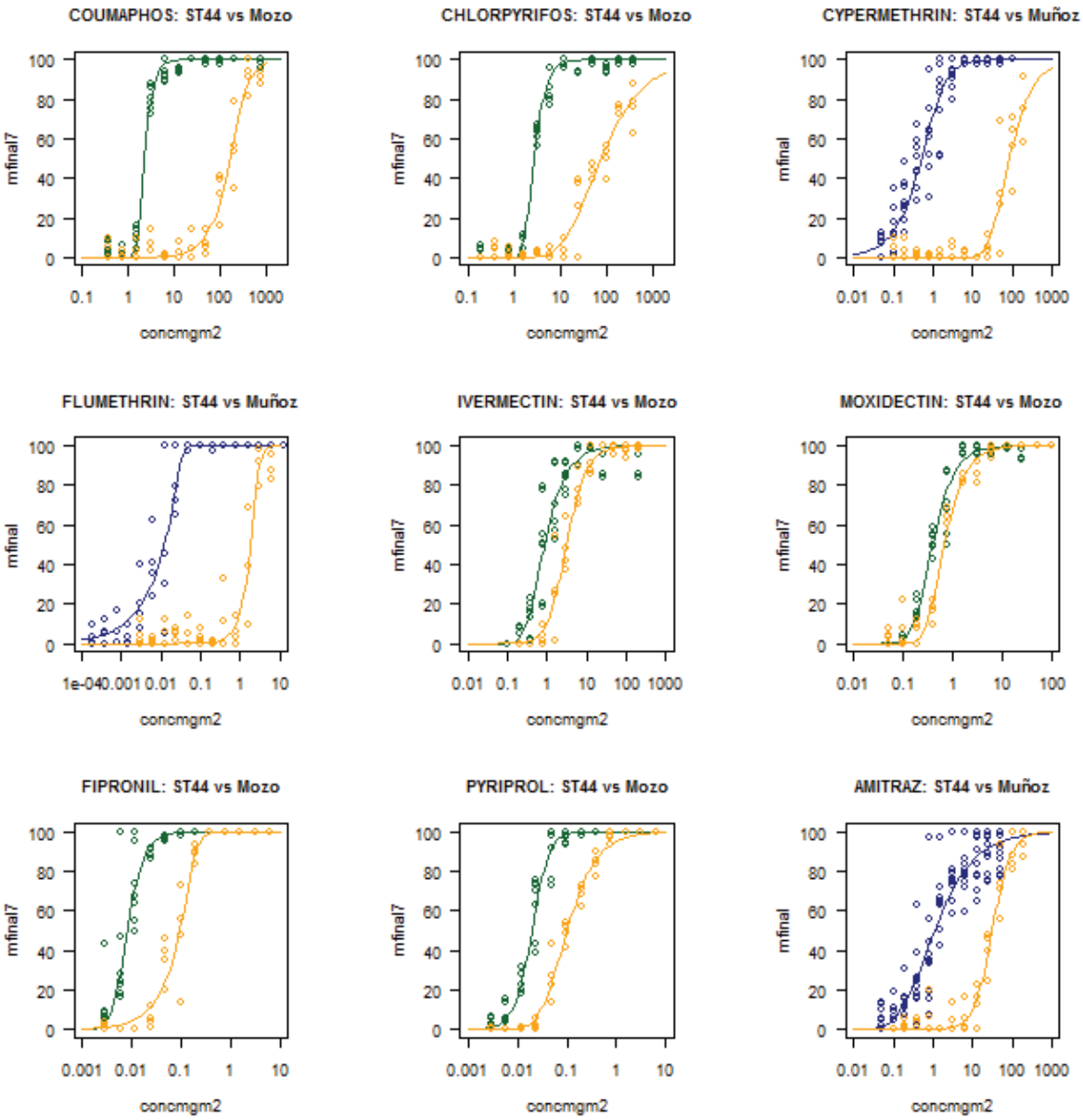


Fig. 4. Dose-response mortality obtained with the LTT for the Brazilian field population ST44 (orange) and for the susceptible Mozo strain (green) or Muñoz strain (blue).

Figure 5: ST45

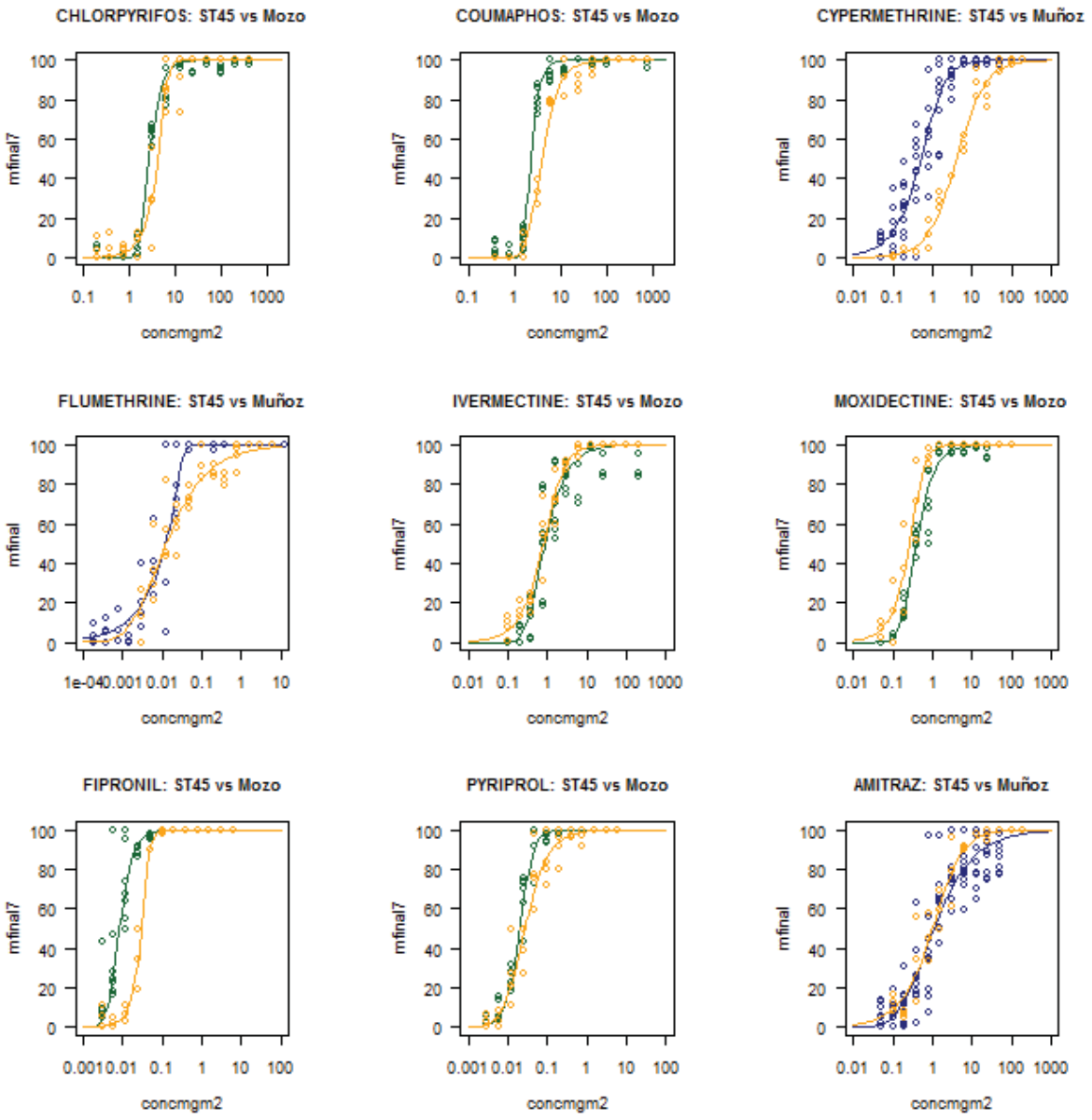


Fig. 5. Dose-response mortality obtained with the LTT for the Brazilian field population ST45 (orange) and for the susceptible Mozo strain (green) or Muñoz strain (blue).

Figure 6: ST46

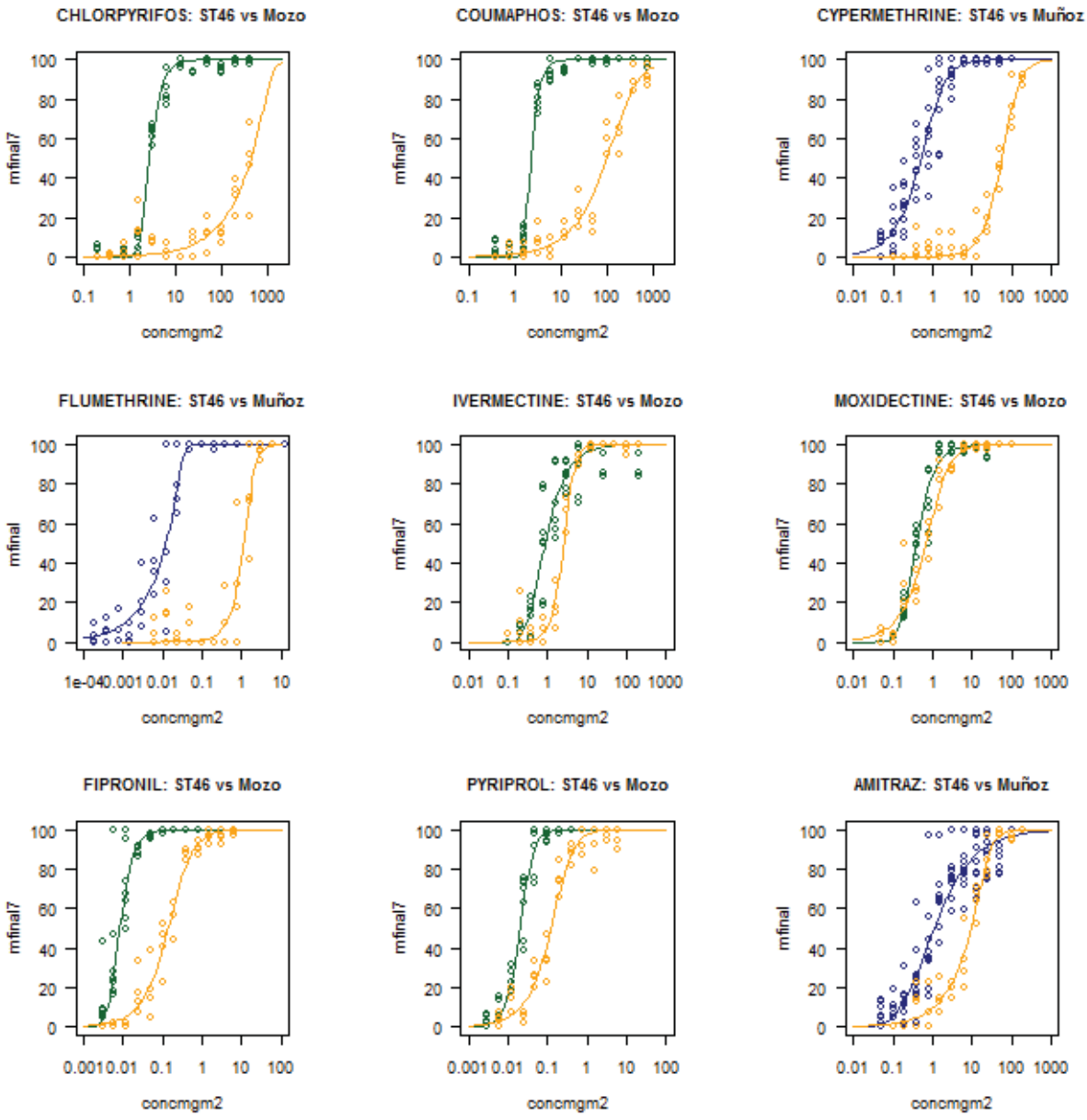


Fig. 6. Dose-response mortality obtained with the LTT for the Brazilian field population ST46 (orange) and for the susceptible Mozo strain (green) or Muñoz strain (blue).

Figure 7: ST47

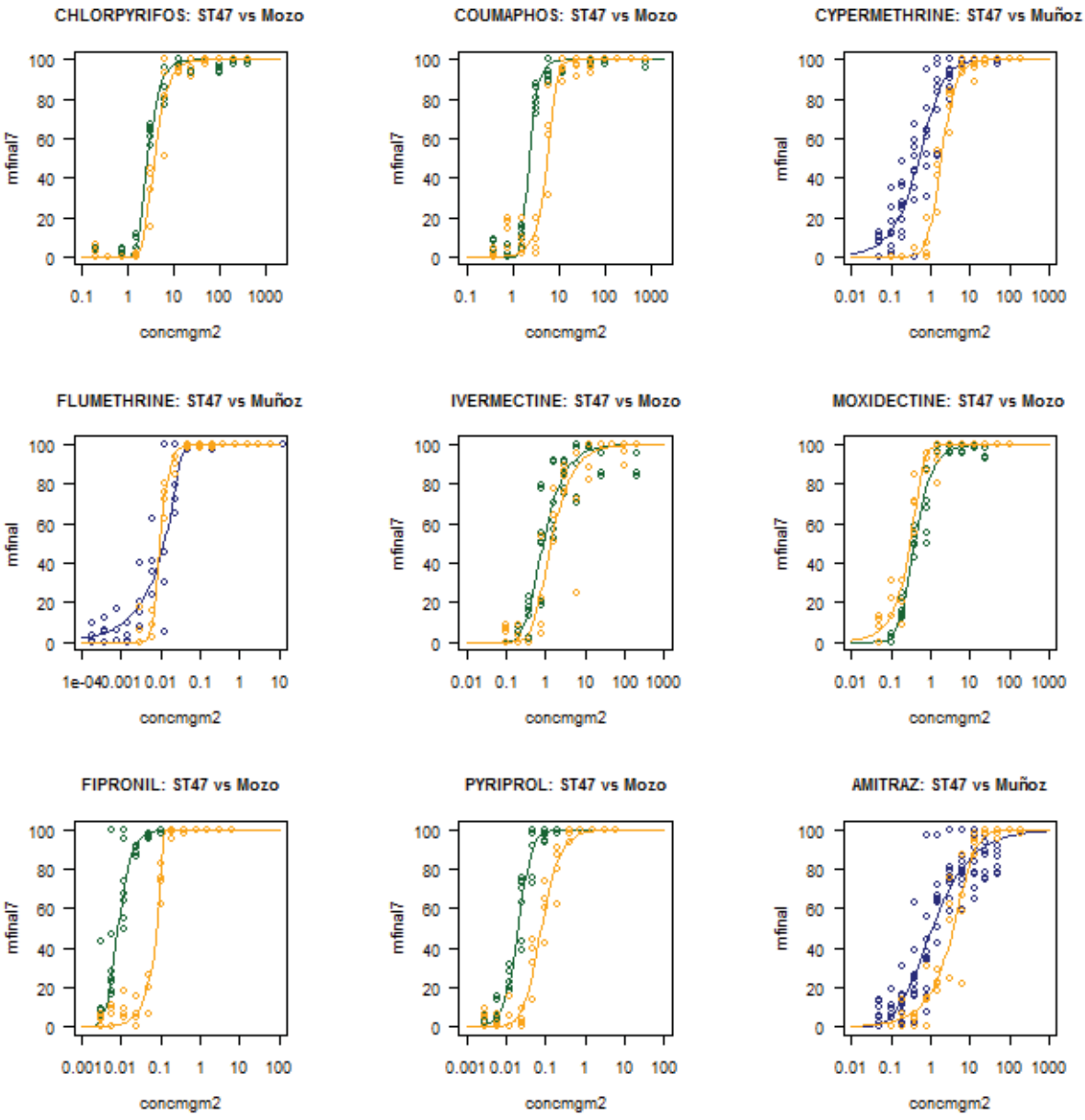


Fig. 7. Dose-response mortality obtained with the LTT for the Brazilian field population ST47 (orange) and for the susceptible Mozo strain (green) or Muñoz strain (blue).

Figure 8: ST48

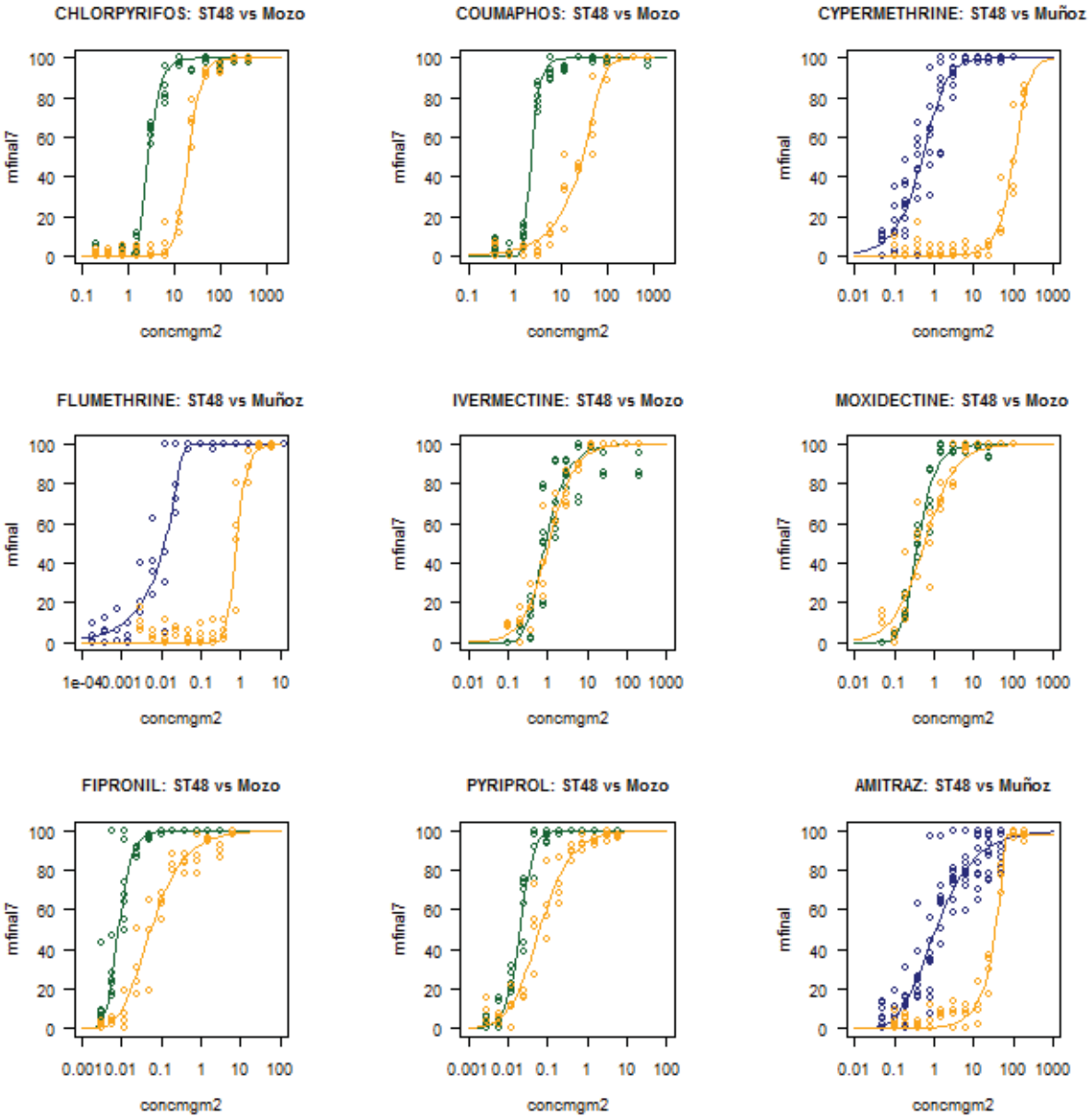


Fig. 8. Dose-response mortality obtained with the LTT for the Brazilian field population ST48 (orange) and for the susceptible Mozo strain (green) or Muñoz strain (blue).

Figure 9: ST49

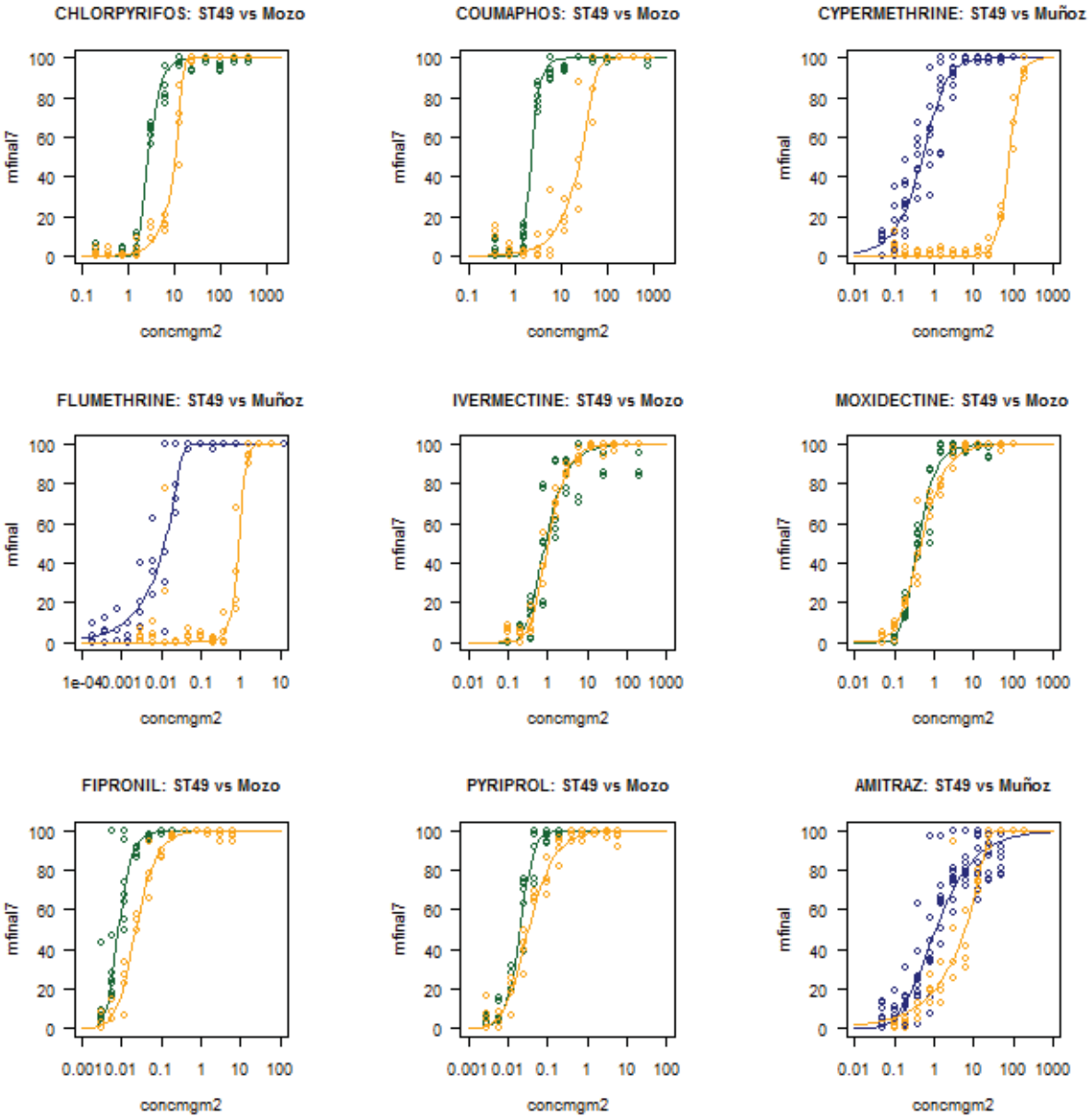


Fig. 9. Dose-response mortality obtained with the LTT for the Brazilian field population ST49 (orange) and for the susceptible Mozo strain (green) or Muñoz strain (blue).

Figure 10: ST50 & ST51

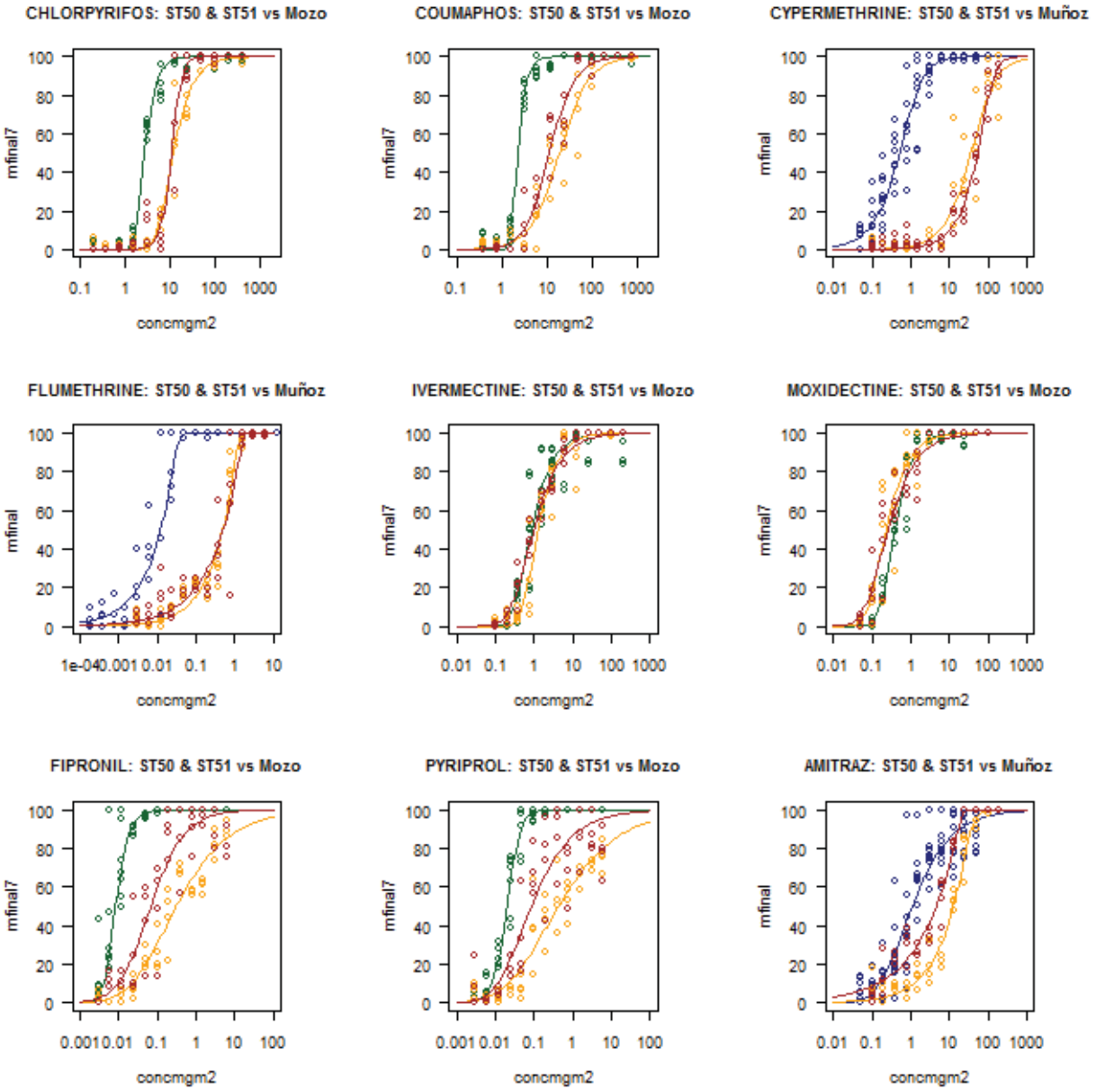


Fig. 10. Dose-response mortality obtained with the LTT for the Brazilian field populations ST50 (orange) and ST51 (brown) and for the susceptible Mozo strain (green) or Muñoz strain (blue).

Figure 11: ST52

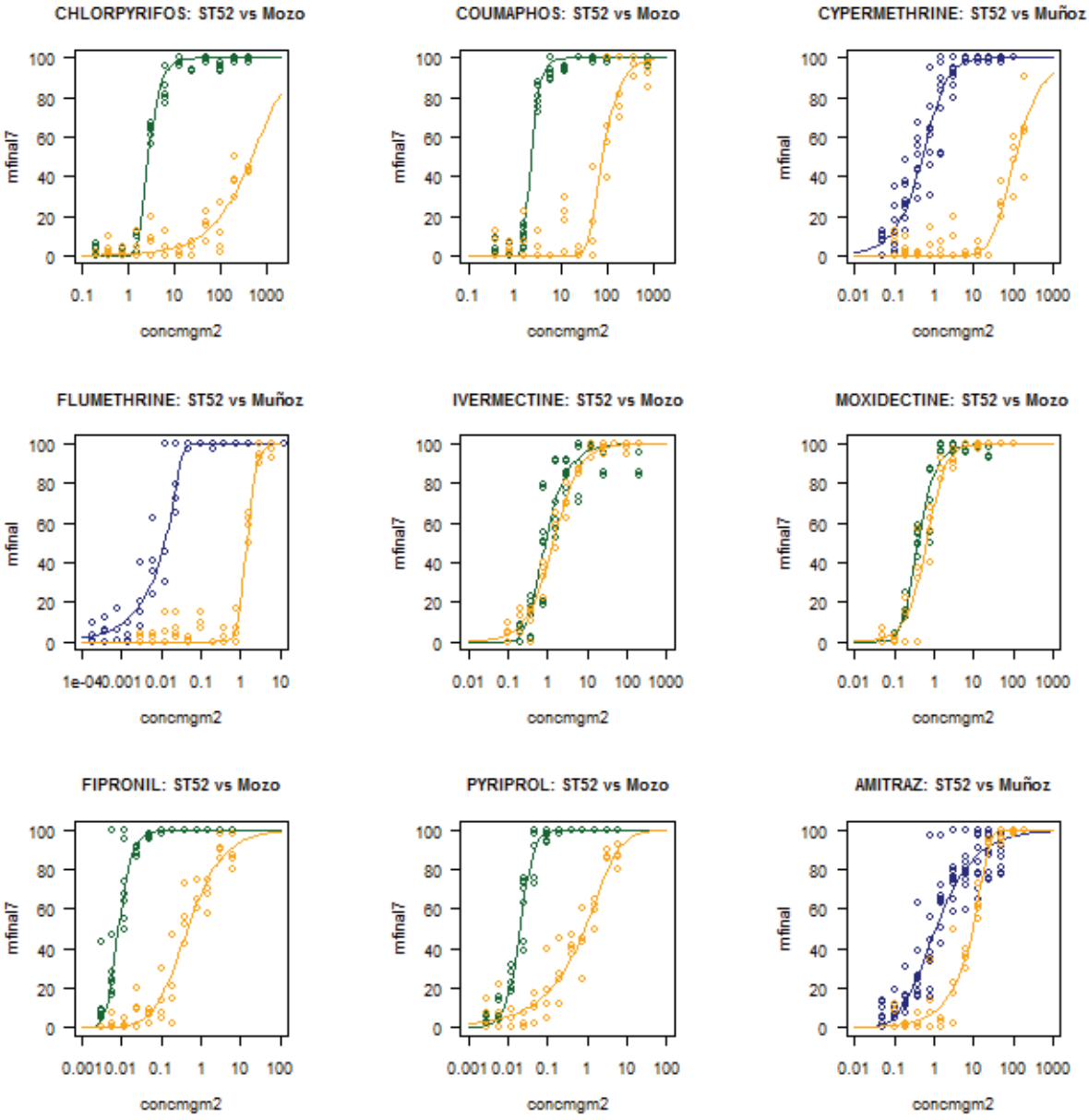


Fig. 11. Dose-response mortality obtained with the LTT for the Brazilian field population ST52 (orange) and for the susceptible Mozo strain (green) or Muñoz strain (blue).

Figure 12: ST53

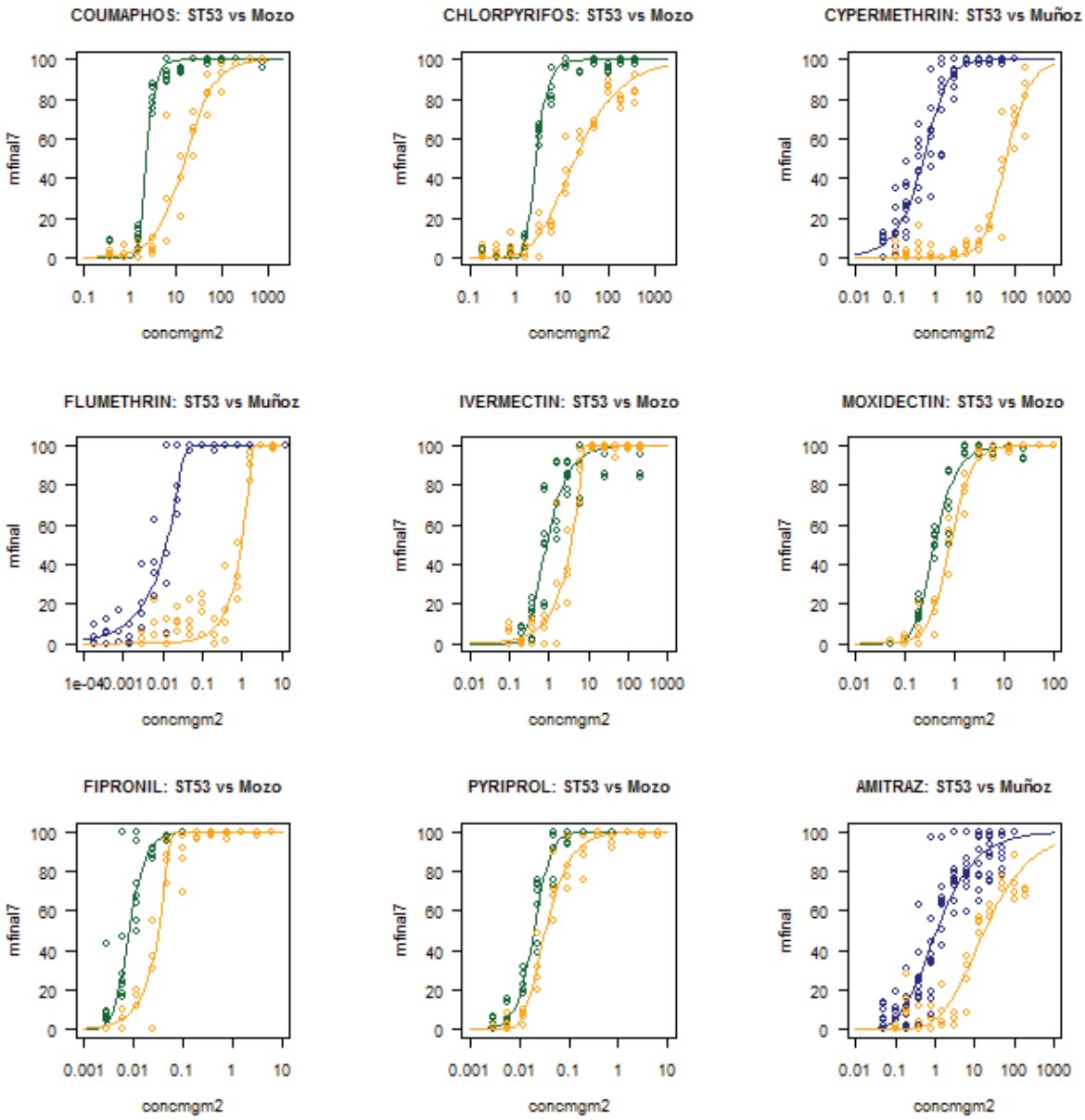


Fig. 12. Dose-response mortality obtained with the LTT for the Brazilian field population ST53 (orange) and for the susceptible Mozo strain (green) or Muñoz strain (blue).

Figure 13: ST55

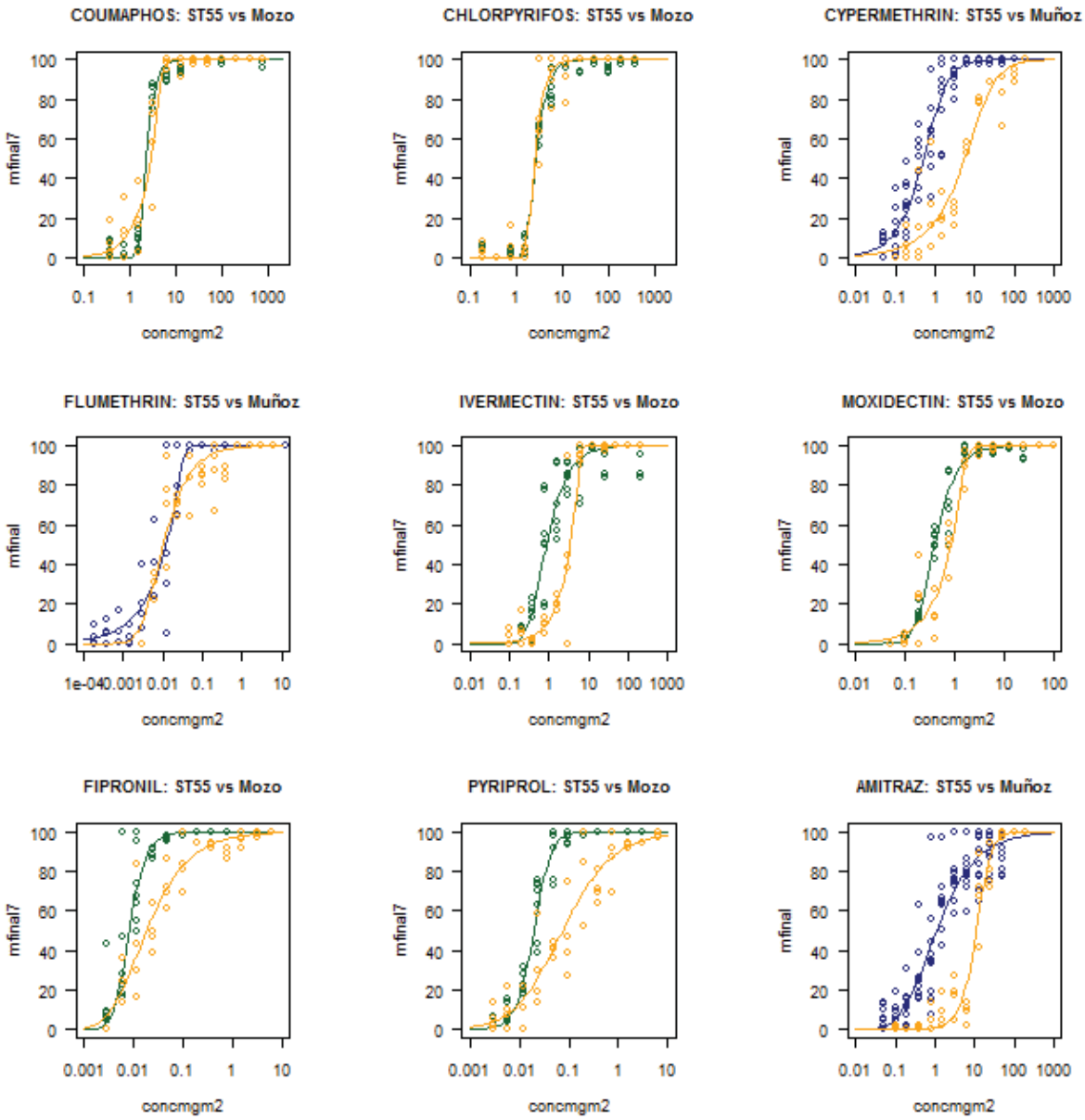


Fig. 13. Dose-response mortality obtained with the LTT for the Brazilian field population ST55 (orange) and for the susceptible Mozo strain (green) or Muñoz strain (blue).

Figure 14: ST57

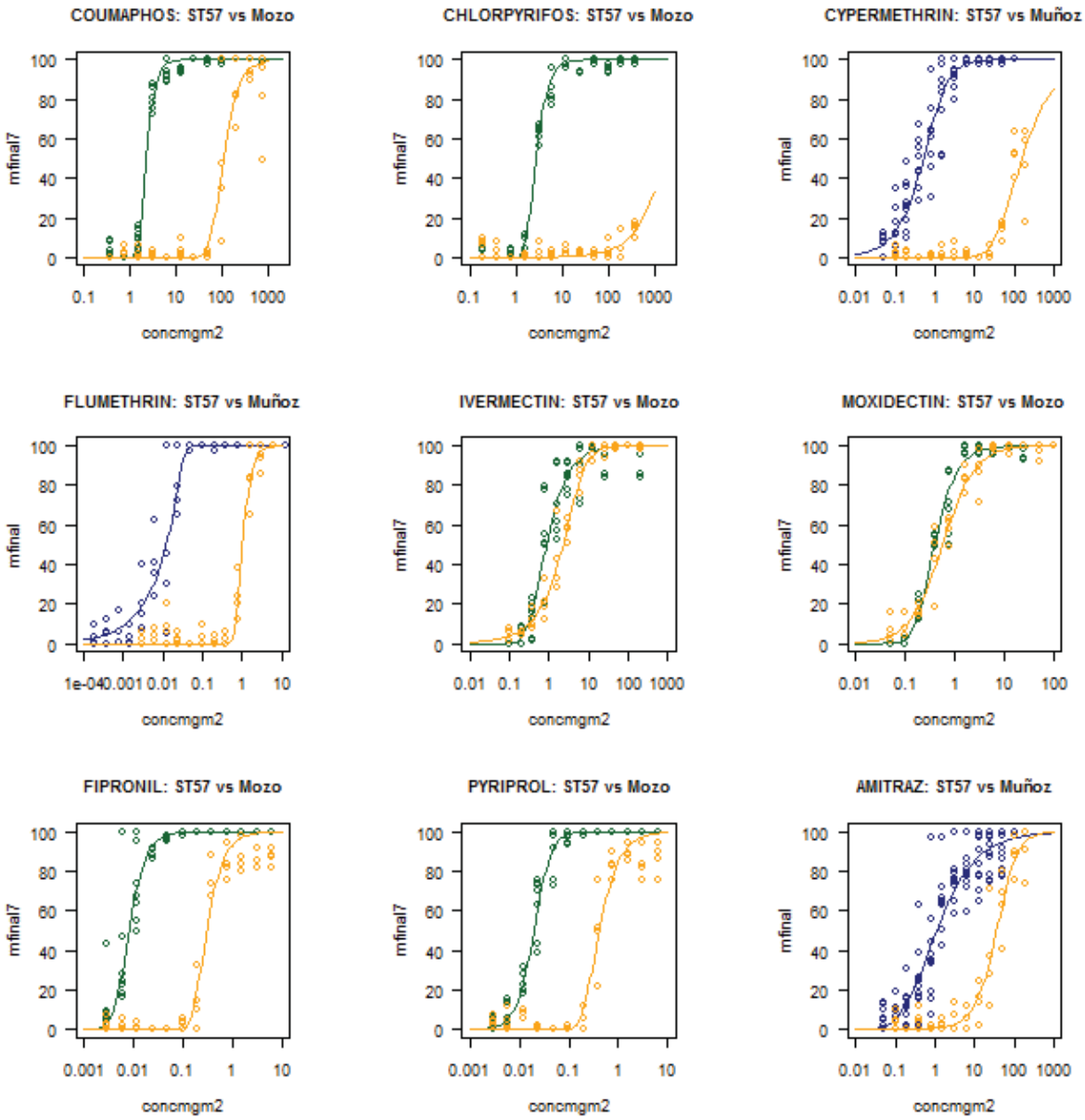


Fig. 14. Dose-response mortality obtained with the LTT for the Brazilian field population ST57 (orange) and for the susceptible Mozo strain (green) or Muñoz strain (blue).

Figure 15: ST58

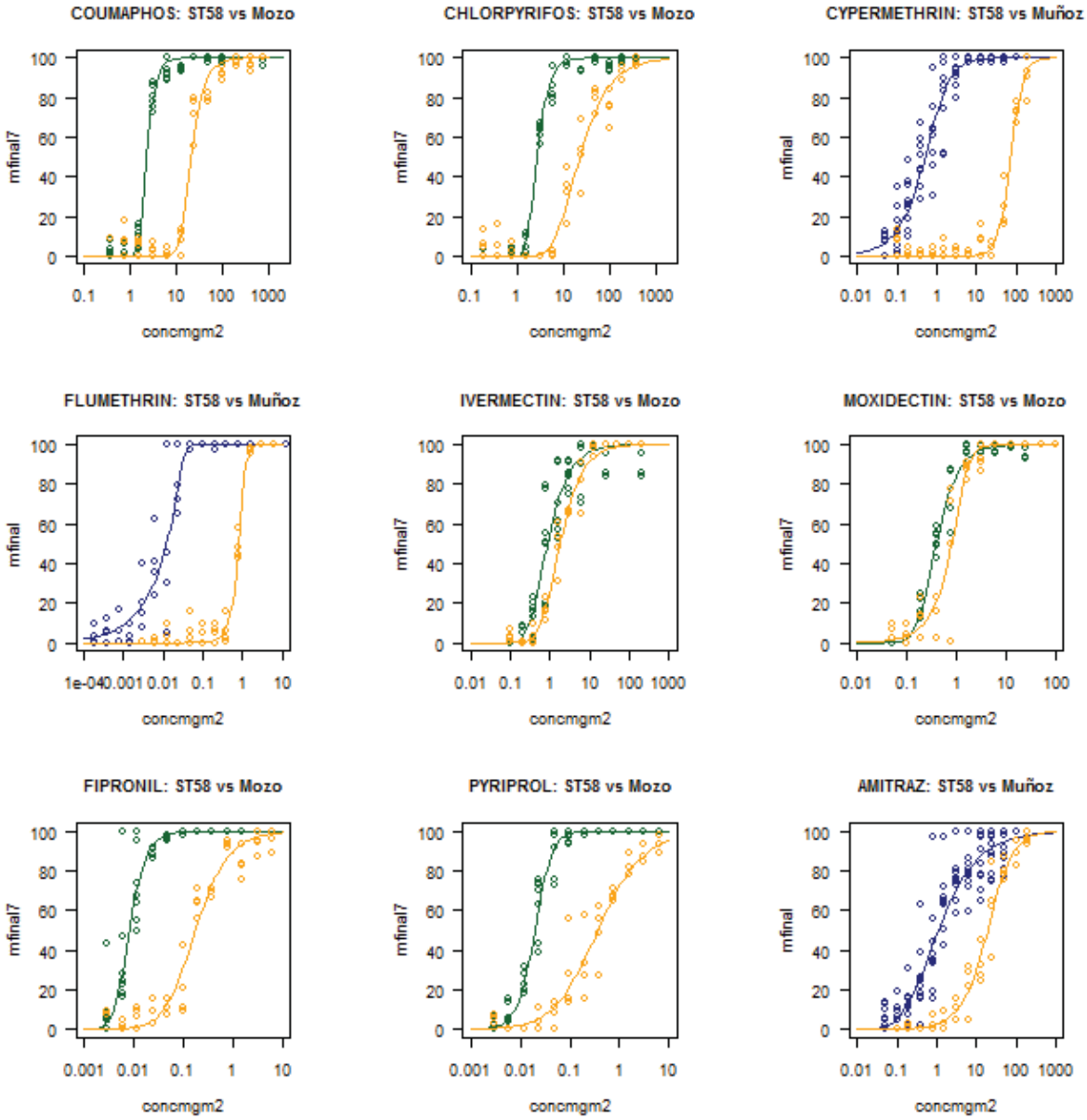
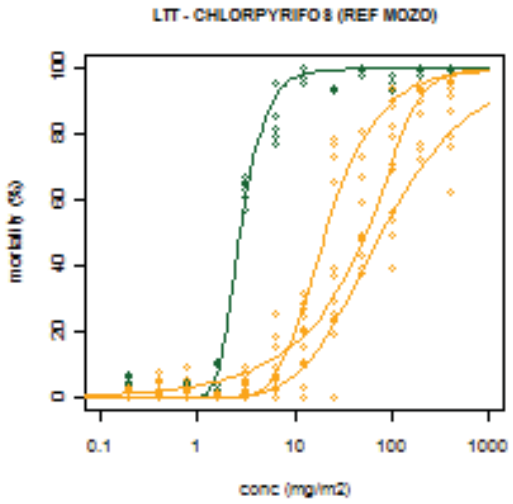
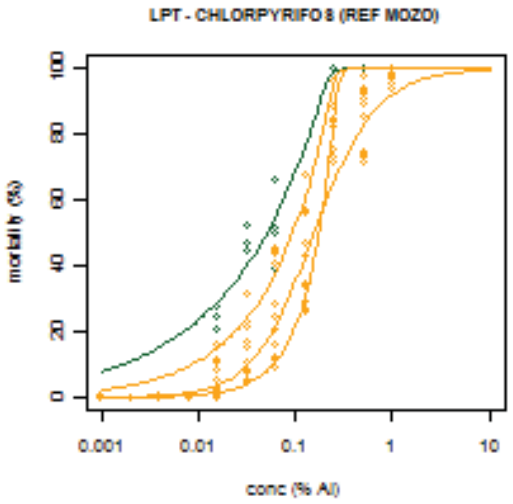
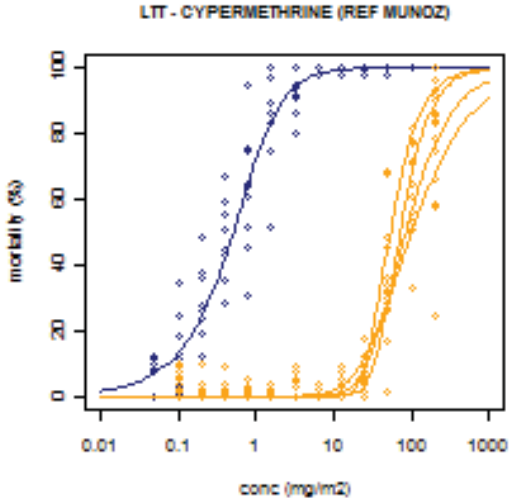


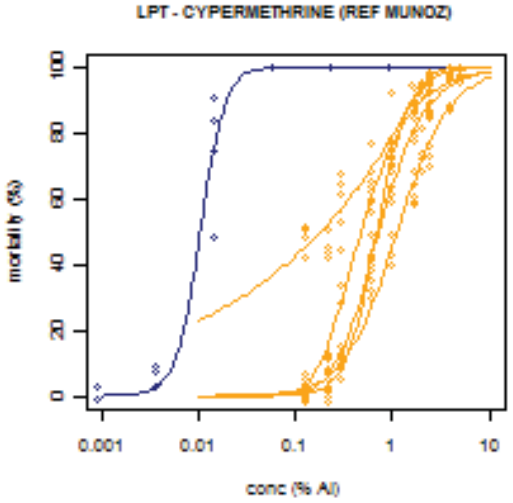
Fig. 15. Dose-response mortality obtained with the LTT for the Brazilian field population ST58 (orange) and for the susceptible Mozo strain (green) or Muñoz strain (blue).

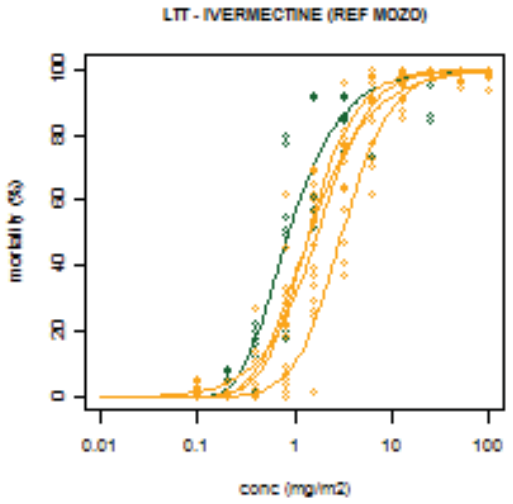
8.10. Appendice 10: Comparison of the dose-response mortality obtained with the LPT and the LTT (Brazilian populations)

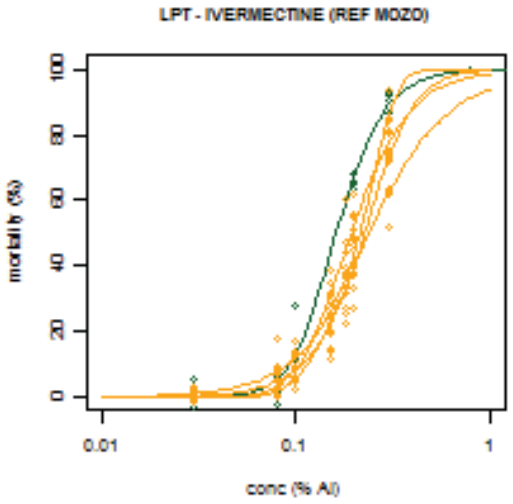












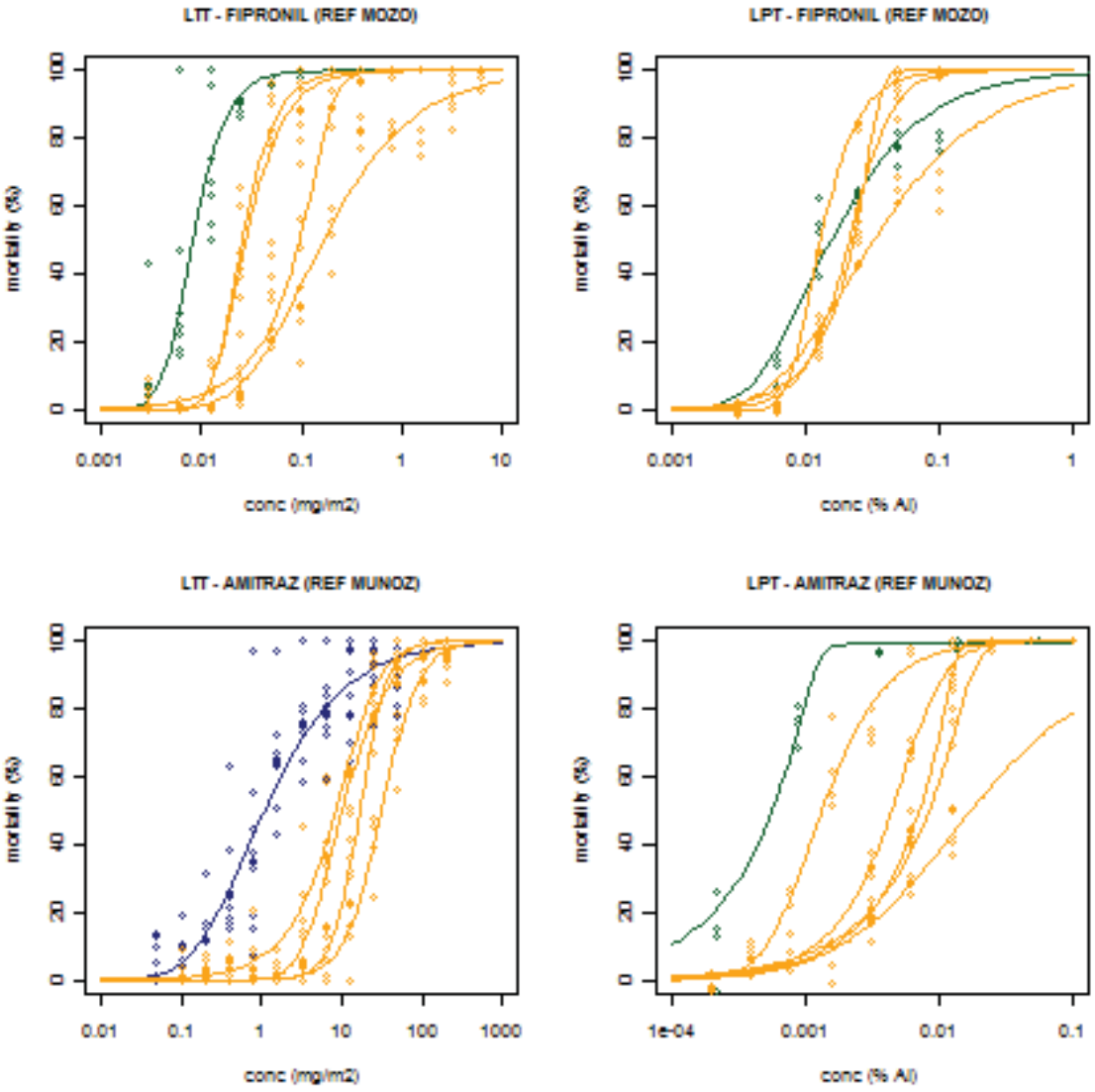


Figure 1: Dose-response mortality obtained with the LTT (left) and the LPT (right) for the four Brazilian field population (ST40, ST41, ST41, ST44) (orange) and for the susceptible Mozo (green) or Muñoz strain (blue).

8.11. Appendice 11: Dose-response mortality obtained with the LTT for the USDA strains

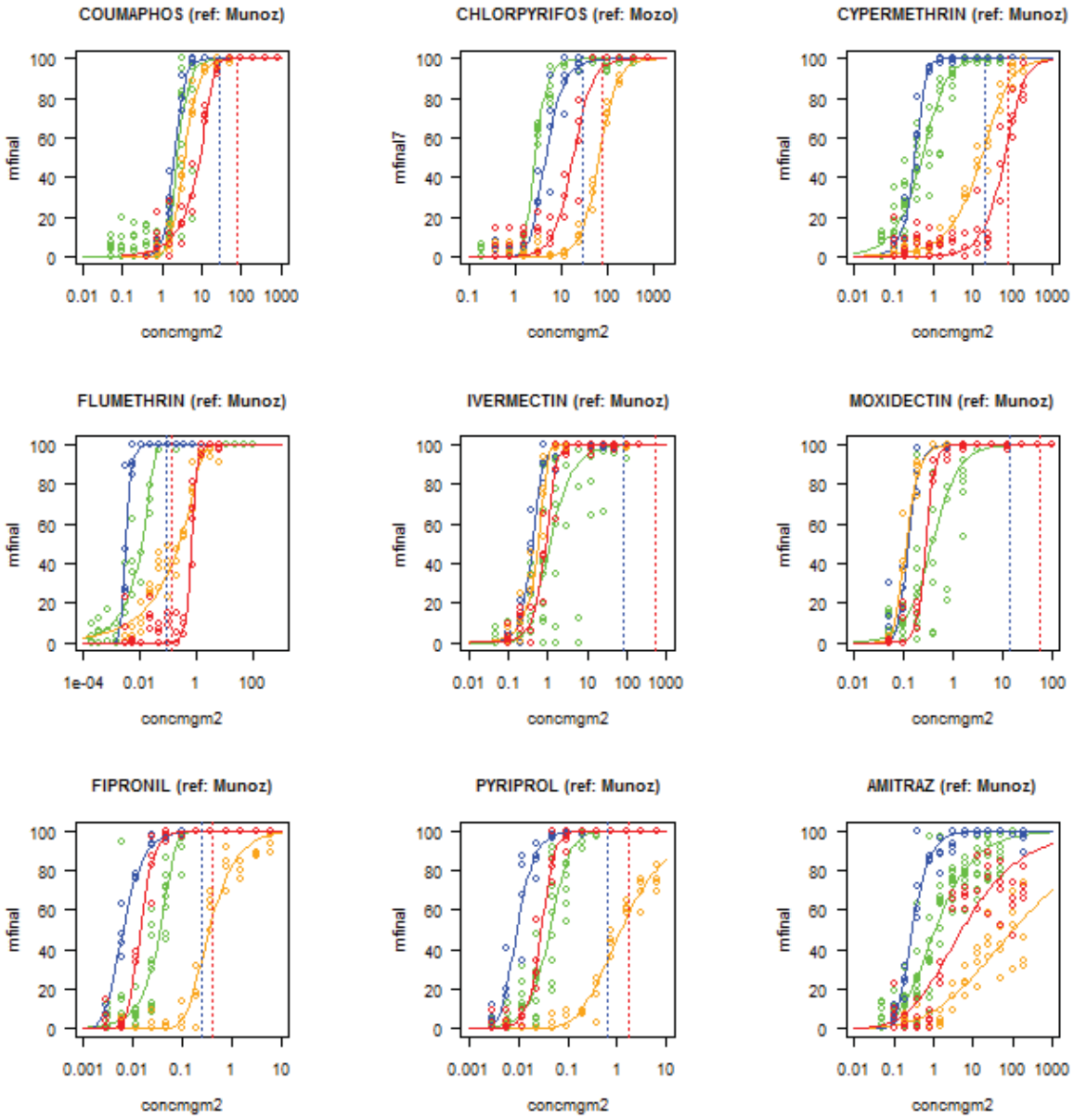


Figure 1: Dose-response mortality obtained with the LTT for the USDA strains vs Munoz (green): Deutch (blue); Fipronil resistant strain (orange); Santa Luiza (red). Dashed lines indicate the concentrations corresponding to 2x LD_{99} (blue) and 2x $LD_{99.9}$ (red) of the susceptible strain.