DETERMINING RESISTANCE LEVELS AND MECHANISMS IN *RHIPICEPHALUS SANGUINEUS* (LATREILLE) TO ACARICIDES USED TO TREAT DOMESTIC ANIMALS AND RESIDENTIAL ENVIRONMENTS

By

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To my family and friends, I am forever grateful for your support

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TABLE OF CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AI	Active ingredient
CDC	Center for Disease Control
DEM	Diethyl maleate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FAO	Food and Agriculture Organization
GABA	Gamma-aminobutyric acid
GST	Glutathione *S*-transferase
KCl	Potassium chloride
LC	Lethal concentration
LD	Lethal dose
LPT	Larval packet test
MSF	Mediterranean spotted fever
PBO	Piperonyl butoxide
PPM	Parts per million
RR	Resistance ratio
RMSP	Rocky Mountain spotted fever
SR	Synergist ratio
TCE	Trichloroethylene
TPP	Triphenyl phosphate

Abstract of Dissertation Presented to the Graduate School
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DETERMINING RESISTANCE LEVELS AND MECHANISMS IN *RHIPICEPHALUS SANGUINEUS* (LATREILLE) TO ACARICIDES USED TO TREAT DOMESTIC ANIMALS AND RESIDENTIAL ENVIRONMENTS

By

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Chair: Phillip Kaufman
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From September 2010 until October 2013 studies were conducted to evaluate the presence and levels of resistance to permethrin and fipronil in *Rhipicephalus sanguineus* (Latreille), the brown dog tick. Permethrin is a commonly used acaricide for tick control on domestic animals and in residential environments and fipronil for on-animal treatment. Field-collected ticks from kennels and residential facilities were challenged with technical grade permethrin and fipronil to create dose response curves, which were compared to a susceptible strain. Permethrin resistance was screened in seven populations, all of which were resistant with a resistance ratio greater than 10. Fipronil was tested on four populations, which were found to be tolerant, with resistance ratios below 3.8. This is first documentation of *R. sanguineus* permethrin resistance and fipronil tolerance in the United States.

An acaricide-susceptible *R. sanguineus* strain was used to develop a discriminating concentration, which is a single acaricide concentration that can quickly and affordably diagnose resistance in newly encountered field populations. The discriminating concentration, 2x LC_{99}, was used to screen field-collected tick strains.

Permethrin resistance of up to 53-fold was found in Florida collected populations. Fipronil discriminating concentrations were lower, with tolerance found at 2-fold the susceptible strain.

Resistant *R. sanguineus* were evaluated for acaricide resistance mechanisms. Synergists evaluated included those that inhibit esterase, cytochrome P450, and glutathione-*S*-transferase activity. Esterase was the most prominent metabolic resistance mechanism in permethrin resistant ticks. Cytochrome P450 activity was determined to play a likely role in permethrin resistance. Glutathione-*S*-transferase inhibition yielded the smallest levels of change in mortality, suggesting this mechanism was not amplified in the resistant ticks. None of the experiments demonstrated high levels of change in mortality when synergists were combined with fipronil. Using gel electrophoresis, esterase activity was further confirmed as a mechanism for permethrin resistance. Twelve permethrin-resistant strains were tested and increased esterase activity was confirmed in eight strains. These results will be used to guide future brown dog tick control management plans.

CHAPTER 1
LITERATURE REVIEW OF THE BROWN DOG TICK *RHIPICEPHALUS SANGUINEUS* (LATREILLE)

The brown dog tick, *Rhipicephalus sanguineus* (Latreille), is found worldwide as a common ectoparasite of dogs, *Canis lupus* L. (Goddard 1989). It is taxonomically classified in: Kingdom: Animalia, Phylum: Arthropoda, Subphylum: Chelicerata, Class: Arachnida, Order: Ixodidae, Family: Amblyommidae, Subfamily: Rhipicephalinae, Tribe: Rhipicephalini (El-Sayed 2012).

Taxonomy

The order Ixodidae consists of hard ticks that are characterized by the presence of an anal groove, a scutum, and ticks pass through only one nymphal instar. Ixodidae feed on a broad range of hosts including reptiles, birds, and mammals and have an extensive geographic range from tropical to temperate zones (Klompen et al. 1996). Ixodidae currently has 702 described species within 14 genera, including *Rhipicephalus* (Guglielmone et al. 2010).

Guglielmone et al. (2010) placed 82 species within the genus *Rhipicephalus*. This genus is found worldwide and can be characterized by a hexagonal basis capituli (Nicholson et al. 2009). Several species of medical and veterinary importance are classified under *Rhipicephalus*, including the species *R. sanguineus, Rhipicephalus appendiculatus* Neumann*, and Rhipicephalus bursa* (Canestrini and Fazago)*,* and the five tick species that were formally classified under *Boophilus*. Former *Boophilus* ticks include *Rhipicephalus annulatus* (Say, 1821), *Rhipicephalus decoloratus* Koch, *Rhipicephalus geigyi* Aeschlimann and Morel, *Rhipicephalus kohlsi* (Hoogstraal and Kaiser), and *Rhipicephalus microplus* (Canestrini) (Guglielmone et al. 2010).

Boophilus has been reclassified with *Rhipicephalus* as a paraphyletic group (Murrell and Barker 2003). Murrell et al. (2003) used the significant similarities between the genera in the 12S rRNA mitochondrial and the cytochrome *c* oxidase subunit I gene sequences as a basis for reclassification. Alternatively, Beati and Keirans (2001) suggest that *Boophilus* is a monophyletic group that arose out of *Rhipicephalus*. However, biogeographical distribution and morphological characteristics provide additional supporting evidence for classifying *Boophilus* and *Rhipicephalus* in the same genus (Murrell and Barker 2003). Differences between former *Boophilus* and *Rhipicephalus* ticks include biological parameters such as that *Boophilus* ticks are one-host ticks whereas *Rhipicephalus* are mostly three-host ticks, with a few two-host tick species (Murrell and Barker 2003). The relatedness of these former tick genera is important to understand because most of the comparative research in my dissertation with *R. sanguineus* has been completed with *R. microplus.*

Within *Rhipicephalus, R. sanguineus* recently has been genetically compared to assess potential speciation due to morphological, biological, and genetic variations found in populations from different regions (Oliveira et al. 2005, Szabó et al. 2005). Moraes-Filho et al. (2011) used mitochondrial 16S rDNA from *R. sanguineus* collected in different regions of the world in their assessment of speciation. Authors found that New World *R. sanguineus* can be separated into two species, and there may be a species unique to Old World regions as well. Nomenclature to distinguish these different species has not been determined to date.

Rhipicephalus sanguineus crossbreeding experiments were conducted to evaluate the reproductive success of populations from different parts of the world. Levin

et al. (2012) mated populations of *R. sanguineus* from North America, the Mediterranean, and Africa. When the African cohort was mated with the North American or Mediterranean cohorts the resultant progeny were infertile. The North American and Mediterranean populations were able to be mated without impacting the fecundity of the offspring (Levin et al. 2012). This suggests a speciation event between the African and both the North American and Mediterranean populations. Lui et al. (2013) compared the mitochondrial genome of *R. sanguineus* from China and the United States. Authors reported an 11.23% difference in the sequences, suggesting these populations of ticks are closely related species, rather than the same species.

Life History

Dogs are the preferred host for *R. sanguineus*, but cases of human parasitism have been documented (Goddard 1989, Demma et al. 2005). *Rhipicephalus sanguineus* is a three host tick that can complete its entire life cycle indoors and due to this capability, it may infest homes (Demma et al. 2005, Dantas-Torres et al. 2006). Pesticide resistance in tick populations has increased the difficulty of controlling *R. sanguineus* infestations (Miller et al. 2001). Due to the small size of the brown dog tick, an infestation may go undetected for extended periods of time. Female brown dog ticks have been known to oviposit up to 4,000 eggs under laboratory conditions (Koch 1982). Reproduction rates of this magnitude can lead to a severe infestation in a residential environment. Additionally, brown dog ticks spend 94 to 97% of their time off the host, often within cracks and crevices, further confounding detection (Needham and Teel 1991).

Although humans are not the preferred host, human attachment by *R. sanguineus* has been documented (Goddard 1989, Demma et al. 2005). *Rhipicephalus*

sanguineus also has been documented to feed on small mammals, including rodents, under various conditions (Rhodes and Norment 1979). Nelson (1969) demonstrated that *R. sanguineus* has a decreased preference for feeding on human hosts when compared to dogs and guinea pigs. When *R. sanguineus* were placed on humans, 5% or fewer remained attached after 24 hours, whereas 20% or more remained attached to dogs and guinea pigs, demonstrating a preference for nonhuman hosts (Nelson 1969).

Several more recent studies have indicated that some strains of *R. sanguineus* may be developing a greater affinity for human feeding. When surveying Air Force bases in Texas and Oklahoma between May 1986 and September 1988, Goddard (1989) documented nine cases of human parasitism by brown dog ticks. Goddard suggested the ticks were adapting to humans as hosts based on the increased number of cases documented during his study, compared to the number of historically reported cases. A surveillance study conducted by Carpenter et al. (1990) documented nine additional cases of human parasitism by brown dog ticks, with the majority occurring in the same geographical region in which Goddard (1989) conducted his surveillance.

In addition to the human-assisted brown dog tick range expansion that has occurred over the centuries, the climate change hypothesis predicts changes in their distribution range (Gray et al. 2009). Currently, the brown dog tick is rarely found in temperate regions but has been known to establish short-term populations because it can live indoors and will thrive anywhere that is heated (Gray et al. 2009). In laboratory studies, brown dog ticks had the greatest longevity at 10°C but temperatures <10°C were not well tolerated (Sweatman 1967). Engorge females were incapable of ovipositing at ≤15°C. Dantas-Torres et al. (2010) found brown dog tick eggs held at 8°C

were unable to survive. It is predicted that an average climatic temperature increase of 2 to 3°C would promote long-term establishment in temperate zones (Gray et al. 2009).

The optimal conditions for brown dog tick reproduction and survival varies. Srivastava and Varma (1964) reported 99% survival of brown dog tick eggs at 25°C and 100% RH when compared to 80% survival at 29°C and 80% RH. At higher temperatures, eggs hatched more quickly and time to larval molt was reduced, but survival for unfed larvae decreased from four months at 25°C and 100% RH to two months at 29°C and 80% RH. Nymphs required a blood meal around 4 days after emergence and had increased feeding rates on rabbits compared to guinea pigs. Adult unfed females and males held at 25°C and 80% RH were able to survive unfed for 7 months and 4 months, respectively. Data were not given for survival at 29°C.

Mated adult females held at 25°C began feeding 7 days after molting and dropped off the host within 7 to 15 days (Srivastava and Varma 1964). Male brown dog ticks require a blood meal before they can reach sexual maturity and respond to female attractant hormones (Kiszewski et al. 2001). The male brown dog tick also requires a blood meal between multiple mating opportunities and will stay on a host where it will mate multiple times with females are present. When females are not present on a host, males have been shown to switch hosts, increasing the probability of pathogen transmission (Little et al. 2007).

The presence of tick feeding on a host may attract other ticks. Guanine, a pheromone present in tick excrement, has been found to induce aggregation, regardless of the concentration present (Yoder et al. 2013). Tick attraction was greatest under 33% RH, which is within the humidity range of a typical home.

Pathogen Transmission

The brown dog tick has been shown to vector several canine pathogens, including *Babesia spp., Rickettsia spp.*, and *Ehrlichia spp.*, and may be involved in the transmission of *Leishmania chagasi* (Murphy et al. 1998, Coutinho et al. 2005, Demma et al. 2005, Oyamada et al. 2005). Although the pathogens typically impact the dog's health, occurrence of *Ehrlichia canis* and *Rickettsia rickettsii* in humans suggests that these pathogens are zoonotic and that the tick may pose a risk to humans (Maeda et al. 1987, Perez et al. 1996, Demma et al. 2005). Multiple pathogens can exist simultaneously in dogs, but such a circumstance would be less likely to occur in humans, as they infrequently are parasitized by multiple ticks and multiple tick species (Breitschwerdt et al. 1998, Kordick et al. 1999).

Babesia spp. are protozoan parasites that include species that cause species-specific forms of babesiosis in many mammalian species. Symptoms of babesiosis in dogs may include fever, weakness, lethargy, jaundice, red or orange urine, anemia, anorexia, and organ failure (Reviewed by Wulansari et al. 2003). In immunocompromised animals the symptoms are generally more severe. In Sudan, a prevalence survey of 78 dogs was conducted wherein 44 dogs were found infested with *R. sanguineus* (Oyamada et al. 2005). Five dogs tested positive for *Babesia canis rossi* and two dogs with *Babesia canis vogeli*. In humans, the general symptoms of babesiosis include fever, chills, body aches, anorexia, nausea, and headache (Meer-Scherrer et al. 2004, CDC 2013). Babesiosis can cause more severe complications in people with compromised immune systems. However, few cases of *Babesia canis* have been documented in humans.

Rocky Mountain spotted fever (RMSF) is a medically-important disease caused by the bacteria, *Rickettsia rickettsii.* Although *R. sanguineus* is not the primary vector, *R. rickettsii* can be transmitted to humans by the brown dog tick (Demma et al. 2005). Clinical symptoms in humans may include fever, vomiting, nausea, headache, myalgia, and loss of appetite, arthralgia, rash, and possible gastrointestinal symptoms. Around 2,000 cases of RMSF were reported in 2010 in the United States (CDC 2013).

A few RMSF outbreaks have been linked to *R. sanguineus*. One research study identified 16 human cases in Arizona that likely were likely the result of feeding by the brown dog tick (Demma et al. 2005). Patients in all confirmed RMSF cases reported contact with *R. sanguineus*, with one patient having an actively feeding tick still attached. Researchers cultured the pathogen from *R. sanguineus* confirming the presence of *R. rickettsii*. Following the identification of the outbreak, a pathogen prevalence study of ticks in Arizona was conducted using a sample population of dogs and found five of 20 brown dog ticks harboring rickettsial DNA (Eremeeva et al. 2006). Vector competency studies are still needed to in order to verify transmission.

Internationally, *R. sanguineus* vectors the bacteria *Rickettsia conorii* that causes Mediterranean spotted fever (MSF), also known as boutonneuse fever (Segura-Porta et al. 1998). Mediterranean spotted fever occurs throughout Europe, Asia, and Africa (Mumcuoglu et al. 1993). The distribution of *R. sanguineus* has expanded to northern regions of Europe, including the Netherlands, increasing the range of clinical cases observed (Beugnet and Marie 2009). Prevalence surveys in southwest Spain have found *R. sanguineus* to be a vector of *Rickettsia massiliae*, which is believed to cause symptoms similar to MSF (Marquez et al. 2008). In southeastern Spain, Segura-Porta

(1998) sampled serum from 150 asymptomatic human patients and found that 12 had been exposed to MSF, likely due to feeding by *R. sanguineus*. In parts of Israel up to 7% of dog owners tested positive for MSF, with 90% of dogs seropositive for *Rickettsia* (Mumcuoglu et al. 1993). These case reports demonstrate the importance of *R. sanguineus* as a pathogen vector.

Surveillance studies conducted by Goddard (1989) and Carpenter et al. (1990) found that more cases of human parasitism by *R. sanguineus* occurred in children than in adults. Factors contributing to differences in parasitism rates could include children having awareness of the tick-feeding or that children may come into closer contact with ticks due to behavioral differences and closer interaction with animals. Because children have less developed immune systems, there is a greater concern for pathogens causing more severe illness.

Ehrlichiosis is a disease, caused by several species of bacteria, which can be vectored by *R. sanguineus*, as well as several other tick species. Under experimental conditions brown dog tick nymphs acquired *Ehrlichia canis* and successfully transmitted the pathogen to a second host as adult males, thus demonstrating transstadial transmission (Bremer et al. 2005). The brown dog tick also was able to transmit *E. canis* intrastadially under experimental conditions where males acquired the pathogen from one host and were subsequently introduced to a second host, whereupon they transmitted the pathogen. This study documented a variation in symptoms from canines that were inoculated via syringe with *E. canis* over those exposed from tick feeding. The symptoms of the canines that contracted the pathogen from the ticks were

less severe than the symptoms experienced by the canines that were inoculated (Bremer et al. 2005).

Ndip et al. (2007) conducted a study in Cameroon to determine if *R. sanguineus* could transmit *E. canis, Ehrlichia ewingii,* and *Ehrlichia chaffeensis*. They collected 92 brown dog ticks from a survey of 104 dogs, of which 51 dogs presented with *R. sanguineus*. It was determined that 30% of these ticks harbored ehrlichial DNA, including all three species. Two of these positive ticks were co-infected with two *Ehrlichia* species. Of the dogs infested with *R. sanguineus, E. canis* was the predominant pathogen with 15 dogs*,* two ticks were positive for *E. ewingii*, while all of the dogs were negative for *E. chaffeensis,* (Ndip et al. 2007).

Dogs have been documented as a likely reservoir for several species of ehrlichiosis (Ristic et al. 1988, Unver et al. 2001, Liddell et al. 2003). Brown dog tick transmission of *Ehrlichia* spp. is becoming more frequently reported in human populations (CDC 2013). Symptoms that are seen in humans include fever, chills, headache, myalgia, diarrhea, and sensitivity to light in some human patients (CDC 2013). Typically, human infections of *Ehrlichia* spp. are vectored by the lone star tick, *Amblyomma americanum* (L.), but *R. sanguineus* has been found to harbor *E. canis,* and *E. ewingii* (Murphy et al. 1998). However, the vector competence of the brown dog tick for *E. ewingii* has not been determined.

Ehrlichia ewingii and *E. chaffeensis* are the two *Ehrlichia* that most frequently impact human populations, while human infections with *E. canis* have been documented, but only rarely (Maeda et al. 1987, Perez et al. 1996). In human populations, *E. chaffeensis* was identified during the early 1990's (Anderson et al.1991)

and *E. ewingii* in the late 1990's with four confirmed cases of *E. ewingii* in Missouri (Buller et al. 1999). Surveillance data collected by the Centers for Disease Control and Prevention have shown a rise in human ehrlichiosis cases in the United States from 100 to over 700 between the years 1999 to 2010 (CDC 2013). It is possible that zoonotic transmission is a contributing factor to the rapid rise in the frequency of human cases, as proposed with the rickettsial pathogens. Additionally, improved serological testing methods and increased awareness may aid in indentifying cases, thereby generating higher prevalence data. Therefore, it is important to reduce the potential risk of pathogen transmission to dogs by *R. sanguineus* through monitoring and controlling their populations.

Prolonged tick feeding increases the likelihood of pathogen transfer to potential hosts. Piesman et al. (1987) compared exposure time on a host with *Borrelia burgdorferi* transmission by *Ixodes scapularis* Say and found that while 92% of rodents became infected with *B. burgdorferi* after 72 hours of tick attachment, only 7% pathogen transmission occurred in hosts when ticks were removed 24 hours after initial contact. Therefore, feral or outdoor dogs could potentially harbor multiple pathogens if prompt physical removal of ticks or treatment with an acaricide is not performed.

Host Immunity

Some hosts that are subject to multiple-feedings by ticks have been documented to elicit an immune response. Trager (1939) found a decrease in the number of American dog tick, *Dermacentor variabilis* (Say), larvae that were fully engorged after feeding on a guinea pig with previous tick feeding exposure. Additionally, the immune response from the host was non-specific and provided some protection against other tick species. Ferreira and Silva (1998) found that guinea pigs developed a protective

immune response to tick-feeding after consecutive feedings from *R. sanguineus*. However, mice and dogs did not have the same protective immune reaction. Ali and Sweatman (1966) found that increased estrogen levels in laboratory mice provided protection against brown dog tick larvae. They also determined that increased cortisone levels lead to increased larval attachment rates. Cortisone, a steroid hormone, reduces the immune system response, possibly allowing ticks to host-feed for a longer time period. Ali and Sweatman (1966) also found that larval brown dog ticks that were fed on immature male mice had a greater number of engorged ticks and higher rates of survival than those fed on older mice and female mice. Male mice that were treated with estrogen became more resistant to tick attachment, indicating female hormones may provide some protection against tick feeding.

Other vertebrate hosts have been documented to develop an immunity to tick feeding after a blood meal has been taken. Randolph (1979) compared the success of larval and nymphal *Ixodes triangulicepts* Birula, feeding capability on their natural hosts *Apodemus sylaticus* L., (wood mouse), and laboratory reared mice. The laboratory reared mice developed an immunity and ticks failed to attach after exposure to approximately 10 ticks. In comparison, ticks feeding on *A. sylvaticus* did not demonstrate a change in tick survival or attachment was observed, suggesting the lack of an immune response (Randolph 1979).

Canine vaccine development has been hampered by the lack of an immune response exhibited by dogs following multiple exposures to *R. sanguineus*. Dogs were inoculated with brown dog tick concentrated crude proteins using three subcutaneous injections at 15 day intervals (Bechara et al. 1994). Dogs did not demonstrate any level

of resistance to *R. sanguineus* feeding and introduced ticks did not show any change in reproductive and feeding performance. Later studies isolated gut extract proteins and inoculated dogs using the same immunization schedule as the previous study (Szabó and Bechara 1997). Gut extract immunizations yielded some decrease in tick engorgement and fecundity when paired with a proper adjuvant. Jittapalapong et al. (2000) inoculated dogs with *R. sanguineus* salivary gland or midgut immunizations three times at 21 day intervals. With their formulation they found a decrease in feeding and engorgement with salivary gland extracts and a decrease in fecundity factors from midgut extract immunizations. Rodríguez-Mallon et al. (2012) used a novel *R. sanguineus* protein that was formulated in a vaccine and tested it on rabbits. They found that ticks that fed on vaccinated rabbits demonstrated a decrease in the size of molting larvae and nymphs, as well as reduced reproductive success. Continued studies on novel proteins and vaccine development are ongoing.

Biological Control

Several species of entomopathogenic fungi have been evaluated as biological control agents for ticks. This group of fungi effectively penetrates the arthropod cuticle and does not require ingestion. The enzymes that break down the cuticle are produced less than 24 hours after initial contact and perform similarly with insects and ticks (St. Leger et al. 1986). Cuticle breakdown is initiated by esterases and the proteolytic complexes such as protease, aminopepidase, and carboxypeptidase, followed by chitinases. Mortality is caused by cuticular penetration that damages the tick exoskeleton and the hyphal bodies spread throughout the arthropod body (Hajek and St. Leger 1994).

Beauveria bassiana (Balsamo) Vuillemin and *Metarhizium (anisopliae) brunneum* (Petch) were evaluated as biological control agents for *Rhipicephalus appendiculatus* Neumann and *Amblyomma variegatum* (Fabricius) that were actively feeding on rabbits. Kaaya et al. (1996) found both fungal species resulted in 30% greater mortality in adults ticks. Adult ticks also demonstrated decreased fecundity, engorgement weight, egg mass, and egg eclosion rates (up to 99% under both field and laboratory conditions). In laboratory experiments, ticks were place in closed stockings around rabbit ears. After two days ears were sprayed with a spore suspension. Under field conditions naturally tick-infested cattle were sprayed with a spore suspension under an ear sock. The exposure of adult *R. appendiculatus* to these fungal species resulted in 100% larval mortality out of 900 ticks, 76 to 95% nymphal mortality with 600 ticks, and 36 to 64% mortality out of 600 adult ticks. *Beauveria bassiana* and *M. brunneum* were not impacted by exposure to organophosphate acaricides for over 120 hours, therefore, chemical and biological control measures could be combined effectively. Although researchers did not indicate why the use of biological control was not commercialized, it was indicted that spores persisted on cattle for one week and three weeks for *B. bassiana* and *M. brunneum*, respectively.

Zhioua et al. (1997) found *M. brunneum* significantly increased larval and adult mortality of *I. scapularis*. A concentration of 10^6 fungal spores per mL resulted in significant rates of mortality. Gindin et al. (2001) found that *M. brunneum* was significantly more effective than *B. bassiana* in controlling the cattle tick. *Metarhizium brunneum* also effectively increased mortality in unfed and engorged larval, nymphal, and adult brown dog ticks (Samish et al. 2001).

The formulation of entomopathogenic fungi can impact mortality levels. Kaaya and Hassan (2000) found oil-based formulations of *M. brunneum* and *B. bassiana* were more effective in initiating adult tick mortality than were aqueous formulations. Spraying the formulations in the environment to control resting ticks can be more effective than host-based control measures. The efficacy and commercialization of entomopathogenic fungi depend on the development of effective formulations. Mishra et al. (2013) evaluated the effectiveness *B. bassiana* formulated as bait, encapsulated, and emulsion on *M. domestica*. They determined advanced emulsion and encapsulated formulas provided a fungal delivery system that could be effectively manufactured to provide persistent mortality.

Entomopathogenic nematodes have been used under laboratory conditions against several species of ticks, including the brown dog tick (Kocan et al. 1998). The nematodes cause mortality and tissue destruction when ticks develop an infection from bacteria released by the nematode. Although some nematode species caused mortality in 30 to 100% of ticks, their application in natural settings may be limited due to specific environmental conditions required to promote tick and nematode interactions. Moisture levels under laboratory conditions were critical for nematodes to be effective biological control agents, which would be difficult to ensure under field conditions (Kocan et al. 1998).

Biological control agents that target ticks specifically will minimize the impact of mortality on other non-target arthropods (Samish et al. 2004). However, biological control methods generally require more time to be effective. Efficacy is also highly dependent on environmental conditions (Samish et al. 2004). Finally, homeowners are

generally resistant to the concept of releasing additional organism in their home to control the target pest.

Chemical Control

On-Animal Control

Preventing and eliminating tick infestations frequently requires acaricide treatment. There are several commercially-available products designed to control ticks on companion animals including collars, shampoos, sprays, and spot-on treatments. Spot-on treatments are individually-packaged doses of acaricide that are administered topically between the shoulder blades. Some spot-on treatments for companion animals include K9 Advantix® II (Bayer Healthcare LLC, Animal Health Division, Shawnee Mission, KS) active ingredient (AI) permethrin 44%, imidacloprid 8.8%, pyriproxyfen 0.44%, Frontline® (Merial, Duluth, GA) AI fipronil 9.8%; Certifect® (Merial, Duluth, GA) AI fipronil 6.4%, (*S*)-methoprene 5.8%, amitraz 7.6%; Vectra 3D® (Ceva, Lenexa, KS) AI dinotefuran 4.95%, pyriproxyfen 0.44%, permethrin 36 or 58%; and Promeris® (Fort Dodge Animal Health, Overland Park KS) AI metaflumizone 14.34%, amitraz 14.34%. Acaricide impregnated collars on the market include Preventic® (Virbac, Carros, France) amitraz 9% and Seresto™ (Bayer AG, Shawnee Mission, KS) flumethrin 4.5%, imidacloprid 10%. The number of different acaricide active ingredients currently approved for use on companion animals is quite limited, leading to repeated use of products with the same chemical class.

Mehlhorn et al. (2003) tested K9 Advantix® on *Ixodes ricinus* (L.) and determined that this product was repellent to ticks, reducing tick attachment in all life stages, in addition to the acaricidal activity of permethrin on directly exposed ticks. Effects were measured with both *in-vitro* studies, in which hair samples were taken from treated dogs

and placed in Petri dishes with ticks, and *in-vivo* studies, in which adult ticks were placed on dogs previously treated with the K9 Advantix® product. In both studies, mortality was assessed following new tick placement at 7, 14, 21, and 28 days post-treatment (Mehlhorn et al. 2003). Nearly all exposed ticks died or were paralyzed and did not recover within a 48-hour period after exposure to either treatment regimen.

Frontline® was tested as a pour-on formulation on cattle at concentrations of 0.25, 0.5, and 1% fipronil and the index of reproduction was measured on *R. microplus* (Davey et al. 1998). Fipronil was 100% effective in killing ticks for 8 weeks at the 1% concentration, but at 0.5% AI, 100% protection lasted only 4 weeks (Davey et al. 1998). Bonneau et al. (2010) evaluated the efficacy of Frontline® and Effipro®, (10% fipronil wt/vol) (Virbac, Carros, France) spot-on treatments against *I. ricinus* infestations of dogs. Both products were effective within 48 hours of tick exposure, which is rapid enough to minimize transmission of *Borrelia burgdorferi*, the pathogen that causes Lyme disease (Piesman and Dolan 2002). Both Effipro® and Frontline® provided >90% protection against ticks for 5 weeks and 3 weeks, respectively (Bonneau et al. 2010).

Brianti et al. (2010) evaluated the efficacy and the effects of water on Frontline Plus® applications against all stages of *R. sanguineus*. Twenty-four dogs were treated according to manufacture recommendations. Ticks were counted on 7, 14, 21, and 28 days post-treatment. The ticks present were naturally infesting the dogs in an outdoor kennel. The mean tick load for the dogs prior to the study was approximately 150 per dog. In this study, one-half of the treated dogs were washed with detergent-free water on day 14, while the other dogs were not bathed. The efficacy of the product did not decrease following exposure to water and there was >90% protection against all life

stages during the 4-week trial period (Brianti et al. 2010). The effects of detergent were not evaluated.

When K9 Advantix® was compared with Frontline Plus® as a protectant against *R. sanguineus*, it was determined that the permethrin in K9 Advantix® was a more effective repellent for ticks than the fipronil in Frontline Plus® (Young et al. 2003). Additionally, Otranto et al. (2005) determined that the active ingredients in Frontline Plus® fell below effective levels at 28 days resulting in only 72% tick mortality. K9 Advantix® resulted in 98% mortality at 28 days post-treatment, and was considered more effective at inducing mortality in larval and nymphal *R. sanguineus*. Endris et al. (2000) also compared the efficacy of a 65% permethrin product (Defend®, Exspot® Treatment for dogs, Merck, Whitehouse Station, NJ) and a 9.7% fipronil product (Frontline®) against *I. ricinus*. The researchers found permethrin provided 87% mortality at 28 days post-treatment, while fipronil provided no more than 61% protection during the 41-day evaluation.

On dogs experimentally infested with *I. ricinus*, spot-on treatments containing 65% permethrin (Defend®) were compared against those containing selamectin (Revolution™, Pfizer Animal Health, New York, New York) (Endris et al. 2002a). Selamectin provided less than 10.9% repellency (percent live and dead ticks in the cage unattached to the dog) compared to the 63 to 80% repellency provided by permethrin. No acaricidal effects were measured with the use of selamectin, but control mortality was measured at 90% with the use of 65% permethrin. Jernigan et al. (2000) evaluated the use of selamectin as a spot-on treatment against *R. sanguineus* and *D. variabilis* on dogs and found effective control of experimentally-introduced ticks increased when the

spot-on treatment was administered every two weeks. With bi-monthly administration, 97 to 100% tick control for *R. sanguineus* and *D. variablis* was achieved; however, with once-a-month applications, control dropped to 90% in some dogs when tick counts were completed 5 days post-treatment. Greater than 95% tick control was achieved when treatment was applied for consecutive months, compared to treatment for one month alone that provided only 90% or greater tick mortality (Jernigan et al. 2000).

Promeris® (20 mg/kg dose metaflumizone and amitraz) efficacy studies on *R. sanguineus* demonstrated >90% tick control 35 days post-treatment on dogs (Rugg and Hair 2007). Promeris® was withdrawn from the market due to reports of pustular acantholytic dermatitis in dogs (Oberkirchner et al. 2011).

Amitraz formulated in collars has demonstrated excellent control against *R. sanguineus, I. ricinus,* and *Ixodes scapularus* Say in dogs (Estrada-Peña and Rème 2005). Three experimental groups were used: an untreated control collar, a 9% amitraz collar, and a 9% amitraz and 0.5% pyriproxyfen collar, each applied as one collar per dog. Collars with amitraz alone provided 94 to 97% protection against *R. sanguineus* and 85 to 96% protection in tested *Ixodes* species. However, surviving female ticks were able to successfully oviposit. Collars impregnated with 9% amitraz and pyriproxyfen provided up to 95 to 98% protection against *R. sanguineus* and 86 to 95% protection in tested *Ixodes* species. However, in this case, females that survived and oviposited did not produce viable eggs (Estrada-Peña and Rème 2005). Amitraz also has been found to have increased mortality against *R. sanguineus* when combined with fipronil (Prullage et al. 2011).

Spinosad is naturally derived from the aerobic fermentation of the bacterial species *Saccharopolyspora spinosa*. In a study evaluating systemic acaricide treatments, two dog groups were administered oral spinosad treatments containing either 50 or 100 mg/kg to determine efficacy against *R. sanguineus.* Tick numbers were reduced by 94 to 97% 24 hours post-treatment. Control of ticks remained effective up to nine days post-treatment and no difference in tick mortality was documented between the treatment and control group from 16 to 30 days (Snyder et al. 2009).

A few plant essential oils have been evaluated for *R. sanguineus* and *R. microplus* control, including oils derived from *Drimys brasiliensis* Miers, *Chaemaecyparis nootakensis* (D. Don) Spach, and *Calea serrata* Less (Ribeiro et al. 2008, 2011, Flor-Weiler et al. 2011). All three plant-derived essential oils were proven to be effective at causing mortality in larval tick populations in laboratory studies and may be the target of future research. To date no work has been published on the formulation of these essential oils for use in tick control and extracts have not been evaluated to determine how they will impact non-pest arthropods.

Burridge et al. (2004) conducted efficacy testing comparing several acaricides against *R. sanguineus* using contact bioassays. Fipronil provided the highest mortality followed by carbaryl, cyfluthrin, and permethrin. Pyrethrins had moderate effectiveness, and selamectin and chlorpyrifos killed the fewest *R. sanguineus*.

Residential and Kennel Facility Control

Pyrethroids are used heavily for residential and on-animal tick control. In the 1990s indoor pest control treatment recommendations included carbamates (bendiocarb, carbaryl, and propoxur) and organophosphates (chlorpyriphos, diazinon, and malathion), in addition to pyrethroids (St. Aubin 1990). After enactment of the 1996

Food Quality and Protection Act, most carbamate and organophosphate insecticide use was limited and pyrethroids became relied upon heavily (EPA 2013).

Most labels of pyrethroid products include indoor crack and crevice or spot-treatment applications, greatly increasing the number of products that ticks could contact. If the treatment site area is listed on the label it is a legal application even if ticks are not specifically listed in the “pest” section. However, several different pyrethroids have been formulated into products that include brown dog ticks specifically on the label. Examples include, Onslaught® microencapsulated insecticide (McLauglin Gormley King, Minneapolis, MN), AI esfenvalerate 6.4%, which is labeled for brown dog tick control at an end-use rate of 0.05%. Published research on esfenvalerate for tick control is unavailable, but studies have been conducted on resistance development in *Leptinotarsa decemlineata* (Say), the Colorado potato beetle. Huang et al. (1994) found a significant increase in LC_{50} values after repeat applications during the potato growing season, indicating resistance selection.

NyGuard® Plus Flea & Tick Premise Spray (McLauglin Gormley King, Minneapolis, MN), AI sumithrin 0.40%, and MGK 1.60%, a proprietary cytochrome P450 synergist, is used for indoor application to control brown dog ticks. Published resistance research on sumithrin-based products for ticks limited. However, sumithrin is also labeled for use against the human head louse, *Pediculus capitis* (DeGeer), and resistance development has been documented (Picollo et al. 1998).

Cy-kick® CS (Prescription treatment® brand, BASF, St. Louis, MO) AI cyfluthrin 6.0%, is formulated for use at 0.05% or 0.10%. Cyfluthin was evaluated for control of *Ixodes* (*scapularus*) *dammini* Spielman and *A. americanum* during a study that applied

the product to 35 field plots at 0.41% (Solberg et al. 1992). Adult and nymphal populations were reduced up to 87% using a granular formulation, two months post-treatment and up to 100% two months post-treatment, after liquid application. Cyfluthrin was evaluated in an *I. scapularis* a laboratory contact assay and demonstrated greater toxicity than esfenvalerate (Maupin and Piesman 1994). Resistance to cyfluthrin has been found in *Blattella germanica* (L.) and *Haematobia irritans* (L.) (Cochran 1989, Farnsworth et al. 1997). Contact assays were completed in *B. germanica* to evaluate cyfluthrin resistance in 45 field-collected strains within a few years after cyfluthrin's introduction to the marketplace (Cochran 1989). Of the 45 strains tested, one strain demonstrated a resistance ratio greater than three. Comparatively, four strains demonstrated resistance ratios greater than three for permethrin and fenvalerate. Although all three compounds are pyrethroids, the different chemical structures were suggested to cause variations in resistance. A survey evaluated 99 farms for cyfluthrin resistance in *H. irritans* using a discriminating concentration and found 40% mean mortality (Farnsworth et al. 1997). In this study, resistance was confirmed for all pyrethroids tested, which included deltamethrin, cyhalothrin, cypermethrin, and flumethrin, but cyfluthrin produced the lowest mortality. Kaufman et al. (2010) found permethrin and β-cyfluthrin resistance in five *M. domestic* populations collected from four Florida dairies. Two populations that were tested from the same farm, 22 months apart, demonstrated survival at higher concentrations of both permethrin and β-cyfluthrin during the latter collection date.

Pest control operators have reported indoor brown dog tick treatment protocols using indoor applications spraying or spot-treatments with pyrethroid-based products

including but not limited to Masterline® Bifenthrin 7.9, AI bifenthrin 7.9% (0.02 to 0.06% label rate; Univar®, Austin, TX), Kicker®, AI permethrin 6.0% (0.1% label rate) and piperonyl butoxide 60.0% (1.0% label rate) (Bayer Environmental Science, Research Triangle Park, NC), CB-80™, AI pyrethrins 0.5% and piperonyl butoxide 4.0% (FMC Corperation, Agriculture Product Group, Philadelphia, PA), NyGuard® Plus Flea & Tick Premise Spray, AI d-phenothrin 0.4%, N-octyl bicycloheptene dicarboximide 1.6%, and pyriproxyfen 0.1% (MGK®, Minneapolis, MN), ready-to-use, Prescription Treatment® brand Ultracide®, AI pyriproxyfen 0.1%, pyrethrins 0.05%, n-octyl bicycloheptene dicarboximide 0.4% and permethrin 0.4% (Whitmire Micro-Gen Research Laboratories, Inc., St. Louis, MO), and Precor 2000® Plus, AI (S)-methoprene 0.085%, permethrin 0.35%, phenothrin 0.3%, N-octyl bicycloheptene dicarboximide 2.0% and piperonyl butoxide1.4%, ready-to-use, (Wellmark International, Schaumurg, IL). Many of these products are labeled to treat other indoor pests such as German cockroaches and fleas, which may lead to residual amounts of pesticides present in the home prior to treatment for brown dog ticks. Residual levels of pesticides may induce increased metabolic enzymes, in turn conferring resistance (Yu 2008).

A study conducted by Stout et al. (2009) that surveyed 1,131 housing units throughout the United States to determine the frequency of residual pesticides present, found that in 448 home, 88% had residual levels of permethrin present. Other pyrethroids were detected, including but not limited to cypermethrin (46%, 480 homes), bifenthrin (33%, 466 homes), deltamethrin (27%, 469 homes), and sumithrin (22%, 468 homes). In addition, fipronil, which is not labeled for indoor spray applications, was found inside 40% of 478 homes, although the amounts detected were low (Stout et al.

2009). Fipronil is labeled for flea and tick control in products for companion animals and also is used in baits and gels to control ants and cockroaches. Products include Maxforce® FC Magnum, AI fipronil 0.05% (Bayer Environmental Science, Research Triangle Park, NC), COMBAT®, AI fipronil 0.03% (Combat Insect Control Systems, Scottsdale, AZ) and outdoor treatment for termites such as Termidor® SC Termiticide/Insecticide, AI fipronil 9.1% (label use 0.06 to 0.125%) (BASF Corporation, Research Triangle Park, NC). These results demonstrate the ubiquity of pyrethroids and fipronil in the environment, which may lead to brown dog tick exposure to sublethal concentrations that may aid in resistance selection.

Stout et al. (2009) also found residual DDT levels present in 41% of 422 homes. DDT exposure has been linked to pyrethroid cross-resistance (Scott and Matsumura 1981, Brengues 2003). Brown dog ticks have had many avenues of exposure to acaricides that could lead to pyrethroid resistance development because they can complete their entire life cycle indoors.

Permethrin

Permethrin, which is found in many flea and tick treatment formulations, is a pyrethroid that acts on the sodium channel, causing neuroexcitation that eventually leads to death. Sublethal doses have been shown cause adverse effects in *Amblyomma americanum* (L.) by causing increases in gas exchange and water loss (Zheng et al. 2013). Permethrin also works as an arthropod repellent, perhaps reducing exposure when arthropods avoid contact (Schreck et al. 1980, Endris et al. 2000, Mencke et al. 2003). However, in some species such as *Hyalomma dromedarrii* (Koch), the camel tick, permethrin has been shown to enhance feeding behavior and increase neurosecretory and salivary gland activity (Mohamed et al. 2000).

In part, due to the widespread use of permethrin across a 30-year period, many arthropods have demonstrated resistance including *Musca domestica* L., *H. irritans*, *Stomoxys calcitrans* (L.), *Anopheles gambiae* Giles, and *R. microplus*, (Scott and Georghiou 1985, Mcdonald and Schmidt 1987, Cilek and Greene 1994, Chandre et al. 1999, Miller et al. 2007a). Two strains of *H. irritans* that were selected for permethrin resistance demonstrated resistance ratios of up to 11,300-fold in comparison to the laboratory susceptible strain (Guerrero et al. 1997). *Rhipicephalus bursa* field collected populations expressed cypermethrin resistance up to 6.9-fold higher than the maximum dose used to treat ticks in the field; levels which lead to control failures (Enayati et al. 2010). After two generations of high dose selection with permethrin, *R. microplus* demonstrated resistance ratios ranging from 9.5 to 263-fold the susceptible colony. Additionally, one population of field-collected *R. sanguineus* from a dog kennel facility in Panama exhibited a 65-fold resistance ratio.

Resistance can be the result of decreased cuticular penetration, increased metabolic activity, and target site mutations (Scott 1990). Increased levels of esterase activity, which leads to metabolic detoxification of permethrin, have been found in resistant strains of *R. microplus* and *R. sanguineus* (Miller et al. 2001, 2007a). Within other populations of *R. microplus,* metabolic resistance to permethrin has been conferred by increased levels of esterase and mixed function oxidase activity (cytochrome P450s) (Hernandez et al. 2002). Over expression of cytochrome P450 also has been found to be an important detoxification mechanism that confers permethrin resistance in *Culex pipiens quinquefasciatus* Say (Kasai et al. 1998) and *B. germanica* (Pridgeon et al. 2003). A permethrin and deltamethrin resistant *B.*

germanica population demonstrated an increase in production of cytochrome P450 between each life stage. In permethrin-resistant *Sarcoptes scabiei* De Geer increased levels of glutathione-*S*-transferase (GST) were determined to be the metabolic mechanism that conferred resistance (Mounsey et al. 2010). Additionally, in the aforementioned resistant strain of *R. bursa* elevated levels of esterase, P450, and GST may have contributed to increased levels of resistance (Enayati et al. 2010).

In addition to metabolic resistance to permethrin, target-site mutations in the sodium channel can confer resistance. As reviewed by Rinkevich et al. (2013) over 30 pyrethroid-resistance mutations in the sodium have been identified in various arthropods, indicating different evolutionary paths. Permethrin resistant *Heliothis virescens* (Fabricius), the tobacco budworm, has a thymine to adenine DNA base pair substitution that results in a single amino acid change from leucine to histidine, which in turn confers resistance (Park and Taylor 1997). *Haematobia irritans* permethrin resistance has been linked to two different point mutations (Guerrero et al. 1997). The first mutation resulted in a methionine to threonine amino acid substitution that provided up to a 68-fold increase in permethrin resistance. The second mutation, found in the super-resistant colonies with up to 11,300-fold resistance ratios, was a leucine to phenylalanine substitution (Guerrero et al. 1997). Dong (1997) reported that *B. germanica* strains, which expressed knockdown resistance, had a single amino acid change from leucine to phenylalanine in the *para* sodium channel gene and that this mutation was not found in susceptible strains. Pyrethroid resistance in *Cimex lectularis* L. conferred by a sodium channel target-site mutation was evaluated across 17 states in a broad scale study (Zhu et al. 2010). Out of 93 populations tested, 78 possessed the

pyrethroid resistance mutation. In *R. microplus,* three point mutations have been documented that are linked to pyrethroid resistance (Lovis et al. 2012). One mutation is found in the Domain III of the sodium channel and two mutations in Domain II, and all result in amino acid shifts.

Cross-resistance to permethrin has been linked to previous exposure to DDT. Scott and Matsumura (1981) evaluated the effects of permethrin on a DDT-resistant strain of *B. germanica* that had no permethrin exposure. Using electrophysiological studies they determined the DDT-resistant cockroaches demonstrated a decreased sensitivity to permethrin in comparison to the susceptible strain. *Aedes aegypti* (L.) also has been shown to express cross-resistance to permethrin from prior DDT exposure and the mechanism was due to a point mutation in the sodium channel (Brengues et al. 2003). Laboratory-selected DDT-resistant *Anopheles stephensi* Liston presented a decreased susceptibility to permethrin, suggesting a cross-resistance mechanism was present (Davari et al. 2007).

Fipronil

Fipronil is in the phenylpyrazole chemical family and is the active ingredient in Frontline®, a topical treatment registered in the United States in 1996 for companion animals to control ticks and fleas. Fipronil acts on gamma-aminobutyric acid (GABA)-gated chloride channels where it blocks the flow of chloride ions, leading to neuroexcitation (Zhao et al. 2003). After over ten years of commercial use, it is important to determine if fipronil resistance has developed in brown dog ticks, or fipronil containing products are retaining effectiveness. Fipronil acts not only as an acaricide, but also has been shown to reduce fertility rates of *R. sanguineus* (Oliveira et al. 2009). In this study, oocytes were examined for damage after ticks were subjected to a 2

minute immersion assay in fipronil at concentrations of 1, 5, and 10 ppm. Fipronil concentrations of 1 ppm demonstrated damaging effects on cell cytoplasm and the plasmic membrane, but most other cell structures were not affected. Treatment at 5 ppm lead to more significant damage to the cytoplasmic areas and 10 ppm of fipronil resulted in total cell destruction (Oliveira et al. 2009). Not only does fipronil impact reproduction rates, but it was hypothesized by Pereira et al. (2009) that effects observed on salivary glands may reduce pathogen transmission. In their study, brown dog tick salivary glands had increased apoptotic activity on both unfed and semi-engorged female ticks when exposed to fipronil at 1, 5, and 10 ppm (Pereira et al. 2009). These two studies demonstrate specific physiological effects of fipronil on *R. sanguineus* that convey important benefits of this compound that extend beyond the acaricidal capabilities.

After many years of use, fipronil resistance has been documented in several arthropod species. In Uruguay, populations of *R. microplus* have demonstrated resistance to fipronil, with resistance ratios ranging from 3.3 to 3,635 (Castro-Janer et al. 2010). The resistant tick populations had been treated 3 to 5 times a year with fipronil for 3 to 7 years. However, the highest resistance ratio was found in a population that received only seven treatments, but may have been exposed previously to fipronil used as a miticide. Interestingly, other populations that had lower resistance expression had received up to 18 treatments. Resistance also may be linked to cross-resistance from dieldrin exposure. Additionally, resistance to fipronil has been documented in *B. germanica*, *M. domestica,* and *Chilo suppressalis* (Walker) (Holbrook et al. 2003, Kristensen et al. 2004, Li et al. 2007).

Brown dog tick populations, along with many other arthropods, have been exposed to various acaricides/insecticides for decades, oftentimes to the point of selecting for resistant populations toward specific compounds. Many newer compounds that act on similar pathways to these older pesticides are subject to reduced activity when exposed to arthropod populations with stable resistance mechanisms. Cross-resistance to acaricides that were previously used for tick control may impact the effectiveness of products on the market today. An *An. stephensi* population that expressed dieldrin resistance was shown to be cross-resistant to fipronil (Kolaczinski and Curtis 2001). Brooke et al. (2000) found that dieldrin resistance was maintained in an *Anopheles gambiae* Giles population at high frequencies in the absence of selection pressures for several years and conferred cross-resistance to fipronil. Holbrook et al. (2003) reported that field-collected, fipronil-naïve *B. germanica* demonstrated survival at 10x the LC_{99} of the laboratory-susceptible colony. This resistance expression was attributed to cross-resistance that had developed from prior dieldrin exposure. *Rhipicephalus microplus* was shown resistant to dieldrin over 50 years ago (Stone and Haydock 1962). If dogs supporting dieldrin-resistant brown dog tick strains were treated with fipronil the control measure may not be effective if this tick shares cross-resistance similarities with the aforementioned insects.

Although the dieldrin-fipronil cross-resistance issue has been observed across many species, fipronil cross-resistance has been shown with other pesticides. In *C. suppressalis*, cross-resistance to fipronil was believed to be due to prior exposure to abemectin (Li et al. 2007). Kristensen et al. (2004) evaluated cross-resistance to fipronil in the house fly, *M. domestica*, wherein fly strains that were resistant to γ-

hexachlorocyclohexane (lindane) were not killed by fipronil applications. Thereafter, these field-collected house flies were found to be resistant (or tolerant) to fipronil without known prior exposure. Overall, the authors concluded that these house flies may have been resistant to fipronil due to either elevated metabolic detoxification or cross-resistance at the target-binding site and that the resistance was conferred by the lindane resistance mechanism (Kristensen et al. 2004).

Target site mutations associated with fipronil resistance have been reported in various arthropod species. Two genetic mutations were found in the GABA receptor of a laboratory-selected fipronil-resistant *Drosophila simulans* Sturtevant strain (Le Goff et al. 2005). Both mutations occurred in the *Rdl* GABA subunit, the first being an alanine to glycine (A2'N) substitution in the M2 domain and the second a theonine to methionine mutation, which occurred in the C-terminal end of the M3 segment. Nakao et al. (2010) reported that fipronil resistant *Sogatella fucifera* Horvath, the plant hopper, also had an A2'N mutation in the GABA receptor. The A302S *Rdl* mutation also has been found in resistant populations of *B. germanica* (Gondhalekar and Scharf 2012, Ang et al. 2013).

Fipronil resistance is frequently linked to a target site mutation, as opposed to the up-regulation of a metabolic pathway. Newborn Norway rat, *Rattus norvegicus* (Berkenhout) and *Periplaneta americana* (L.), neurons were used to measure the blocking ability of fipronil sulfone, a metabolite of fipronil, on the mammalian gamma-aminobutyric acid (GABA) receptor and the insects GABA and glutamate receptors. Glutamate-gated chloride channel receptors are not found in mammals. Fipronil sulfone was found to have greater binding affinity than the parent compound, fipronil, in mammalian GABA receptors (Zhao et al. 2005). In insect neurons, fipronil sulfone was

as effective as fipronil at blocking GABA receptors and had greater binding affinity to glutamate receptors than fipronil. Zhao et al. (2005) concluded that because fipronil binds to two target sites and fipronil sulfone binds to three targets sites, development of target site resistance may take longer to evolve when compared to compounds that have a single binding site. Additionally, fipronil metabolic resistance should occur less frequently due to the metabolite creating greater toxicity than the original compound.

In *Ostrinia nubilalis* (Hübner), the European corn borer, fipronil is metabolized into fipronil sulfone through cytochrome P450 microsomal monooxygenases (Durham et al. 2002). This metabolic pathway generates increased toxicity of the parent compound. Toxicity levels of fipronil metabolites were greater than fipronil for both *C. suppressalis* and *S. inferens*, when tested on larvae (Fang et al. 2008). The high level of toxic effects observed in *C. suppressalis* is believed to be the result of increased metabolic activity producing higher amounts of fipronil sulfone. However, in a laboratory-reared strain of *C. suppressalis* a 45-fold resistance to fipronil was reached after selecting for six generations (Huang et al. 2010). The relatively short time before this high level of resistance developed suggests that there was a heritable factor present that conferred resistance. Following the selection of a resistant strain, the effects of piperonyl butoxide (PBO) as a synergist demonstrated an increase in mortality in comparison to the susceptible strain, which had a decrease in mortality (Huang et al. 2010). Bioassays were completed by adding PBO to the stock solution and applying it to thoracic region using a microapplicator. Therefore, in a fipronil-resistant field population PBO may be a useful synergist; however, if susceptible insects exist in sufficient numbers in the population, PBO could possibly make a treatment less effective.

Esterase activity may play a role in fipronil resistance in *C. suppressalis* and *S. fucifera* (Li et al. 2007, Tang et al. 2010). Although esterase does not metabolize fipronil, the use of TPP as a synergist may increase insecticide penetration or blocking of fipronil by sequestration (Scott 1990, Soderlund and Bloomquist 1990). Glutathione-*S*-transferase (GST) is known to metabolize fipronil (Scharf et al. 2000). However, there is little evidence that GST metabolism increases resistance to fipronil. Kristensen et al. (2004) found increased GST levels in both fipronil-susceptible and tolerant strains of *M. domestica* and Li et al. (2007) found no significant differences in GST levels between susceptible and resistant strains of *C. suppressalis*.

Determining resistance mechanisms before they become problematic may assist in developing more effective pest management strategies that could delay the onset or severity of arthropod resistance. Physiological comparisons on the effects of acaricides that act on similar target sites within the arthropod nervous system can increase the understanding of how resistance could develop and lead to proactive control efforts. (Sayyed and Wright 2004).

Forms of Resistance

Resistance to acaricides and insecticides is not found in all populations of arthropods but resistance traits increase in frequency, as populations are subject to selection pressure over time. *Rhipicephalus microplus* inheritance studies found that resistance due to the transformation of the sodium channel is passed to subsequent generations as a recessive trait (Aguilar-Tipacamú et al. 2008). Homozygous resistant (RR) and susceptible (SS) strains were bred and heterozygous (RS) progeny were intermediate in their resistance levels. In *Plutella xylostella* (L.), diamondback moths, Sayyed and Wright (2004) found fipronil resistance to be a recessive trait. Incomplete

recessive (RS) moths presented lower levels of fipronil resistance as compared to those with complete recessive (SS) gene inheritance expressing high resistance levels.

When comparing reproductive fitness between cattle ticks that are resistant or susceptible to organophosphate, pyrethroid, and formamidine insecticides, the most notable difference was within organophosphate resistant strains, which produced 30% fewer eggs than the susceptible strain (Davey et al. 2006). The reproductive fitness in the pyrethroid- and formamidine-resistant strains did not significantly affect oviposition period, egg output, incubation period, and viability. It was predicted that under real world conditions, in the absence of acaricide selective pressure, the proportion of organophosphate-resistant ticks in a population would eventually decrease due to fitness costs (Davey et al. 2006).

Host resistance is increasingly recognized as a control measure for cattle production systems allowing for a reduction in chemical control. Kongsuwan et al. (2010) found that cattle skin thickness and certain lipids and proteins provided effective barriers that prevented parasitism by the cattle tick. Louly et al. (2010) looked at host resistance by evaluating *R. sanguineus* infestations on tick-resistant beagles and tick-susceptible cocker spaniels. The cocker spaniels had five times the number of ticks present. Hair samples of both dog breeds were evaluated with an olfactometer to determine if *R. sanguineus* demonstrated preference between them. The olfactometer ruled out behaviors exhibited by the dogs that may have influenced tick choice. Their results documented that *R. sanguineus* most often selected the susceptible host. Findings from these studies supported the idea that the brown dog tick utilized host-

based chemical attractants and may be repelled by chemical signals generated by more resistant dog breeds (Louly et al. 2010).

A study was conducted to evaluate possible host resistance development following brown dog tick feeding on previously tick-exposed beagles. Inokuma et al. (1997) found that the number of brown dog ticks attached to dogs previously exposed to tick feeding was significantly lower than the number of ticks that attached to unexposed individuals. When ticks were attached, there was no difference between size, feeding patterns, or reproductive variables of engorged females that fed on hosts that had or had not been previously infested (Inokuma et al. 1997).

Acaricide Resistance Testing

In order to determine if brown dog tick populations are resistant to acaricides, as has been demonstrated in several arthropods, bioassays must be completed. Acaricides resistance has been evaluated using several methods. Stone and Haydock (1962) developed the larval packet test method that has since been adapted by the Food and Agriculture Organization (FAO). This method places approximately 100 larval ticks and into acaricide treated filter paper packets and assesses mortality after 24 hours. In some instances, the larval packet test may require modifications to allow for screening of compounds with unique attributes. Miller et al. (2002) modified the larval packet test by using nylon instead of filter paper as the substrate to evaluate *R. microplus* resistance to amitraz because results were inconsistent.

Larval tick acaricide resistance experiments also can be conducted using the larval immersion test. This method involves submergence of ticks into an acaricide solution for 10 minutes, placement into an untreated containment area, and assessment of mortality after 24 hours (Shaw 1966). The larval immersion test has not been

approved for standardized use by the FAO. Drummond et al. (1973) developed the adult immersion test using the similar principles. The adult immersion test, which has been approved by the FAO, serves as an alternative for rapid resistance analysis when financial resources are not available for obtaining larvae.

In order to evaluate the metabolic resistance mechanisms previously discussed, including glutathione-*S*-transferase, cytochrome P450, and esterase activity, enzyme inhibitory synergists can be added to larval packet test acaricide solutions (Miller et al. 1999). As reviewed by Scott (1990), glutathione-*S*-transferase can be inhibited by diethyl maleate. Cytochrome P450 activity inhibitors include piperonyl butoxide and Sesamex, and esterase activity can be inhibited by adding triphenyl phosphate or *S, S, S*-tributyl phosphorotrithioate (DEF).

Research Objectives

One research study on *R. sanguineus* resistance at a single location has been published and it was not completed in the United States. After numerous reports of difficult to control brown dog tick infestations, it is clear an evaluation of resistance and resistance mechanisms should be completed. It is suspected that control failures are a result of resistance after years of selection pressure. Research was conducted to characterize the resistance status of Florida brown dog tick populations, which can help redefine tick control treatment plans. Field-collected tick populations were used to evaluate resistance levels and look for resistance mechanisms. Laboratory reared susceptible ticks were used for comparison and to set a discriminating concentration that can be used to rapidly screen for resistance in new field-collected populations. The specific objectives include:

1. Determine presence and level of *Rhipicephalus sanguineus* resistance to permethrin and fipronil using the FAO larval packet test.

2. Develop discriminating concentration for permethrin and fipronil resistance in brown dog ticks.

3. Determination of metabolic and target site resistance mechanism in pyrethroid and fipronil resistant brown dog ticks.

CHAPTER 2
DETERMINE PRESENCE AND LEVEL OF *RHIPICEPHALUS SANGUINEUS* (LATREILLE) RESISTANCE TO PERMETHRIN AND FIPRONIL USING THE FAO LARVAL PACKET TEST.

Introduction

The brown dog tick, *Rhipicephalus sanguineus* (Latreille), is a canine ectoparasite that can be considered structural pests due to its ability to infest homes, a high reproductive rate leading to large numbers of ticks, and the ability to complete its life cycle indoors (Demma et al. 2005, Dantas-Torres et al. 2006). Brown dog ticks have three blood-feeding life stages: larva, nymph, and adult. A fully-engorged adult female may oviposit up to 4,000 eggs (Koch 1982). Brown dog ticks spend 94 to 97% of their lifespan off the host. Due to their small size and cryptic behavior, indoor infestations can be difficult to detect, allowing populations to rapidly increase (Needham and Teel 1991). Generally, infestations are discovered when ticks are observed crawling up walls and curtains (Lord 2001).

There are several chemical control methods used to prevent or eliminate brown dog tick populations from both homes and domestic dogs. Products containing amitraz, fipronil, and permethrin appear in the largest number of acaricide formulations uses in the prevention and treatment of tick infestations on dogs. Various acaricide formulations used for indoor pest control and topical treatments for pets have been reported by homeowners and kennel operators as failing to prevent or control *R. sanguineus* infestations (P. Kaufman, unpubl. data). Currently, there is no published documentation of the resistance levels in the brown dog tick to permethrin and fipronil in Florida.

Pyrethroids are a class of pesticide that includes several active ingredients used to control indoor pests. Pyrethroids act by disrupting the function of the sodium channel (Narahashi 1986). Permethrin has been registered for use in topical treatments applied to dogs for over 20 years (EPA 1996). It is inexpensive, available over-the-counter, and thus likely the most commonly used product on the market. Mehlhorn et al. (2003) found that K9 Advantix™, containing 10% imidacloprid and 50% permethrin, provided protection to dogs from *Ixodes* ticks for up to 28 days. *Haemaphysalis longicornis* Neumann, a dog tick found in Japan, demonstrated avoidance behavior by not walking onto treated filter paper when exposed to 10% imidacloprid and 50% permethrin (Hagimori et al. 2005).

Fipronil, in the phenylpyrazole chemical class, is the active ingredient in Frontline®, a topically-applied product registered for tick control on companion animals in the United States since 1996. Fipronil acts on the GABA-gated chloride channel and blocks the flow of chloride ions leading to neuroexcitation (Zhao et al. 2003). A year-long study in Africa documented that fipronil treatment provided a 96% protection against transmission of *Ehrlichia canis*, a bacteria vectored by *R. sanguineus* (Davoust et al. 2003). Burridge et al. (2004) concluded fipronil was the most effective acaricide for controlling *R. sanguineus* when compared to carbaryl, cyfluthrin, chlorpyrifos, permethrin, pyrethrins, amitraz, and selamectin.

In part, due to its widespread, heavy use for 30 years as a general purpose insecticide, many pests have demonstrated resistance to permethrin including, *Musca domestica* L., *Haematobia irritans* (L.), *Stomoxys calcitrans* (L.), *Anopheles gambiae* Giles, and *Rhipicephalus microplus* (Canestrini) (Scott and Georghiou 1985, Mcdonald

and Schmidt 1987, Cilek and Greene 1994, Chandre et al. 1999, Miller et al. 2007a). More specifically, permethrin has been used extensively in indoor pest control for decades, thereby exposing brown dog tick populations to this active ingredient.

Fipronil resistance has been documented in several arthropod species including *Blattella germanica* (L.), *M. domestica, Chilo suppressalis* (Walker), and *R. microplus* (Holbrook et al. 2003, Kristensen et al. 2004, Li et al. 2007, Castro-Janer et al. 2010). Miller et al. (2001) reported limited fipronil resistance in a population of *R. sanguineus* in Panama that had previously demonstrated resistance to permethrin, as well as DDT, coumaphos, and amitraz. However, that particular population of *R. sanguineus* had had a minimal level of exposure to fipronil prior to the Miller et al. (2001) study.

Both permethrin and fipronil have been used extensively against fleas, including on-animal, prophylactic, monthly treatments, increasing the probability of exposure of brown dog ticks. Resistance to permethrin and fipronil is suspected in Florida brown dog ticks due to reports from homeowners and pest control companies citing repeated, unsuccessful acaricide applications targeting in-home and dog kennel tick populations. Many factors can contribute to an apparent lack of acaricide efficacy, including improper acaricide application, pest reinfestation, improper post-treatment animal handling such as washing animals with shampoo, tick acaricide resistance, or a combination of factors. Miller et al. (2001) reported that acaricide resistance in their subject tick population increased the difficulty of controlling *R. sanguineus* infestations. A project was initiated to determine the permethrin and fipronil resistance status of brown dog tick populations obtained from across Florida residential and dog kennel operations, because resistance data were not available for the United States.

Materials and Methods

Ticks

Susceptible brown dog ticks

Acaricide-susceptible brown dog ticks were obtained from Ecto Services, INC (Henderson, NC). The susceptible tick colony was established in March 2008, with new ticks introduced annually to improve genetic diversity. Ticks used in this study were obtained through four shipments between January 2012 and April 2012. Appendix A contains information on the susceptible colony collection history. Susceptible strain larvae used in evaluations were purchased as needed in shipments containing between 1,000 to 2,000 ticks from the same female. These larvae were held for 14 to 16 days post eclosion and challenged with permethrin and fipronil to provide baseline data.

Field-collected brown dog ticks

Brown dog ticks evaluated for permethrin and fipronil susceptibility were collected from 29 residential homes and two commercial dog kennels in Florida and Texas. Under certain circumstances, submitted tick strains were not evaluated for resistance. These included a submission with insufficient numbers of engorged females, females that died prior to ovipositing or produced eggs that did not hatch, or adults could not be reared. All tick sources were self-identified clientele who contacted the University of Florida veterinary entomology program. Following a consultation with program personnel, clientele appearing to have sufficient brown dog tick populations were asked to collect and submit ticks. On three occasions, personnel from our program collected ticks and on six occasions, a pest control operator submitted ticks. The remaining 20 strains were mailed to the University of Florida by clientele. Ticks received included blood-fed females, flat males and females and on one occasion, bloodfed nymphs. In

each case, clientele reported difficulty in eliminating tick infestations with the standard treatment regimens recommended by pest control companies. Whenever possible, a pesticide use history for both the dog(s) and the home/facility was taken at the time of tick submission (Appendix A).

If sufficient numbers (n ≥5) of engorged females were collected or received, the larvae from the eggs that hatched were tested in the bioassay described below. All non-engorged females and same-strain males were bloodfed on a rabbit host to obtain eggs from that tick strain (UF IACUC Protocol 200802142, 201102142, Appendix B). All eggs oviposited by engorged ticks collected from the same residence were placed together in one container and gently mixed using a watercolor paint brush in order to improve genetic diversity during testing. Eggs were weighed into vials of either 250 mg or 500 mg aliquots. Once larvae had emerged, containers were held for 14 to 16 days in the incubator described below where after larvae were placed into the acaricide bioassay.

Nomenclature of tick strains dictates that the larvae tested were the F1 of the collected blood-fed females. However, three tick strains were submitted with both blood-fed and flat adults. In these instances, eggs were collected from the blood-fed ticks and challenged, while the flat adults were fed on the rabbit host to obtain additional eggs. Additionally, eggs were reared and larvae were challenged with acaricides. Nomenclature used to separate these two sub-strains are F1 and F1a from larvae that eclosed from bloodfed adult female eggs that were oviposited upon arrival from collection site and flat adults that were bloodfed in the laboratory, respectively. In a few

instances, the F1 and F1a generations were reared through adulthood and subsequently laid eggs. These larvae are referred to as the F2 generation.

Tick Rearing

Brown dog tick strains were held at 25 ± 1°C, with a photoperiod of 12:12 (L:D). Relatively humidity was maintained by either a saturated solution of potassium nitrate salt (ScienceLab Inc, Houston, TX) and distilled water placed in glass holding chambers that maintained 92% RH (Winston and Bates 1960) or ticks were held in a sealed chamber (Sanpia) with four 500 mL containers of distilled water that maintained 85% RH. Each active life stage was fed on New Zealand white rabbits to obtain a blood meal (Appendix B). For more complete rearing information and tick feeding chamber construction see Appendix C.

Bioassays

The FAO larval packet test (LPT) developed for acaricide testing of tick populations was used to screen tick strains for acaricide resistance. The LPT was first described by Stone and Haydock (1962). This method has been used to determine resistance status in *R. sanguineus* and several strains of *R. microplus* (Miller et al. 2001, Sabatini et al. 2001, Castro-Janer et al. 2009). Technical grade permethrin (98.8%, *cis*, *trans*, 40.1:58.7) (ChemService Inc., West Chester, PA) and fipronil (98.3%) (ChemService Inc., West Chester, PA) were used separately to conduct the LPT. Acaricides were dissolved into a solution of two parts trichloroethylene (TCE) (Sigma-Aldrich, St. Louis, MO) and one part olive oil (Spectrum, Gardena, CA). For fipronil solutions, 1 mL of acetone was substituted for TCE to aid in the dissolving process. For both solutions, the technical grade acaricide was placed in the flask, TCE was added to ensure proper acaricide breakdown then olive oil added as the final step.

The solution was vortexed to ensure even acaricide distribution. The chemical solution was serially diluted into a TCE and olive oil mixture that included 6 to 10 treatment concentrations, depending on the number of ticks available, and a non-acaricide treated control. Serial dilution concentration differences ranged from 30% to 60% for permethrin and 40% to 50% for fipronil, depending on the predicted resistance level. Each dilution was used to produce two to four replicates provided sufficient numbers (250 mg of eggs) of larval ticks were available for testing. Given the treatment history, the highest dose of the serial dilutions tested on all newly-acquired tick strains ranged from 5% to 30% for permethrin and 0.1 to 1% for fipronil. Permethrin evaluations were given priority and if sufficient ticks were available, fipronil susceptibility was evaluated.

Filter papers (Whatman® No. 1, Maidstone, England) were cut into 7.6 x 8.9 cm pieces and 1 mL of a test dilution was applied to a paper. The solvent-only control filter papers were treated with a 2:1 TCE and olive oil solution. Following dilution application, acaricide-treated and control filter papers were suspended from an aluminum restaurant ticket order holding bar (Alegacy®, Santa Fe Springs, CA) under a fume hood in a constant airflow for 2 hours to allow the TCE to evaporate, leaving the acaricide and olive oil on the filter paper (Fig. 2-1).

After the drying period each filter paper was folded in half. Thereafter, the filter paper sides were clipped using Boston Number 2 bulldog clips (Sparco™, Atlanta, GA) (Fig. 2-2). Approximately 100 larval ticks were placed in each packet using a water color paint brush. The open end of the packet was secured with a third bulldog clip. Once assembled, the packets were suspended on a metal rod and held in a humidified incubator at 25 ± 1°C, 92% RH, with a photoperiod of 12:12 (L:D) for 24 hours (Fig. 2-

3). The humidifying incubator consisted of a 38 liter glass aquarium that was sealed with weather stripping and a glass cover. Relative humidity was maintained by a potassium saturated nitrate salt solution on the bottom of the aquarium (Winston and Bates 1960).

Following a 24-hour incubation period, packets were removed and mortality was assessed by counting the number of live and dead ticks in each packet (Fig. 2-4). Dead ticks were characterized by lack of movement after being disturbed and appeared flat and desiccated. Moribund ticks that were on their dorsum and demonstrated leg movement but were incapable of locomotion also were categorized as dead. Tick behavioral changes occurred after exposure to permethrin, that should be considered when scoring mortality. Live ticks exposed to high doses of permethrin tended to present themselves in a non-mobile state. Their appearance was similar to an untreated tick in that they did not have the desiccated and flattened look of the dead ticks. Permethrin treated ticks in the quiescent state remained inactive until they were disturbed, sometimes by breathing on them, but typically by probing or physical disruption. During experiments with fipronil, live ticks exposed to high doses occasionally required human breath to induce activity, but they did not enter the quiescent stage like the permethrin exposed ticks. Occasionally, there was tick mortality due to ticks being crushed under the bulldog clips during the packet sealing process. Ticks that were found dead in areas of the filter paper that were indented by the clips were not included in the overall count. Larval ticks that survived high doses of an acaricide were place in an empty vial and stored at -80°C for later polymerase chain reaction (PCR) testing.

Statistics

The acaricide-susceptible strain was used to generate complete permethrin and fipronil concentration-response curves for all tick strains. Bioassay data from replications within a tick strain were pooled and analyzed by standard Probit analysis (Finney 1971) using Abbott's transformation (Abbott 1925) to correct for control mortality and all hypotheses were tested by the likelihood ratio test (Savin et al. 1977). Probit analysis was conducted using PoloPlus© (LeOra Software Company®, Petaluma, CA) (LeOra 1997). Mortality regression models were generated for permethrin and fipronil. Resistance ratios (RR), based on the susceptible laboratory strain, were determined using the LC_{50} and LC_{90} values (Robertson and Preisler 1992). Resistance ratios were calculated as: LC_x of resistant strain / LC_x of susceptible strain to determine the level of resistance for each field strain. Strain susceptibility to an acaricide was considered significantly different when there was no overlap between the 95% confidence interval of each field strain and the laboratory susceptible strain at an LC_{50} or LC_{90} value. Tick strains with resistance ratios ≤10 were considered acaricide tolerant and populations with resistance ratios >10 were classified as resistant (Mota-Sanchez et al. 2008).

Results

In total, 29 residential units and two dog kennels submitted ticks for testing. Of these tick submissions, seven were assessed for permethrin resistance (Table 2-1, Figs. 2-5, 2-6, 2-7) and four were assessed for fipronil resistance (Table 2-2, Fig. 2-7). When an insufficient number of doses were tested for statistical analysis due to low tick numbers, survival data were presented only in Figures demonstrating percent survival without statistical analysis. The Gilchrist and Webb, TX strain had two tick generations

that were tested for permethrin resistance, while the Palm Beach-2 strain had two generations tested for fipronil resistance.

All strains tested demonstrated permethrin resistance, with most of the strains exhibiting such high resistance levels that the mortality data did not fit the statistical model requirements for accurate LC-value generation (Table 2-1). Of the strains where an accurate LC-value could be determined, the Palm Beach-2 submission resulted in the highest LC_{50} and LC_{90} values of 0.775% and 5.86%. This can be compared to the acaricide-susceptible ticks that had a LC_{50} of 0.027%, and the LC_{90} of 0.051%. These values resulted in a RR_{50} of 29 at the LC_{50} (Table 2-1). Accurate LC-values could not be calculated for the St. Johns, Gilchrist F1, Gilchrist F2, Palm Beach-2 F1a, Duval, Broward, and Sarasota-2 strains due to high tick survival at the highest permethrin levels tested of 5% and 10% (Fig. 2-5). Although specific LC-values were not calculable, 28% survival was observed in the Gilchrist F2 strain when challenged with a 30% permethrin concentration, indicating that this strain was highly resistant (Fig. 2-6). Although still highly resistant, the lowest RR was found in the Webb, TX strain with a F1 generation RR_{50} of 26 and a F2 generation RR_{50} of 29. The Palm Beach-2 strain had the largest difference in RRs within F1 generation testing with the F1 presenting with a RR_{50} of 29 while F1a resistance ratio was unable to be calculated due to high survival at high levels of permethrin, but is estimated to be >300 (Fig. 2-7).

Brown dog tick susceptibility to fipronil was evaluated in four strains and compared against the acaricide-susceptible tick strain (Table 2-2, Fig. 2-8). In the Palm Beach-2 strain, sufficient ticks were submitted to evaluate two groups within the F1 and generation. All strains tested expressed tolerance against fipronil exposure with

resistance ratios at the LC_{50} and LC_{90} below 3.8. The St. Johns strain had the highest LC_{50} at 0.088%. However, the highest LC_{90} value was observed in the Palm Beach-2 F1 strain at 0.174%. The LC_{50} and LC_{90} values of the susceptible strain are significantly lower than the LC_{50} and LC_{90} values of the St. Johns and Palm Beach-2 F1 strains. The St. Johns strain RR_{50} was the highest at 3.52, while the Collier-1 strain had the lowest RR_{50} of 1.72. The RR_{90} for the Collier-1 strain was nearly equivalent to both Gilchrist and Palm Beach-2 F1a strains. Overall, resistance ratios for fipronil were much lower than what was found for permethrin (Fig. 2-9). All fipronil tested strains were considered tolerant, while permethrin resistance was high.

Discussion

This study provides the first laboratory-confirmed permethrin resistance and fipronil tolerance data for brown dog ticks from the United States. The only other documented report of acaricide resistance in *R. sanguineus* was to permethrin, DDT, and coumaphos at the Corozal Army Veterinary Quarantine Center in Panama (Miller et al. 2001). The Panama brown dog tick resistant strain had an estimated LC_{50} of 4.2% active ingredient. With the strains tested in the United States that provided statistically-accurate responses, the Palm Beach-2 F1 and Webb, TX F1 and F2 demonstrated lower levels of resistance (0.7%) in comparison to the Panama strain. However, the remaining six strains and Palm Beach-2 F1a strain had much higher estimated LC_{50} values that could not be conclusively determined, but ranged from >8.9% to well over 30% permethrin concentrations. Many of the comparative tick resistance studies have been completed on *R. microplus,* which is a member of the same genus as brown dog ticks. Because of *R. microplus'* pestiferous relationship with cattle, it has been exposed to several classes of acaricides. Permethrin resistant *R. microplus* have demonstrated

levels of resistance so elevated that LC values are unable to be determined because of high survival at the highest dose evaluated (30% active ingredient) (Miller et al. 1999), similar to what was demonstrated in the Gilchrist strain (Fig. 2-6). If the St. Johns tick strain had successfully survived through to the F2 generation, low mortality was predicted at the 30% permethrin concentration due to the <2% mortality observed at the 10% permethrin concentration (Fig. 2-5). For comparison, the Gilchrist F2 strain expressed 15% mortality at the 10% permethrin concentration and 28% mortality was observed at the 30% permethrin concentration (Fig. 2-6). Additional tick strains showed low mortality at 10% permethrin concentrations including, Duval at 35%, Broward at 28%, and Sarasota-2 at 50% (Fig. 2-5).

The most likely explanation for permethrin resistance development in these tick strains is exposure at the site of the tick infestation. The Duval, Gilchrist, and Sarasota-2 strain reported the use of store purchased permethrin home habitat treatments. The Gilchrist, Sarasota-2, and Broward strain reported the permethrin exposure history from veterinary prescribed topical tick control products. Timing of applications differed, wherein the Gilchrist and Sarasota-2 had been using on-animal pyrethroid products inconsistently for at least five years. The Broward and Duval infestations reported on-animal permethrin use for less than one year. Therefore, the high permethrin resistance levels expressed in the Broward and Duval strains either developed rapidly in the home or were already present in the tick population when the ticks were introduced to the home. Given the slow life cycle of the brown dog tick, high resistance expression development in a single year due to in-home acaricide applications seems the less likely cause.

Burridge et al. (2004) concluded that fipronil was highly effective for brown dog tick control. Miller et al. (2001) reported fipronil susceptibility in a Panamanian brown dog tick population that demonstrated DDT, permethrin, coumaphos and amitraz resistance. The four tick strains tested in our study had fipronil exposure history through companion animal topical treatments. The fifth strain was collected from a kennel with unknown, but potential exposure history. At the time that the Miller et al. (2001) study was published, fipronil had been registered for less then five years. The cost for fipronil use was considerably higher than permethrin as well, so the likelihood of fipronil use was low. Accordingly, increased, but still limited fipronil use would be expected at the time of the Burridge et al. (2004) publication. Fipronil use on companion animals is no longer under patent protection and comparatively inexpensive formulations are available. Thus, as the product is used more, the likelihood for resistance in brown dog ticks is expected to increase.

In comparison to the permethrin resistance reported in the current study, fipronil RR_{50} values were relatively low ranging from 1.72 to 3.52 (Fig. 2-9). The St. Johns strain, which expressed the greatest permethrin resistance, also had the highest fipronil LC_{50} value (0.088% AI) among ticks evaluated with both active ingredients. Interestingly, this tick strain, which originated at a dog kennel, reportedly received topical spot-on treatments containing permethrin and not fipronil (Appendix A). However, this was based on the regimen of the kennel operators for long-term animal boarding, but dog acaricide treatment history was not available for short-term boarded animals. The Gilchrist strain originated from a home with 19 dogs and had an exceedingly large brown dog tick population, which demonstrated resistance levels to

permethrin and tolerance to fipronil. The ticks from this location had a treatment history of both permethrin and fipronil topical spot-on treatments, however, the owners reported inconsistent use of these products (Appendix A).

Although no published reports of fipronil resistance exist for the brown dog tick, in Uruguay, populations of *R. microplus* have demonstrated fipronil resistance, with resistance ratios ranging from 3.3 to 3,635 (Castro-Janer et al. 2010). The resistant tick populations had been treated with fipronil, which had been used in this situation for agriculture purposes for 3 to 7 years. However, the highest resistance ratio was found in a population that received only seven treatments, while other strains with lower resistance ratios had received up to 18 treatments. Cyclodienes, including dieldrin were historically used in agricultural settings. The authors suggest that the rapid resistance development with this strain may have originated from a dieldrin cross-resistance mechanism already present in *R. microplus*. Cross-resistance between cyclodienes and fipronil has been documented in *B. germanica* (Holbrook et al. 2003). Brown dog ticks are not likely to have recent or regular exposure to dieldrin or other cyclodienes, because they are not currently approved for use in homes or on companion animals. Chlordane has been used for indoor tick control (Mallis 1954), which may increase the likelihood of cross-resistance as a mechanism of fipronil resistance. Bloomquist and Robinson (1999) detected fipronil-tolerance in chlordane resistant *B. germanica.* Fipronil has been formulated for use as a spot-on treatment on companion animals since 1996, which has allowed ample time for resistance development in situations where it has been used aggressively. Given that fipronil is now marketed as a generic

product, it is expected that *R. sanguineus* populations will receive considerably more fipronil exposure, thus accelerating resistance expression.

Between the F1 and F1a tick cohorts that were tested, the Palm Beach-2 strain expressed increased permethrin and fipronil resistance levels. These ticks were collected 10 days after the home had been treated with esfenvalerate, Onslaught® (McLaughlin Gormley King Company, Minneapolis, MN) (Appendix A). In addition to the pyrethroid application to the home, the dogs had been treated with Frontline Plus® (Merial, Duluth, GA), which contains fipronil, within the previous 30 days. The Palm Beach-2 F1 larvae came from owner-collected engorged females, which were likely exposed to both of these chemicals. The Palm Beach-2 F1a strain consisted of larvae produced from flat male and female ticks obtained in the same shipment as the blood fed females and subsequently blood fed and mated under laboratory conditions without exposure to acaricides. A third group of ticks (F2) were reared from F1a larvae through to adulthood, mated, bloodfed and the F2 resultant larvae were tested in the LPT. This process required one year to complete. It is unclear why the F1a and F2 generation had differential survival as compared to the F1 ticks when collection was from the same population, and no acaricide exposure or selection had occurred (Table 2-1, Appendix A).

Three populations of permethrin resistant cattle ticks found in Mexico demonstrated varying levels of resistance ranging from resistance ratios of 166 in one strain to greater than 1,000-fold in two other strains (Miller et al. 1999). High levels of variation in permethrin resistance were found within the different populations of *R. sanguineus* that were tested in the current study. The *R. microplus* strain with 166-fold

resistance was determined to have increased esterase and cytochrome P450 activity, leading to metabolic detoxification. The two strains with higher levels of resistance did not show any indication of metabolic resistance, and were later determined to have sodium channel point mutations (Miller et al. 1999, Guerrero et al. 2001). Based on those results, it is suspected that various mechanisms of permethrin resistance in *R. sanguineus* are present. Field-collected permethrin-resistant *R. microplus* from an infestation in the United States had a resistance ratio of 9.5 and following two generations of selection at 5.9% and 9.7% permethrin concentrations, the resistance ratio had increased to 263 (Miller et al. 2007a). This population was determined to have a target site mutation on the sodium channel and the gene frequency in the population increased after the high concentration selection (Miller et al. 2007a). Although permethrin-resistant, this tick population was susceptible to coumaphos and amitraz and the tick was eliminated from the cattle ranch. Such an occurrence highlights the diversity of information that needs to be gathered in *R. sanguineus* by characterizing resistance and its resistance mechanisms.

Some resistant brown dog tick strains had no history of exposure to acaricides from the collection site, indicating resistance must have evolved in the tick populations prior to their appearance in the home. Brown dog ticks have an average of two to four generations per year in the field (Dantas-Torres 2008). Therefore, resistant tick populations have been selected for through years of ongoing acaricide exposure. Georghiou and Taylor (1986) determined it typically requires 10 to 15 generations to select for resistance. Therefore, depending on field-conditions, brown dog ticks under selection pressure may develop resistance between 2.5 to 7.5 years.

In ~1978 a survey was conducted in 958 Indiana homes to determine the frequency of pesticide use (Bennett et al. 1983). Of these homes 77% used pesticides, 17% of which were pyrethroids, the most frequently used chemical class. Historically, chlorpyrifos (Dursban™, Dow AgroSciences, Indianapolis, IN) and pyrethroids were heavily used for indoor control of cockroaches and ants (St. Aubin 1990). Since the Food Quality and Protection Act in 1996 (EPA 2013), the use of chlorpyrifos became restricted for indoor pest control and pesticide use has shifted toward pyrethroid applications (Goddard 2011). Pyrethroid-resistant arthropods have been found to demonstrate cross-resistance to several pyrethroids after being under selection pressure with a single pyrethroid.

A study evaluated 20 residences in Boston public housing facilities for pesticide presence using a 24-hour air sample and three wipe samples from each home. Permethrin was detected in 38% and cypermethrin in 24% of the wipe samples. Of the 20 indoor air samples, the pyrethroids, tefluthrin, and cyhalothrin were found in the greatest concentrations (Lu et al. 2013).

In another study, residual permethrin levels were found in 88% of 448 homes, and fipronil in 40% of 478 homes (Stout et al. 2009). Brown dog tick populations that are newly introduced to residential areas may be subjected to sublethal acaricide exposure prior to tick treatment protocol implementation. Sublethal exposure may lead to the induction of metabolic mechanisms that can confer resistance. The exposure of DDT and cyclodienes leads to increased cytochrome P450 activity (Yu 2008), which has been found to confer resistance to pyrethroids in *R. microplus* (Miller 2001).

Acaricide exposure differences that result in differential selection pressure among tick strains is the most likely reason for the varied permethrin resistance levels observed in this study. However, resistance development in arthropods, particularly to permethrin, has been conferred through a variety of resistance mechanisms. Additionally, as these ticks have limited non-host dispersal capabilities, the likelihood of a unified mechanism shared across intermingling sub-populations is unlikely. Cuticular penetration, increased metabolic activity leading to acaricide detoxification, and target site mutations all have been documented as different forms of resistance (Scott 1990). Additionally, permethrin is a repellent, therefore in field-conditions ticks may avoid contact surfaces that have been treated (Bissinger and Roe 2010). Forms of metabolic detoxification include increases in the production of cytochrome P450, esterase, and glutathione-*S*-transferase. Cytochrome P450 and esterase metabolic detoxification through the use of synergists that blocks metabolic activity were evaluated in permethrin-resistant *R. sanguineus* in Panama (Miller et al. 2001). In this instance, increased esterase activity was found to confer permethrin resistance in these ticks.

To date, there has been no published research documenting metabolic resistance to fipronil in ticks, however, an examination of other arthropods provides insight as to possible resistance mechanisms. Although fipronil is acaricidal in its parent form, cytochrome P450 has been found to metabolize fipronil into fipronil sulfone (Durham et al. 2002). The metabolite generated greater toxicity when compared to fipronil, making this metabolic route unlikely to produce resistance (Zhao et al. 2005). Alternatively, increased esterase activity has been suggested to confer fipronil resistance in *Chilo suppressalis* (Walker) and *Sogatella fucifera* Horvath (Li et al. 2007,

Tang et al. 2010). Although a mode of action was not suggested, synergist use may increase pesticide penetration or molecule sequestration (Scott 1990, Suderland and Bloomquist 1990).

The active target site of permethrin is on the neuron at the voltage-sensitive sodium channel (Soderlund and Bloomquist 1989). No publications exist on the expression of a target site mutation for permethrin resistance in *R. sanguineus*. However, three target-site or point mutations that result in amino acid changes and thus permethrin binding site alterations are believed to confer permethrin resistance in *R. microplus* (He et al. 1999c, Morgan et al. 2009, Jonsson et al. 2010). Resistance due to point mutations also has been documented in numerous arthropods including *Heliothis virescens* (Fabricius)*, Blattella germanica* (L.), and *Aedes aegypti* (L.) (Park and Taylor 1997, Dong 1997, Brengues et al. 2003). One or more point mutations could be present in permethrin resistant strains of *R. sanguineus.*

The target site of fipronil is located on the gamma-aminobutyric acid (GABA)-gated chloride channel (Zhao et al. 2003). Point mutations that confer fipronil resistance have not been documented in any tick species. However, fipronil resistant *Drosophila simulans* Sturtevant and *S. fucifera* have been documented to have point mutations on the *Rdl* (resistance to dieldrin) GABA receptor in the chloride channel that are believed to be linked to resistance (Le Goff et al. 2005, Nakao et al. 2010).

Due to limitations in available tick numbers, high-dose laboratory selection for fipronil resistance was unable to be completed. In future studies, laboratory dose selection of resistant ticks could help determine how quickly resistance can be selected for in a population. It also would be of value to understand how resistance develops

with laboratory selection as one could determine how quickly and to what levels resistance is expressed. While under fipronil selection, if tick strains develop only tolerance (resistance ratio <10), but not resistance (resistance ratios >10), current products on the market that exceed tolerance concentrations should remain effective. Davey and George (1998) selected for permethrin resistance over five generations in a strain of *R. microplus*. Resistance ratios increased from 5.4-fold to 20.9-fold the susceptible strain. Ticks from the resistance-selected strain and the susceptible strain were placed on cattle and dipped in 0.057% AI permethrin, as recommended by the label. The susceptible tick has 99.2% mortality, however, the laboratory-selected ticks had only 35.7% mortality (Davey and George 1998). If the acaricide dip in the previous study were to have been used at the labeled rate (0.057%) on dogs for *R. sanguineus* control, few ticks from the current study would succumb to the animal-applied permethrin exposure.

There are few products sold for brown dog tick control with alternative active ingredients because, in addition to being effective for tick control, they must be veterinary approved for use on mammals. The currently approved active ingredients for tick control on dogs include permethrin, fipronil, amitraz, and flumethrin, which has been formulated for use in tick collars. The widespread permethrin and increasing fipronil resistance documented in this study narrows the product selection available for some tick populations. The high level of permethrin resistance detected herein may be due to permethrins ubiquity in commercially-available products and that such high levels were observed may reflect in cross-resistance to flumethrin, another pyrethroid. Pyrethroid effectiveness may vary between insects and ticks due to variation in the binding site

(O'Reilly et al. 2013). A single amino acid change between ticks and insects was found at the pyrethroid binding site in the sodium channel using 3D modeling. Understanding variations in binding sites can aid in the production of more specific acaricides.

Although it is unclear how resistance develops in many populations, behaviors such as not following proper treatment schedules or using improper concentrations, as well as the lack of acaricide alternatives that lead to repeated acaricide use further pressures resistance selection in arthropod populations. Contributing factors may include off-label pesticide applications when control is not achieved at approved doses. Such an approach invariably creates high-dose selection pressure. In the case of on-animal applications, oftentimes the products are delivered at low rates or washed from the pets resulting in low-dose exposure and a failure to control ticks leaving an end user who believes that resistance is occurring. Additionally, even well intentioned pet owners attempting to change acaricides may purchase different product brands not realizing that they are using the same active ingredient, thereby contributing to resistance selection. Many of the crack and crevice applications utilize pyrethroid-based products with the same mode of action as the on-animal permethrin-based products, further exposing tick populations to increased selection pressure. Finally, many of the on-animal products are under patent protections or are available only through veterinary clinics and cost many fold more than over-the-counter permethrin formulations, making consumer choice often based on economics, not best management practices. Through the recent availability of generic fipronil-containing products, this bottleneck may be opening. The extent of fipronil resistance is uncertain at this point.

Heavy exposure to pyrethroids for many years in the home and through the use of on-animal products may have lead to the high levels of resistance presented in this study. In comparison, the labels of fipronil-based products have not been used as long and no indoor applications are approved, which could explain the lack of resistance, and only tolerance demonstrated in *R. sanguineus.* Cross-resistance in resistant and tolerant strains also may be a result of previous selection pressure to DDT for sodium channel mutations and to chlordane for GABA-gated chloride channel mutations.

The knowledge of *R. sanguineus* resistance is important to help guide pest control operator best management practices, as they treat homes with brown dog tick infestations. If infestations are not responding to acaricide applications, resistance may be present and treatment protocols should be adjusted. Education of veterinarians is critical, in that veterinarians provide tick control plans for companion animals. It is quite common for veterinarians to preemptively place client dogs on tick preventatives without documentation of tick presence or even potential exposure. Such prophylactic treatment approaches violate the principle of integrated pest management, where one does not treat without the pest being present or historical evidence of reinfestation.

Residential areas and facilities that house high numbers of dogs can be subject to repeated ineffective chemical control applications if resistant tick populations are present. In addition to the cost of professional treatments, there is increased unnecessary acaricide exposure, wasted resources, stress to the people surrounded by the infestation, stress and blood loss for the animal being exposed to ticks, increased risk of vector-borne disease for humans and animals, and continued selection for resistant tick populations. To prevent further resistance selection and effectively control

resistant populations, different classes of acaricides should be used and rotated during treatment plans and dogs should only be treated when ticks are observed on animals or in the home.

Table 2-1. Toxicity of permethrin to brown dog tick, *Rhipicephalus sanguineus* (Latreille), populations collected from residential homes and dog kennels in Florida and Texas as determined using the Food and Agriculture Organization larval packet test.

Tick strain	n [a] (replicates)	LC_{50} (95% CI) [b]	LC_{90} (95% CI) [b]	RR_{50} [c,d]	RR_{90} [c,d]	Slope (SEM)	χ^2(df)
Susceptible	5,240 (40)	0.027 (0.026-0.028)	0.051 (0.048-0.054)	—	—	4.64 (0.15)	55.4 (34)
Broward[g]	1,945 (28)	ND	ND	>600	>1,000		
Duval[g]	1,442 (36)	ND	ND	>5,000	>300,000		
Gilchrist F1[f]	2,209 (32)	ND	ND				
Gilchrist F2	4,302 (42)	ND	ND	>2,000	>11,000		
Palm Beach-2 F1	1,245 (24)	0.775 (0.646-0.929)	5.860 (4.384-8.451)	29	115	1.46 (0.08)	32.9 (22)
Palm Beach-2 F1a	2,505 (32)	ND	ND	>300	>1,000		
Sarasota-1[g]	2,419 (24)	ND	ND	>500	>900		
St. Johns	1,760 (36)	ND[e]	ND				
Webb, TX F1	1,913 (21)	0.703 (0.569-0.843)	2.71 (2.190-3.567)	26	53	2.19 (0.11)	49.5 (16)
Webb, TX F2	3,221 (36)	0.781 (0.721-0.845)	2.680 (2.38703.054)	29	53	2.39 (0.82)	40.6 (30)

[a] n is defined as the total number of ticks used in the experimental run and replicates represents total number of packets

[b] Values represent percentage of active ingredient (w/v) applied to treated filter papers 7.5 x 8.5 cm

[c] Tick strains were considered statistically different when the LC 95% confidence intervals (CI) from the field-collected strains did not overlap with the laboratory susceptible strain

[d] Resistance ratios at LC_{50} and LC_{90} were calculated by dividing the respective LC_{50} and LC_{90} of each field strain by that of the susceptible strain (Ecto Services, Inc.)

[e] ND: Lethal concentration could not be determined due to <64% mortality at high concentrations

[f] Low mortality at all concentrations beginning at 5% A.I. No effect determined by chemical treatment

[g] Low mortality at 10% active ingredient. Unable to obtain specific levels, but resistance demonstrated

Table 2-2. Toxicity of fipronil to brown dog tick, *Rhipicephalus sanguineus* (Latreille), populations collected from residential homes and dog kennels in Florida and Texas as determined using the Food and Agriculture Organization larval packet test.

Tick strain	n[a] (replicates)	LC_{50} (95% CI)[b]	LC_{90} (95% CI)[b]	RR_{50}[c,d]	RR_{90}[c,d]	Slope (SEM)	χ^2(df)
Susceptible	3,643 (27)	0.025 (0.024-0.026)	0.046 (0.043-0.049)	—	—	4.84 (0.11)	63.6 (25)
St. Johns	3,196 (36)	0.088 (0.084-0.091)	0.120 (0.114-0.126)	3.52	2.61	9.39 (0.47)	12.0 (30)
Gilchrist F1[e]	2,408 (24)	0.054 (0.047-0.063)	0.124 (0.103-0.158)	2.16	2.70	3.56 (0.13)	159 (22)
Palm Beach-2 F1	1,045 (18)	0.047 (0.028-0.076)	0.174 (0.102-0.471)	1.88	3.78	2.25 (0.12)	168 (14)
Palm Beach-2 F1a	2,346 (32)	0.062 (0.054-0.072)	0.151 (0.123-0.196)	2.48	3.28	3.30 (0.13)	118 (26)
Palm Beach-2 F2	2,821 (32)	0.061 (0.054-0.070)	0.123 (0.105-0.154)	2.44	2.67	4.23 (0.16)	190 (26)
Collier-1	828 (18)	0.043 (0.031-0.059)	0.124 (0.088-0.221)	1.72	2.70	2.80 (0.18)	61.1 (14)

[a]n is defined as the total number of ticks used per experiment and replicates represent the number of packets
[b]Values represent percentage of active ingredient (w/v) applied to treated filter papers 7.5 x 8.5 cm
[c] Resistant ratios were considered statistically different when the LC 95% confidence intervals (CI) from the field-collected strains did not overlap with the susceptible strain
[d] Resistance ratios at LC_{50} and LC_{90} were calculated by dividing the LC_{50} and LC_{90} of the field strain divided by the susceptible strain (Ecto Services, INC.)

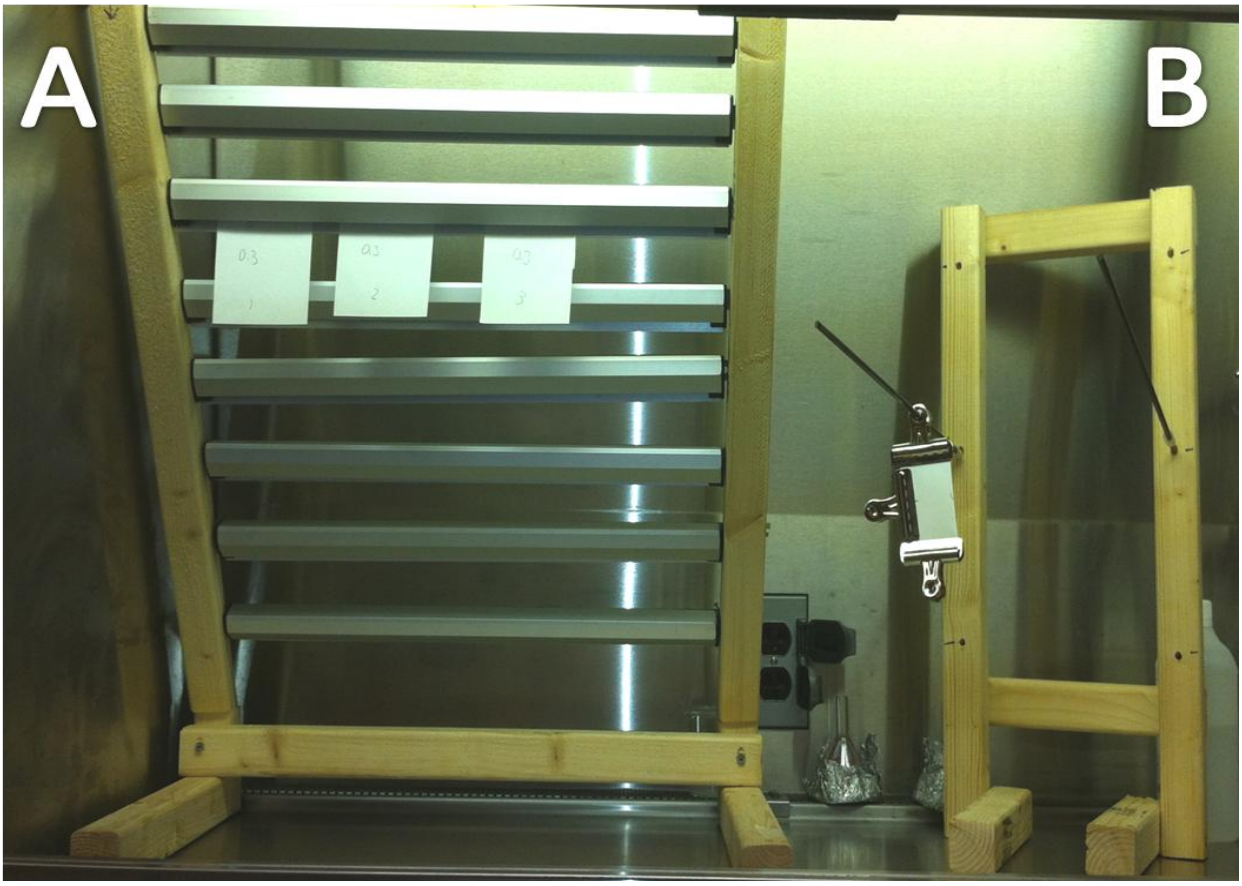


Figure 2-1. Left side: A) Drying device for acaricide treated filter papers. Designed to allow for airflow while preventing the filter papers from contacting each other. Right side: B) Larval packet suspension device, used to hold packets before transfer on the suspension rods to the humidifying incubator for the 24 hour holding period. Photo courtesy of Amanda L. Eiden

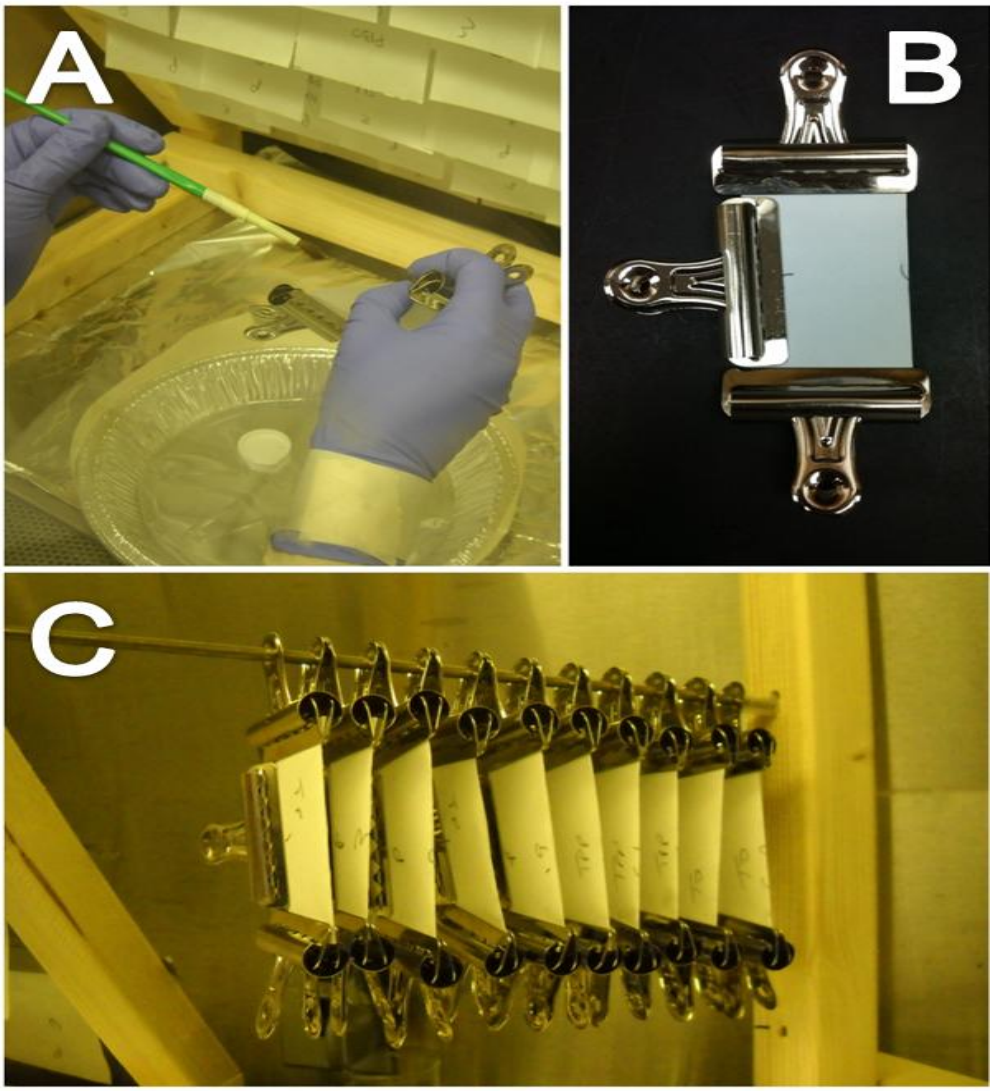


Figure 2-2. Apparatus and procedures used in resistance profiling of permethrin and fipronil to field-collected strains of *Rhipicephalus sanguineus* (Latreille), using the larval packet test. A) After filter papers were treated with varying concentrations of acaricide, dried for two hours and approximately 100 larval ticks were placed within the filter paper. B) The packet was sealed using bulldog clips. C) Filter papers were suspended for 24 hours. Photo courtesy of Amanda L. Eiden.

Figure 2-3. Humidifying incubator for larval packet test. The temperature of 25 ± 1°C maintained the relative humidity at 92% using a potassium nitrate saturated salt solution. Larval packets containing ticks were suspended in the chamber using steel rods. The top of the glass holding chamber was coated with petroleum jelly to prevent tick escape in the event that a tick crawled out of improperly sealed packets. Photo courtesy of Amanda L. Eiden.

Figure 2-4. After packets were suspended in a humidifying incubator for 24 hours, larval *Rhipicephalus sanguineus* (Latreille) mortality was assessed. Photo courtesy of Amanda L. Eiden.

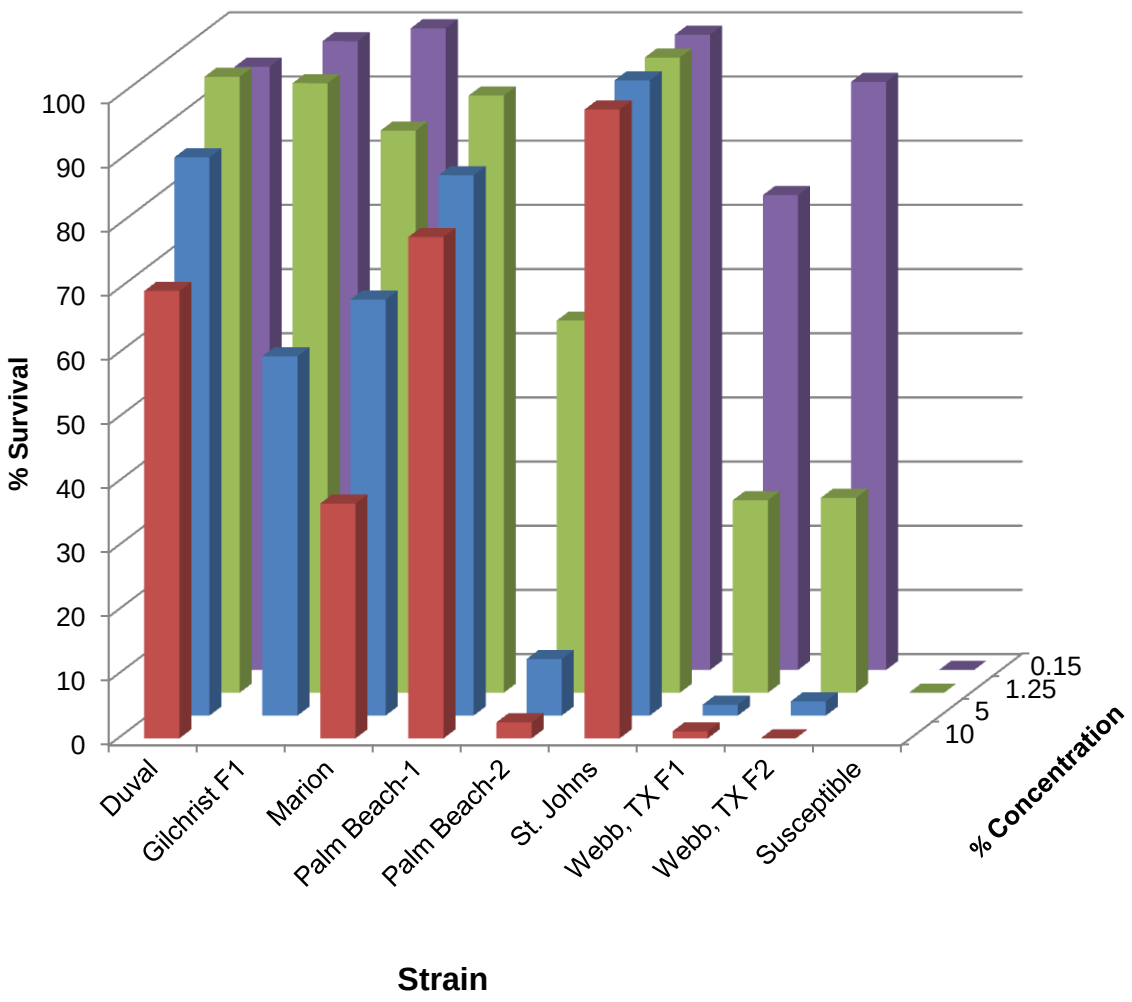


Figure 2-5. Mean percentage survival of *Rhipicephalus sanguineus* (Latreille) field collected strains exposed to four permethrin concentrations using the Food and Agriculture Organization larval packet test and the susceptible tick strain from Ecto Services, Inc, NC. Due to high mortality, susceptible tick mortality was measured only at lower the relatively concentrations of 1% and 0.15%.

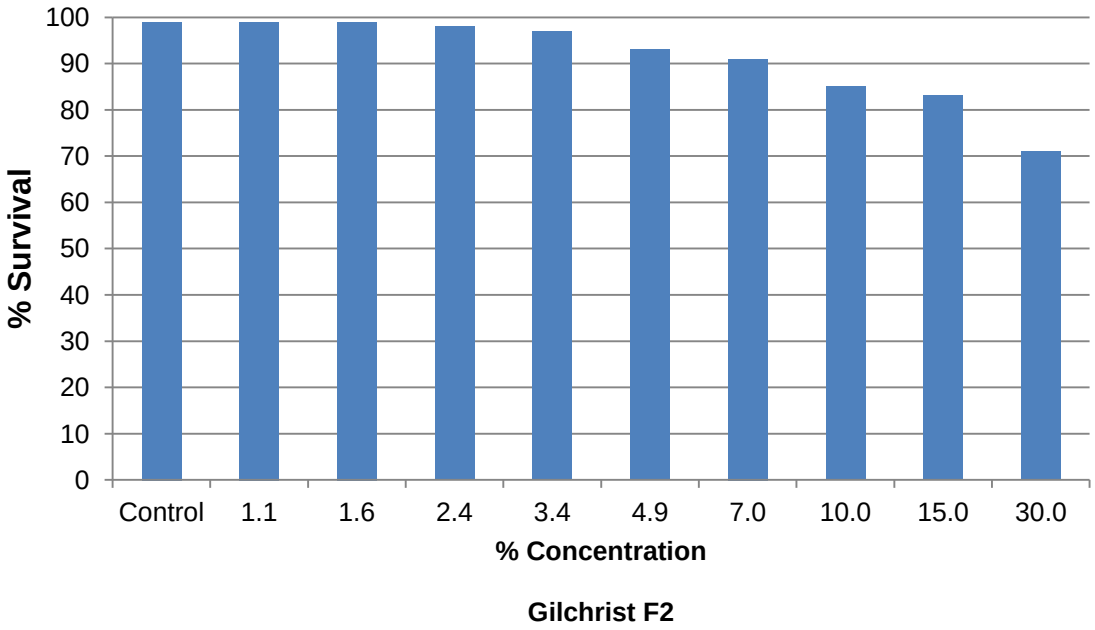


Figure 2-6. Percent survival for the Gilchrist F2 strain of *Rhipicephalus sanguineus* (Latreille), when challenged again different concentrations of permethrin using the Food and Agriculture Organization larval packet test.

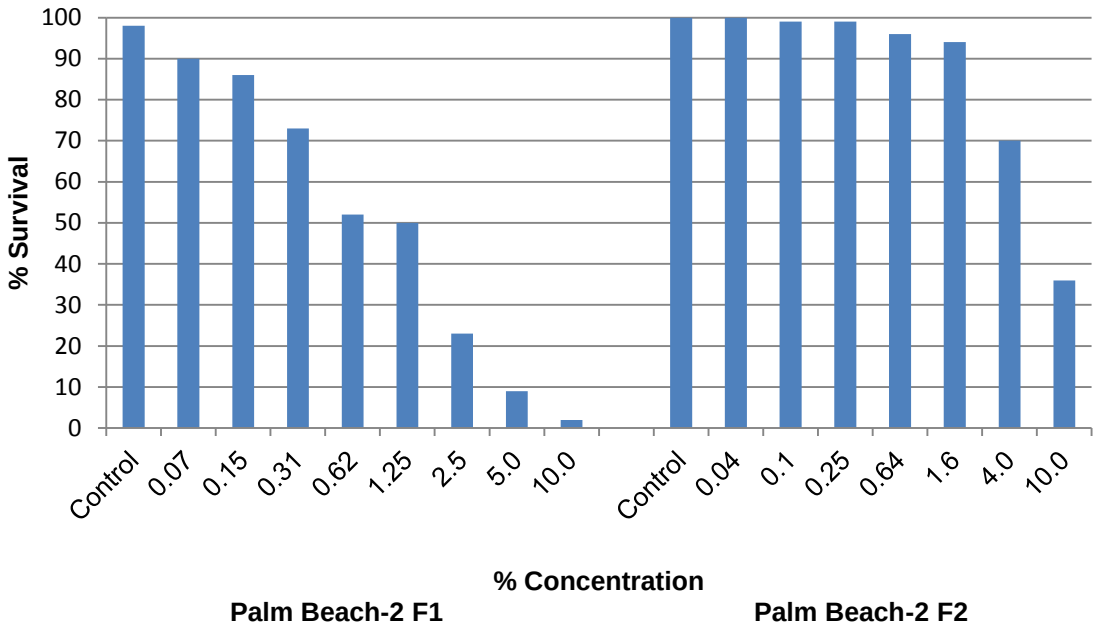


Figure 2-7. The percent survival of *Rhipicephalus sanguineus* (Latreille) at various permethrin concentrations in the Palm Beach-2 strain between generations. Acaricide testing was conducted using the Food and Agriculture Organization larval packet test. Higher levels of survival were demonstrated in the F2 generation.

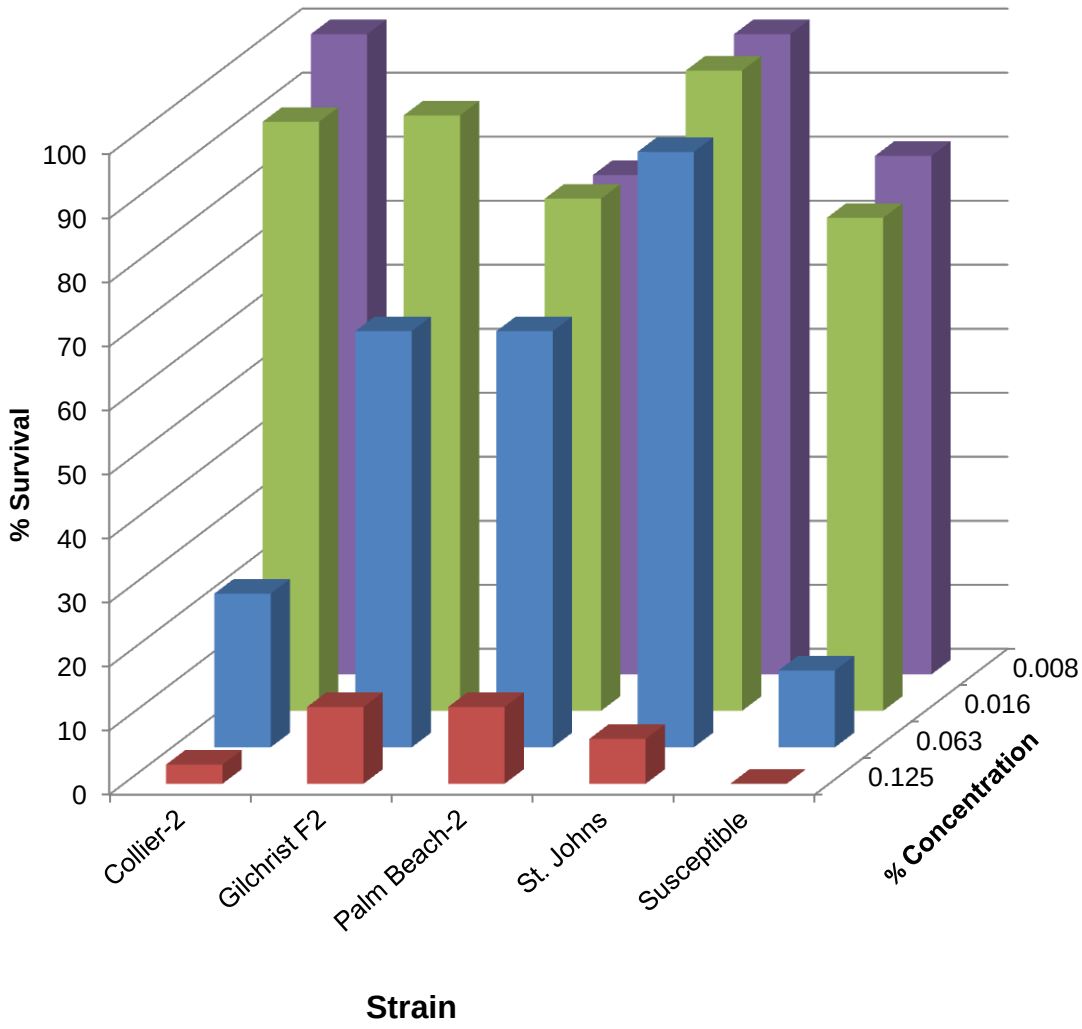


Figure 2-8. Mean percentage survival of *Rhipicephalus sanguineus* (Latreille) field collected strains exposed to four fipronil concentrations using the Food and Agriculture Organization larval packet test and the susceptible tick strain from Ecto Services, Inc, NC.

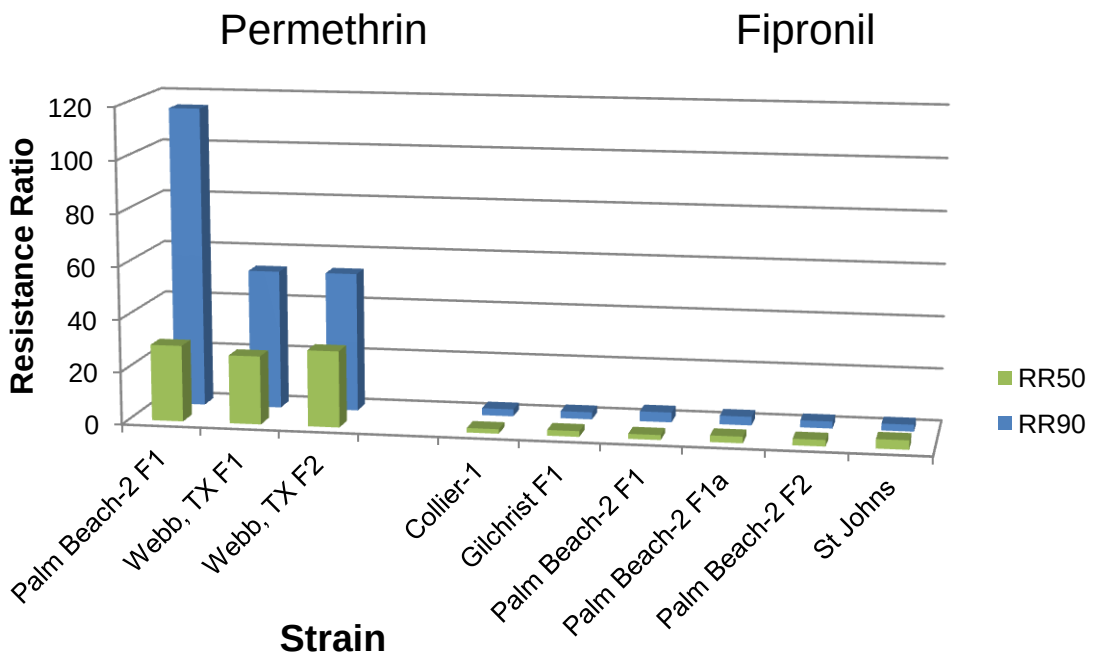


Figure 2-9. Comparison of permethrin and fipronil resistance ratios in different field-collected strains of *Rhipicephalus sanguineus* (Latreille) from data collected using the Food and Agriculture Organization larval packet test. Resistance ratios were calculated using Probit analysis.

CHAPTER 3
DISCRIMINATING CONCENTRATION DEVELOPMENT FOR PERMETHRIN AND FIPRONIL RESISTANCE IN BROWN DOG TICKS

Introduction

Resistance to permethrin and fipronil is present at varied levels in different populations of brown dog ticks, *Rhipicephalus sanguineus* (Latreille) in Florida and Texas (Chapter 2). Prior to this work, published resistance data for *R. sanguineus* in the United States did not exist. As with other pestiferous arthropods, a population's resistance status can be determined using a diagnostic dose or concentration, depending upon the pesticide application technique used. Roush and Tabashnik (1990) described a discriminating concentration as a single concentration of pesticide that will kill a large portion of the susceptible genotypes, while the resistant genotypes remain alive. It is used as a simple yes or no resistance detection assay.

Discriminating concentration methods have been used to rapidly determine the resistance status of several arthropods such as *Tribolium castaneum* (Herbst), *Rhyzopertha dominica* (F.), *Helicoverpa armigers* (Hübner), *Musca domestica* L. *Rhipicephalus* (*Boophilus*) *microplus,* (Canestrini) (Zettler and Cuperusi 1990, Forresters et al. 1993, Sabatini et al. 2001, Kaufman et al. 2001, Castro-Janer et al. 2009). Sabatini et al. (2001) used the larval packet test with *R. microplus* to develop a discriminating concentration for the macrocyclic lactone, cydectin. In their study, they determined the LC_{99} using a Probit analysis calculation for an acaricide-susceptible tick strain and the discriminating concentration was set at 2x LC_{99}.

The use of discriminating concentrations can help predict the effectiveness of currently sold tick control products. Permethrin and fipronil have been formulated into

many different products and sold for *R. sanguineus* control. Commercially-available products currently sold for use on dogs that contain permethrin include, but are not limited to, the topical animal spot-on treatments, K9 Advantix® II (Bayer Healthcare LLC, Animal Health Division, Shawnee Mission, KS) active ingredient (AI) permethrin 44%, imidacloprid 8.8%, pyriproxyfen 0.44% and Vectra 3D® (Ceva Animal Health LLC, Lenexa, KS) AI dinotefuran 4 to 8%, pyriproxyfen 2 to 5%, permethrin 35 to 45%, depending on size of the dog. Although many permethrin-based product formulations are used for indoor residential application including Tengard® (United Phosphorus, Inc., King of Prussia, PA) and Martin's Permethrin® SFR (Control Solutions Incorporated, Pasadena, TX), which contain 36.8% permethrin, the end use labeled rate is often quite similar among products and typically is near 0.5% AI. These products are designed for professional use but may be purchased online. Shampoos for tick control include Sentry® Pro Flea & Tick Shampoo (Sentry Pet Care Products, Inc., Omaha, NE), AI permethrin 0.10%, piperonyl butoxide 0.50%, pyriproxyfen 0.01%. In addition to permethrin-containing products, several other pyrethroid class acaricides are sold, including products containing fenvalerate, lambda-cyhalothrin and bifenthrin. Many of these formulations include the synergist piperonyl butoxide.

Products currently registered for use on dogs that contain fipronil include, but are not limited to, topical treatments for companion animals such as Frontline® (Merial, Duluth, GA) AI fipronil 9.8% and PetArmor® (Fidopharm, Yardley, PA) AI fipronil 9.7%. On-animal sprays sold for tick control include Frontline® Spray Treatment for Pets, AI fipronil 0.29% and Sentry® FirpoGuard® Flea & Tick Spray for Dogs & Cats, AI fipronil

0.29%. Termidor® Foam for indoor void treatments (BASF, St. Louis, MO), AI fipronil 9.1% is sold, but is not labeled for tick control.

The development of a discriminating concentration allows for a much simpler method to screen tick population samples from residential and kennel facilities with a single chemical concentration to determine if they are susceptible or resistant. Additionally, if residents can supply several engorged female ticks, an evaluation of resistance could be conducted without the need to rear ticks. A discriminating concentration forms the basis for an inexpensive and rapid resistance diagnostic technique that can be used to guide tick management plans for animal and environmental treatments. In the currently study, the discriminating concentration for brown dog ticks has been established for permethrin and fipronil and its appropriateness and suitability was evaluated with field-collected brown dog ticks.

Materials and Methods

Ticks

Susceptible brown dog ticks

Ecto Services, INC (Henderson, NC) provided acaricide-susceptible brown dog ticks from a colony that was established in March 2008. Appendix A contains detailed information on the susceptible colony collection history. Between January 2012 and April 2012 four shipments of tick eggs were received for testing and held in an incubator until hatching as described below. After hatching, larvae were challenged with permethrin and fipronil 14 to 16 days post-eclosion to develop the LC_{99} values later used to establish the respective discriminating concentrations.

Field-collected brown dog ticks

Potentially resistant brown dog ticks used in these studies originated from pest control operators who encountered heavy infestations in residential homes, from residents who were struggling to control tick infestations in their homes, or by dog kennels operators. In total, ticks were received from two commercial dog kennels in Florida and Texas and 29 residential units in Florida. Table 3-1 contains a summary of permethrin and fipronil exposure, while Appendix A contains a more detailed acaricide exposure history. If feasible, an interview was conducted with the resident or kennel operator to gather acaricide use history for the submitted tick samples.

Ticks received from each location were held separately and labeled as an individual strain based on the county of collection and the order in which they were received from that county. Larvae were tested from field-collected engorged females when eggs from five or more ticks successfully hatched. Flat male and female ticks were laboratory reared as described below to sustain the colony and obtain larvae for bioassays. To maintain genetic diversity eggs from all ovipositing females within the same strain were mixed and divided for experimental use or rearing.

Tick Rearing

New Zealand white rabbits were used as blood feeding hosts for all tick life stages (UF IACUC Protocol 200802142, 201102142, Appendix B). When off the host, brown dog tick strains were held at 25 ± 1°C, 92% RH, with a photoperiod of 12:12 (L:D). Detailed information regarding rearing and equipment construction are presented in Appendix C. Tick colonies that had different genertations that were tested are labeled as F1 for the first generation and F2 for the second generation that was reared in the laboratory.

Bioassay

Detailed methods for bioassays were described in detail in Chapter 2 but briefly, permethrin and fipronil dose response curves were generated for the acaricide-susceptible brown dog tick strain using the Food and Agriculture Organization Larval Packet Test (LPT) (FAO 2004). In this test, technical grade permethrin (98.8%, *cis*, *trans*, 40.1:58.7) (ChemService Inc., West Chester, PA) or fipronil (93.8%) (ChemService Inc., West Chester, PA) was dissolved in a solution (w/v) composed of two parts trichloroethylene (TCE) (Sigma-Aldrich, St. Louis, MO) to one part olive oil (Spectrum, Gardena, CA). The chemical mixture was serially diluted and filter papers (7.5 x 8.5 cm) (Whatman® No. 1, Maidstone, England) were treated with 1 mL of solution then dried for two hours under a fume hood leaving the acaricide and olive oil on the filter paper. Control filter papers were treated with only TCE and olive oil at the same ratio.

The highest concentrations were chosen to exceed the expected discriminating concentration based on levels that were established in *R. microplus* (Miller, unpublished data, Miller et al. 2007b, Castro-Janer et al. 2009). Susceptible ticks were initially evaluated using a range of permethrin and fipronil concentrations between 0.010 and 1%, each of which was serially diluted with a concentration reduction of 40% across 10 concentrations. The filter papers treated with only TCE and olive oil served as the untreated control. Three or four packets were treated at each concentration with approximately 100 tick larvae exposed per packet. Based on the results of the first experiment, the highest concentration was decreased from 1% to 0.36% for permethrin and 1% to 0.12% for fipronil in the second experiment. Herein, acaricides were serially diluted at 40% until 10 concentrations were generated. The final two experiments were

conducted with the same top concentration, but were serially diluted by 30% to refine tick response results.

After the drying period, approximately 100 larval ticks that were 14 to 16 days post-eclosion were placed in each filter paper packet. Packets were constructed by folding the filter paper in half and clipping the sides together with Boston Number 2 bulldog clips (Sparco™, Atlanta, GA) (Chapter 2, Fig. 2-2). After the ticks were placed in the packet, the open end was secured with a third bulldog clip. Assembled packets were held for 24 hours in a humidifying chamber at 25 ± 1°C, 92% RH, with a photoperiod of 12:12 (L:D) (Chapter 2, Fig. 2-3). Mortality was assessed after the 24 hour holding period. Ticks were counted and the numbers of live and dead ticks were recorded (Chapter 2, Fig. 2-4). Ticks were categorized as dead if they did not respond with a probe or to CO_2 from human breath. Moribund ticks that were unable to walk but demonstrated leg movement were categorized as dead. Testing of the dilution series was repeated on four occasions, each time representing another replicate. Four LPTs were conducted on different days due to equipment and space constraints. Each LPT served as a replicate, with newly mixed stock and dilution solutions. Through these evaluations, the LC_{99} of the pooled data was obtained. This value was the basis for generating the discriminating concentration, which was set at two-fold the LC_{99} of the acaricide-susceptible tick strain.

Resistance Evaluation

Field-collected ticks were exposed separately, using the aforementioned LPT procedures, to four concentrations of permethrin and fipronil in order to screen for resistance. These concentrations were selected based upon both tick responses to serially-diluted acaricides identified in Chapter 2 and pooled results from the susceptible

tick strain experiments described above. Concentrations evaluated against field-collected ticks were 1-, 5-, 16- and 53-times the 2x LC_{99} value. Provided the increased tick susceptibility to fipronil, two lower concentration screening values (LC_{90} and LC_{99}) and two diagnostic concentrations (2x LC_{99} and 4x LC_{99}) were used to evaluate responses of field-collected ticks. These concentrations were based on survival data obtained in Chapter 2. The number of replicates and ticks per packet varied based upon the number of ticks obtained from field collections. Between two and four replicates for each concentration were completed, with between 30 and 100 ticks per packet. With the exception of one experiment, where 295 ticks were tested at three concentrations, no fewer than 500 larval ticks were used in any given experiment, with a maximum of 1,600 used for a full evaluation. Mortality was assessed at 24 hours using parameters described previously.

Data Analysis

The LPT test data for the susceptible tick bioassays were entered into the PoloPlus© (LeOra Software, Petaluma, CA) statistical software program for Logit analysis of permethrin LPT data and Probit analysis of fipronil data (LeOra 1997). Different analyses (Probit or Logit) were required in order to fit the models properly, as Probit and Logit provide with similar but not identical probability curves. Control mortality was corrected for using Abbott's correction (Abbott 1925). Data were considered significant if the t-ratio >1.96. The LC_{50}, $LC_{90,}$ and LC_{99} were obtained from the program output. The discriminating concentration was established as the 2x LC_{99} value of the pooled acaricide-susceptible tick strain response. Thereafter, all field strains were subject to the 2x LC_{99} discriminating concentration for initial evaluation

(Sabatini et al. 2001), with additional concentrations included as feasible to improve clarity in tick responses. Data were recorded as percentage survival at each multiplier of the discriminating concentration.

Results

Discriminating Concentration

To establish the LC_{99} values for the permethrin bioassay, a total of 16,485 susceptible strain ticks, 155 replicates, were tested while an additional 17,085 ticks, 138 replicates, were used in the fipronil bioassay. The permethrin LC_{50} and LC_{99} values for the pooled data of the acaricide susceptible strain were 0.027% and 0.083%, respectively (Table 3-2). Therefore, the calculated discriminating concentration (2x LC_{99}) was 0.19% using the pooled data from the four evaluations. The fipronil $LC_{50,}$ LC_{90} and LC_{99} values for the acaricide susceptible strain were 0.023%, 0.044%, and 0.074%, respectively (Table 3-3). Using 2x LC_{99} from the pooled susceptible experiments, the discriminating concentration was set at 0.15%.

Permethrin Discriminating Concentration Experiments

Brown dog ticks from 30 Florida locations and one Texas kennel were received. Seven of the Florida strains that were obtained from five FL counties provided sufficient numbers of larvae to allow for permethrin resistance evaluation (Gilchrist F2, Palm Beach-2 F2, Palm Beach-3, Collier-2, Sarasota-1, Sarasota-2, and Broward) using the permethrin discriminating concentration evaluation. The Gilchrist, Palm Beach-2, Sarasota-2, and Broward strains also were evaluated in Chapter 2.

Newly acquired tick strains were screened for mortality with permethrin concentrations of 0.18, 0.9, 3 and 10%, that were representative of the 1x, 5x, 16x, and 53x the diagnostic concentrations. All tick strains tested with permethrin resulted in

>89% survival at the discriminating concentration (Fig. 3-1). At the 5x discriminating concentration, survival ranged from 79 to >99%. Six of the seven tick strains were tested at the 53x discriminating concentration, and only the Palm Beach-3 strain had no survival. The Sarasota-1 tick strain was not tested at the 53x discriminating concentration due to a limited number of ticks. Survival among the remaining tick strains ranged from 19% in the Collier-2 strain to 66% in the Broward strain.

Fipronil Discriminating Concentration Experiments

Six tick strains from five Florida counties (Gilchrist F2, Collier-2, Sarasota-1, Sarasota-2, Palm Beach-3, and Broward) were evaluated for fipronil resistance using the discriminating concentration evaluation. Among these strains, only the Gilchrist strain was evaluated in Chapter 2. Tested strains were selected based upon the availability of sufficient numbers of larval ticks.

Based on results from the acaricide-susceptible strain, the fipronil diagnostic concentration was set at 0.15%. However, the fipronil concentrations evaluated included two concentrations that were lower than the 1x discriminating concentration for fipronil because preliminary screening of field strains indicated 100% mortality a 0.3% active ingredient (2x discriminating concentration). Therefore, the concentrations used were 0.04, 0.07, 0.15, and 0.30, which were representative of the LC_{90}, LC_{99}, 2x LC_{99} (1x discriminating concentration), and 4x LC_{99} (2x discriminating concentration).

In the susceptible strain, 15% survival was observed at the LC_{90}, of 0.04% (Fig 3-2). Survival in the field-acquired strains ranged from 65 to 99% at this lowest concentration. At the diagnostic concentration there was no survival in the acaricide-susceptible strain, but survival ranged from 0% in the Sarasota-2 strain to >95% in the Palm Beach-3 strain. Survival following fipronil exposure dropped considerably

between the 1x and 2x discriminating concentration. At the 2x discriminating concentration, the Palm Beach-3 and the Broward strains demonstrated 5% and 6% survival, respectively.

Discussion

The LPT method has been used to screen potentially resistant *R. microplus* populations against different acaricide classes (Miller et al. 2001, Sabatini et al. 2001, Castro-Janer et al. 2009). Miller et al. (2007a) determined the discriminating concentration for *R. microplus* at 0.25% for permethrin (R. J. M. unpublished data). This test also was used in *R. microplus* to determine the discriminating concentration at 0.28% for macrocyclic lactones (Sabatini et al. 2001). The Cattle Fever Tick Research Laboratory in Edinburg, TX has been using discriminating dose tests since 1997 to screen *R. microplus* populations for acaricide resistance. Miller et al. (2007a) identified the first populations of permethrin resistant *R. microplus* in the United States using this screening tool. In our study, the discriminating concentration for permethrin was determined to be 0.19%. Prior to this research, a discriminating concentration for permethrin or fipronil had not been published for *R. sanguineus*.

The advantage of using a discriminating concentration for ticks falls with the ability to collect eggs from the future submission of engorged female ticks. These emergent larvae can be categorized as susceptible or resistant with a single dose. This is important because the cost of rearing ticks is high and it is time consuming. Additionally, when an infestation is present it is important to provide guidance as quickly as possible and without excessive costs.

The availability of a discriminating concentration allows for an inexpensive and rapid resistance diagnostic technique that can be used to guide tick management plans

for animals and aid in the selection of environmental treatment options. My results for permethrin and fipronil discriminating concentration establishment for brown dog ticks allows for a resistant:susceptible screening for future tick submissions from residences and kennel facilities using a single chemical concentration. The single concentration of 0.19% active ingredient will be used with permethrin and 0.15% active ingredient for fipronil. With the standardized use of larval ticks, the typical client-submission quantity of two to three engorged females would provide sufficient tick offspring numbers needed for this method.

There were large ranges of susceptibility among the tested strains of *R. sanguineus*, especially at the highest concentrations. One would expect such differences given the relative lack of non-host dispersal or mixing among these strains, as well as the diversity of pet-owner driven management practices, creating disproportionate acaricide exposure. Among permethrin-tested field strains, the largest difference in survival was between the Broward and Palm Beach-3 strains with 66% and 0% survival respectively, after exposure to 53x the discriminating concentration of permethrin (Fig. 3-1).

Within the tick strains tested for fipronil resistance using the discriminating concentration, the Sarasota-2 strain was the most susceptible of the field strains evaluated, although survival was only 2% higher at the LC_{99} when compared to the susceptible strain (Fig. 3-2). Interpretation of the Sarasota-2 resistance screening results should be viewed with caution, as only 539 ticks were evaluated, the lowest number among all tested strains. The clientele who submitted the Sarasota-2 ticks reported the use of fipronil on their dogs through the use of a topical spot-on treatment

to the dog. However, the reporting individual claimed this product was ineffective for controlling their tick problem. Under these circumstances it is difficult to determine the cause of such a control failure. Often, inconsistent or improper product use or post-application grooming practices may be occurring, which lead to decreased chemical exposure levels for the ticks.

The Collier-2 strain also had <2% survival at 2x LC_{99} and should be considered susceptible. However, at the LC_{99} there was 70% survival, indicating these ticks were more tolerant to fipronil than the Sarasota-2 and susceptible strains (0 and 2.46%, respectively). No history of fipronil exposure was reported for the Collier-2 strain prior to tick submission. The pet owner for the Collier-2 strain reported that permethrin also had not been used indoors at the residence and had not been applied to the dog. Interestingly, this strain demonstrated high levels of permethrin resistance, suggesting that selection had occurred prior to the infestation.

The Sarasota-1 strain had a reported use of fipronil via spot-on treatments on three dogs in the household. This strain demonstrated 67% survival at 2x LC_{99} but no survival at 4x LC_{99} (Fig. 3-2). Additionally, the home recently had been treated with the pyrethroids deltamethrin and lambda-cyhalothrin as a crack-and-crevice application, which could have selected for permethrin resistance in this strain immediately prior to its submission (Table 3-1, Appendix A). Tick survival at the permethrin 16x discriminating concentration was 92%, suggesting a highly permethrin-resistant tick population existed in the home. Unfortunately, sufficient numbers of ticks were not available to conduct the 53x discriminating concentration assay.

Additional testing methods, other than theLPT, have been used for discriminating concentration development including the larval immersion test developed by Shaw (1966), in which ticks were dipped into an acaricide solution. In the field, ticks are not typically submerged in to acaricides for treatment; therefore, a treated surface contact assay may more accurately depict real-world resistance expression in ticks. Sabatini et al. (2001) compared the LPT and larval immersion assays using macrocyclic lactone exposure to *R. microplus*. In these tests the LPT had a discriminating concentration of 0.28%, while the immersion assay generated a discriminating concentration ranging from 0.0007 to 0.006%. The researchers determined that the LPT provided accurate and detailed results for resistance diagnosis, although higher concentrations of acaricide were required in comparison to the larval immersion test.

The adult immersion test, developed by Drummond et al. (1973), also has been used to evaluate resistance. The adult immersion test, much like the larval immersion test, utilizes a method of submerging ticks into a liquid acaricide. There are no data to document the differences in mortality between all three tick life stages to determine if their susceptibility changes when data are adjusted for body mass. The LPT and adult immersion test have been standardized and are accepted by the Food and Agriculture Organization for resistance screening in ticks, however, the larval immersion test is not an approved method (FAO 2004).

All three described testing methods have been used to calculate the fipronil discriminating concentration for *R. microplus.* Castro-Janer et al. (2009) found variation between all methods, including differences between the larval and adult immersion tests, wherein a discriminating concentration at 7.64 ppm and 4.98 ppm were observed,

respectively. This would indicate that larvae are less sensitive to fipronil than the adults. The LPT in their study, as expected, had a much higher estimated discriminating concentration at 2,366 ppm (0.23%), demonstrating a higher concentration of fipronil was required to obtain similar mortality levels with the two immersion tests. The same conclusion was found in Sabatini et al. (2001) with macrocyclic lactones. Should the larval immersion test gain popularity and as data are published and methods standardized, lower amounts of an acaricide would be required in order to verify established resistance. This is especially important when evaluating experimental or patent-protected acaricides, where availability and cost are prohibitive to widespread evaluation.

Adult immersion tests have been used to generate permethrin and fipronil LC_{50} values with semi-engorged *R. sanguineus* (Roma et al. 2009, Oliveira et al. 2011). In these studies, the LC_{50} was 2,062 ppm (0.20%) for permethrin and 9.647 ppm (0.0009%) for fipronil. There are few labels that include rates for dipping dogs into acaricide solutions; therefore, the closest comparison to a real world situation would be a tick shampoo.

Undiluted Sentry® Pro Flea & Tick Shampoo contains AI permethrin 0.10%, piperonyl butoxide 0.50%, pyriproxyfen 0.01%. Piperonyl butoxide is a synergist that blocks cytochrome P450 activity (Casida 1970). However, if cytochrome P450 activity is not the mechanism of resistance present in the tick population, it will not increase the effectiveness of permethrin. With the determined LC_{50} of adult ticks at 0.20% using the adult immersion test, a diluted application of the shampoo to a pet, already maximized at 0.10% in the formulation, would not be effective in killing many ticks. Furthermore, at

the supplied concentration of 0.10%, it is unlikely that tick exposed directly to the concentrated product would die.

The permethrin-containing product Tengard® has been labeled for use at a diluted level equivalent to deliver a 0.0626% AI concentration in a dip solution for dogs. Roma et al. (2009) reported 26% tick mortality seven days following a 0.10% permethrin adult immersion test, using semi-engorged females. Semi-engorged females in Roma et al. (2009) did not demonstrate 100% mortality until immersion in 2.56% permethrin, which exceeds the label rate for permethrin dips used on companion animals.

As demonstrated in the current study, various levels of permethrin resistance have been found in Florida and Texas brown dog tick populations. To put these resistance levels into perspective using expected performance with two consumer products sold today, application rates and expected tick mortality are presented. Most, if not all, permethrin-containing products labeled for indoor crack-and-crevice applications have an upper limit of 0.5% concentrations, with retreatment intervals of 14 to 30 days. For example, Tengard® and Martin's Permethrin® SFR are labeled for indoor crack-and-crevice applications at 0.5% Al or for use as a spray application directly to dogs at a higher rate of 0.6257%, with 1 to 2 fluid ounces used per animal. The environmental and on-animal levels are ~3.5x below the discriminating concentration (0.19% concentration) tested on the field collected brown dog tick strains that I evaluated. At the 5x level, an AI level slightly higher than that allowed by label instructions, all tested ticks demonstrated 79 to 97% survival. Pet owners with ticks expressing permethrin resistance at these levels would likely experience a failure of the product.

Frontline® Spray Treatment for Pets, and Sentry® FiproGuard® Flea & Tick Spray for Dogs & Cats both have an AI of 0.29% fipronil. This level is equivalent to the 4x LC_{99} that was tested. Mortality at this concentration was high in all field strains tested, with the Palm Beach-3 and Broward strains demonstrating approximately 5% survival. The remaining tested tick strains had <1% survival. This product remains effective according to the results of my study. However, as the levels of fipronil present on the dog diminish between application periods, ticks could be exposed to sub-lethal concentrations. If products with different modes of action are not used, the repeated sub-lethal exposure could lead to increased fipronil resistance levels, similar to what has been demonstrated in laboratory resistance selection studies (Miller et al. 1999).

Having baseline data from susceptible and resistant populations is important in determining changes in resistance over time. Due to the difficulty in tracking where tick populations originated, it is difficult to determine how quickly resistance is evolving or what resistance characteristics the strain had when it first arrived at the home. The Gilchrist F2 strain demonstrated resistance to both permethrin and fipronil. These ticks were collected from a home that had been battling an infestation for at least three years. The resident reported using various acaricides for their companion animals including permethrin-based products and fipronil spot-on treatments, but admitted to inconsistent use. They had received professional pest control treatments with pyrethroid-based products, but also had been inconsistent with following a treatment regimen. Under ideal conditions, the brown dog tick can complete an entire life cycle in 3 months (Goddard 1987). That would indicate, if resistance were selected for at the Gilchrist F2

strain location, this population may have evolved resistance to two different classes of chemicals in 12 generations.

The Broward strain demonstrated high levels of permethrin resistance and the highest level of fipronil tolerance from all tested strains. This strain lacked exposure to broadcast acaricide applications in the submitters' home prior to tick submission. A permethrin-based topical spot-on treatment was used on the dog during summer months, but had not been used for eight months prior to the documented infestation. There was no reported history of fipronil use. This resident submitted 240 engorged nymphs, collected over a two night period, indicating a heavy tick load on the five indoor dogs. The dogs had a history of regular groomer visits, which could have been a source of tick exposure. Given the treatment history of this strain, it suggests that the ticks were under selection pressure for permethrin and fipronil resistance prior the infestation at the Broward residence.

Other field-collected pesticide-resistant arthropods lacking previous exposure history have been documented. Field-collected *B. germanica* with no previous fipronil exposure history where resistant, which was attributed to previous cyclodiene resistance conferred by similar mechanisms (Holbrook et al. 2003). Historically, residential areas have been treated with pesticides that have now been found to confer cross-resistance. Mallis (1954) recommended the use of DDT powder on dogs twice a week and pyrethrum dips with piperonyl butoxide. Indoor areas were also treated DDT or pyrethrum with piperonyl butoxide. Chlordane sprays and dusts also were used as limited spot treatments and approved for use on dogs and in kennel areas.

Chlordane and DDT were used frequently for cockroach control (Mallis 1954) increasing the likelihood of residual pesticide in the residential environment. Carpet samples were evaluated from nine homes in Jacksonville, FL, with two samples being collected from two homes, to test for residual pesticides (Whitmore et al. 1994). Dieldrin (mean 2.2 ul/g) was detected in 10 out of 11 carpet dust samples and DDT (mean 1.2 ul/g) was detected in nine samples (out of 11). Dieldrin and chlordane have been found to confer cross-resistance to fipronil (Holbrook et al. 2003) and DDT has been confirmed to confer cross-resistance to permethrin (Brengues et al. 2003).

For current control procedures, pest control operator protocols and recommendations in Goddard (2011) suggested two treatments over a one month period for brown dog tick control. The first treatment should control active tick life stages, while the second serves to treat newly emerged larvae from egg hatch. Some home owners that have submitted brown dog tick samples to the University of Florida for resistance testing reported control failures after professional pest control applications of pyrethroid products in addition to on-animal treatment. The Sarsota-2 population, which demonstrated 56% survival at 53x the discriminating concentration of permethrin and 0% survival at the fipronil discriminating concentration, were collected from a residence that used Frontline® for on-animal control and the home was professionally treated with several pyrethroid products (Appendix A); however, the home owner reported it took one year to control the infestation.

Due to the challenges in determining the existing resistance status when an infestation began, as well as any prior acaricide exposure, further laboratory testing is necessary to determine the rapidity with which resistance can be selected. With the

variation of susceptibility levels to permethrin and to a lesser extent, fipronil, it is believed different mechanisms that confer resistance have evolved and these mechanisms should be studied. Understanding these mechanisms can aid in the development and planning of effective control programs.

Table 3-1. Acaricide exposure in field-collected *Rhipicephalus sanguineus* (Latreille) as reported by pet owners or kennel operators.

Strain	Permethrin	Fipronil
Broward	Sporadic on-animal topical treatment 8 months prior to submission	None
Collier-2	None, but outdoor area had been treated, possibly with a pyrethroid	None
Gilchrist	Inconsistent on-animal topical treatment, nonspecified pyrethroids indoors	Inconsistent on-animal topical treatment
Palm Beach-2	Indoor area treated with non-permethrin pyrethroid	On-animal topical treatment
Palm Beach-3	Unknown	Unknown
Sarasota-1	Indoor treatment with non-permethrin pyrethroids	On-animal topical treatment monthly for one year
Sarasota-2	On-animal topical treatment intermittently and indoor foggers	On-animal topical treatment monthly for two years

Table 3-2. Lethal concentration (LC) values of permethrin and the discriminating concentration 2x LC_{99} using Logit analysis for susceptible *Rhipicephalus sanguineus* (Latreille) purchased from Ecto Services, INC.

Test	n[a] (replicates)	LC_{50} (95% CI)[b]	LC_{99} (95% CI)[b]	Slope (SEM)	χ^2 (df)	Discriminating concentration[b]
1	4,098 (44)	0.028 (0.026-0.029)	0.099 (0.086-0.117)	8.31 (0.34)	61 (38)	0.20
2	4,308 (44)	0.025 (0.024-0.026)	0.082 (0.048-0.073)	8.98 (0.31)	53 (38)	0.16
3	5,240 (40)	0.027 (0.026-0.028)	0.098 (0.089-0.108)	8.20 (0.28)	39 (34)	0.20
4	2,839 (27)	0.027 (0.026-0.028)	0.087 (0.077-0.101)	9.00 (0.36)	52 (22)	0.17
Pooled	16,485 (155)	0.027 (0.026-0.027)	0.093 (0.087-0.099)	8.42 (0.15)	230 (138)	0.19

[a] n represents total number of ticks tested per experiment and replicates represents the total number of packets
[b]Values represent percentage of active ingredient (w/v) applied to treated filter papers 7.5 x 8.5 cm

Table 3-3. Lethal concentration (LC) values of fipronil and the discriminating concentration 2x LC_{99} using Probit analysis for susceptible *Rhipicephalus sanguineus* (Latreille) purchased from Ecto Services, INC.

Test	n[a] (replicates)	LC_{50} (95% CI)[b]	LC_{90} (95% CI)[b]	LC_{99} (95% CI)[b]	Slope (SEM)	χ^2 (df)	Discriminating concentration[a]
1	4,213 (44)	0.020 (0.018-0.021)	0.038 (0.036-0.042)	0.067 (0.060-0.077)	4.30 (0.16)	58 (38)	0.13
2	4,089 (40)	0.024 (0.023-0.025)	0.041 (0.038-0.044)	0.063 (0.057-0.070)	5.55 (0.16)	67 (34)	0.13
3	3,642 (27)	0.024 (0.022-0.025)	0.047 (0.045-0.051)	0.084 (0.076-0.093)	4.22 (0.11)	55 (25)	0.17
4	5,141 (27)	0.024 (0.023-0.025)	0.046 (0.042-0.050)	0.076 (0.068-0.086)	4.68 (0.11)	82 (25)	0.15
Pooled	17,085 (138)	0.023 (0.023-0.024)	0.044 (0.042-0.046)	0.074 (0.069-0.081)	4.67 (0.06)	438 (128)	0.15

[a] n represents total number of ticks tested per experiment and replicates represents the total number of packets
[b]Values represent percentage of active ingredient (w/v) applied to treated filter papers 7.5 x 8.5 cm

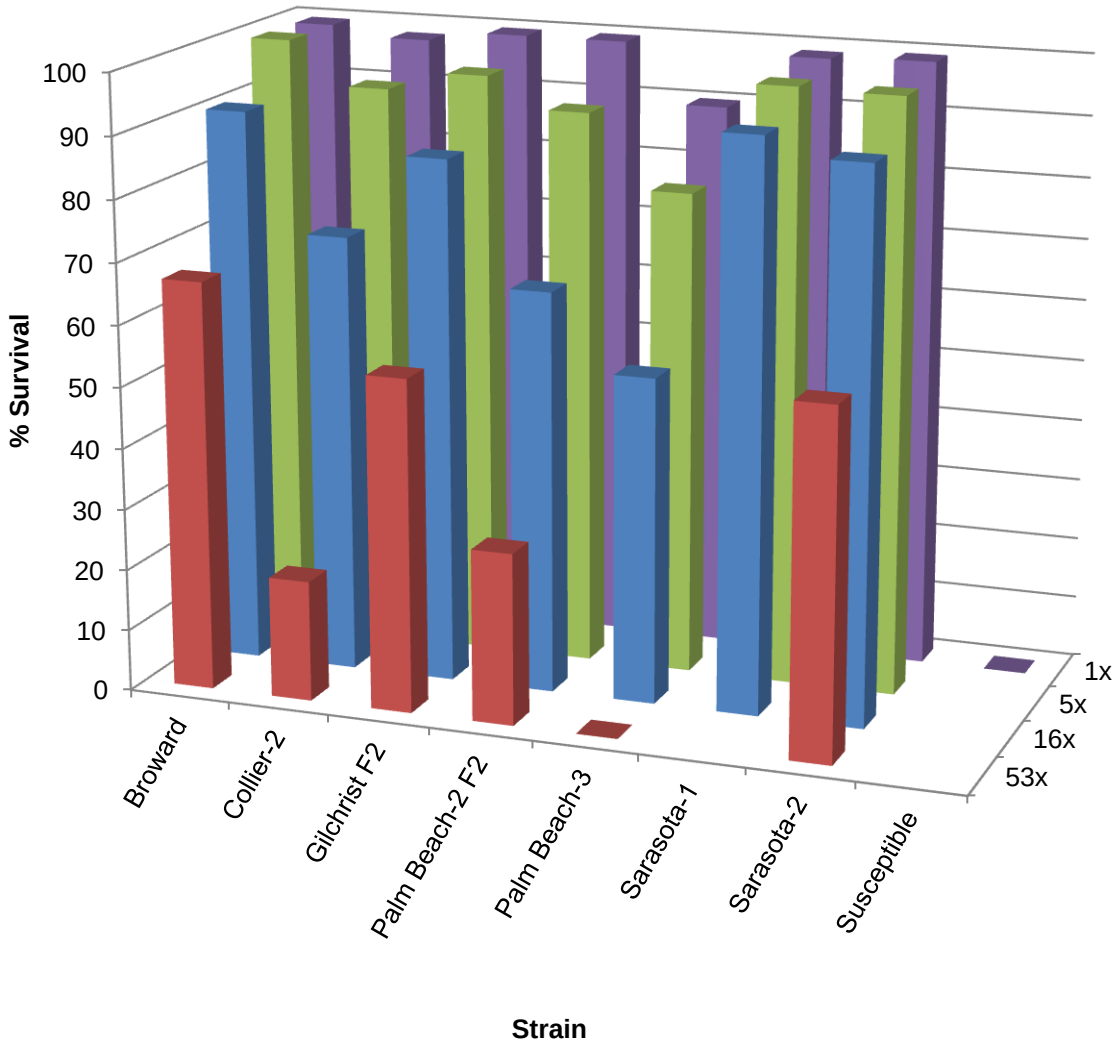


Figure 3-1. Mean percentage survival of *Rhipicephalus sanguineus* (Latreille) strains in Florida exposed to the discriminating concentration of permethrin at 1, 5, 16, and 53 times the LC_{99} value (0.19% concentration) using the Food and Agriculture Organization larval packet test and an acaricide-susceptible tick strain from Ecto Services INC, NC.

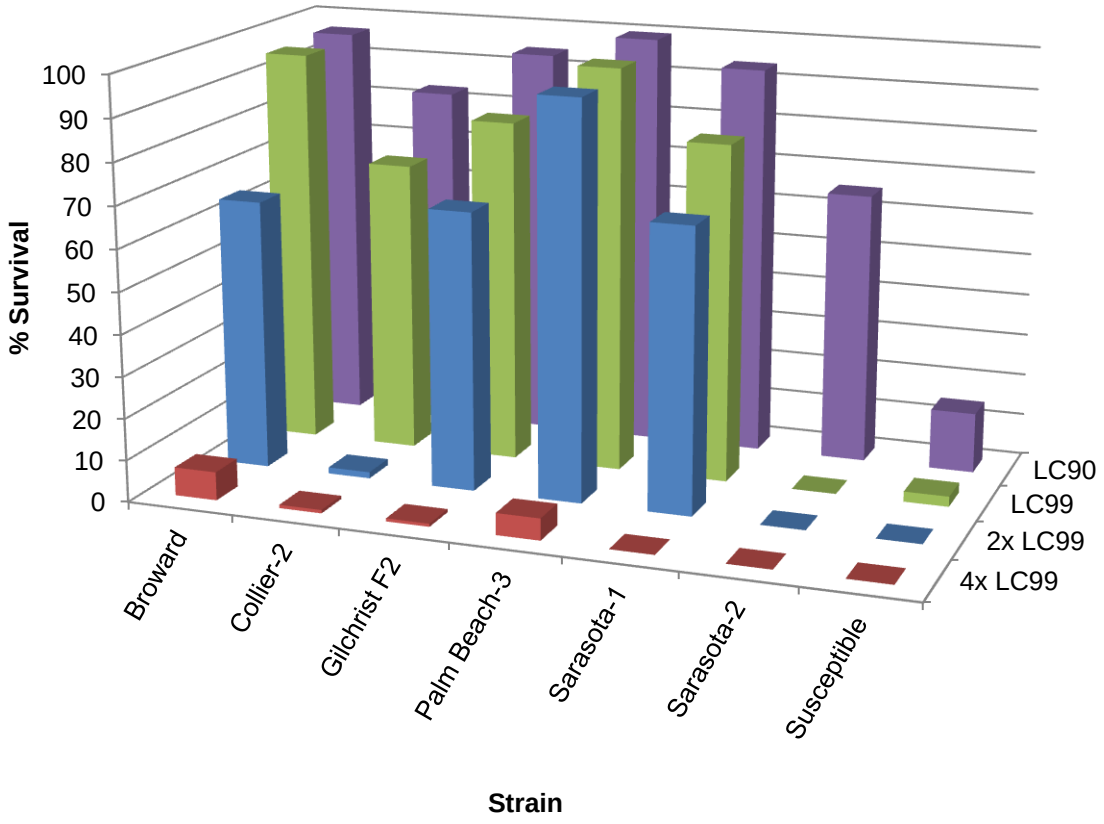


Figure 3-2. Mean percentage survival of six Florida *Rhipicephalus sanguineus* (Latreille) strains exposed to fipronil at the LC_{90}, LC_{99} (0.15% concentration), 2x LC_{99} (the discriminating concentration), 4x LC_{99} using the Food and Agriculture Organization larval packet test and an acaricide-susceptible tick strain from Ecto Services INC, NC.

CHAPTER 4
DETERMINATION OF METABOLIC AND TARGET SITE RESISTANCE MECHANISM IN PYRETHROID AND FIPRONIL RESISTANT BROWN DOG TICKS

Introduction

The brown dog tick, *Rhipicephalus sanguineus* (Latreille), is an arthropod pest that can be brought into a home by canine owners, where it is able to complete its life cycle (Lord 2001, Demma et al. 2005). In susceptible brown dog tick populations, permethrin was shown to provide long-term protection against tick feeding (Endris et al. 2002b). Permethrin is a member of the pyrethroid class of acaricides that acts on the sodium channel causing neuroexcitation that eventually leads to tick death (Narahashi 1986). Permethrin is used for tick control in dogs as a topical spot-on treatment, shampoo, and an on-animal spray. It also is used as an in-home treatment as a residual crack and crevice spray that targets ticks as well as fleas, cockroaches, and bed bugs. However, with several formulations and a long history of use, permethrin resistance may emerge as demonstrated in *R. sanguineus* strains described in Chapters 2 and 3, as well as has been previously demonstrated in several strains of *Rhipicephalus microplus* (Canestrini) (Rosario-Cruz et al. 2009, Morgan et al. 2009). Target site mutations and increase esterase activity both have been found to confer resistance.

Fipronil is in the phenylpyrazole family of chemicals and is the active ingredient in Frontline®, a topical treatment used on companion animals for tick and flea control since the late 1990's. Fipronil acts on gamma-aminobutyric acid (GABA)-gated chloride channels to block the flow of chloride ions, leading to neuroexcitation (Zhao et al. 2003). Fipronil resistance is frequently linked to a target site mutation. Metabolic resistance is unlikely because the metabolites of fipronil retain their toxicity (Zhao et al. 2005). Given

the broad label use and frequent documentation of permethrin resistance, recent generic fipronil formulations sold at reduced prices, which many pet owners have begun to use, may increase fipronil resistance selection.

Increased metabolic activity has been implicated in arthropod pesticide resistance. Cytochrome P450, esterase, and glutathione-*S*-transferase (GST) are important metabolic enzymes found in all organisms. Cytochrome P450s are located within the endoplasmic reticulum and carry out oxidative reactions that confer pesticide resistance in arthropods (Feyereisen 2005, Yu 2008). Cytochrome P450s metabolize compounds by binding to an atom of oxygen from another molecule, which forms water (Bergé et al. 1998). In arthropods, these enzymes catalyze pesticide metabolism via hydroxylation, *N*-demethylation, and *O*-demethylation (Yu 2008). Cytochrome P450s also are important for activation of pro-insecticides such as organophosphates. Important activation reactions include epoxidation, sulfoxidation, and desulfuration.

Increased cytochrome P450 production has been found to occur through different physiological aberrations. Amichot et al. (2004) found DDT-resistant *D. melanogaster* that demonstrated elevated cytochrome P450 production due to a point mutation. Other genetic modifications that have been demonstrated to increase P450 production include gene over-expression, which has been confirmed in *D. melanogaster* (Daborn et al. 2000). Over-expression of the *Cyp6g1* gene increased P450 production, conferring DDT and neonicotinoid resistance, which fall under different chemical classes and modes of action.

Esterase activity detoxifies molecules containing ester bonds. Carboxylesterase and phosphatase are important esterases for pesticide metabolism (Yu 2008).

Pesticide toxicity may be reduced by esterases through enhanced detoxification and sequestration (Devonshire and Moores 1982). In the peach-potato aphid, *Myzus persicae* Sulz, esterase sequestration conferred carbamate and organophosphate resistance. Low levels of organophosphorus and carbamate hydrolysis were found using radiometric methods, but increased esterase was present. Field et al. (1988) found in broadly insecticide resistant *M. persicae,* resistance levels were correlated with the amount of increased gene amplification. Increased esterase activity due to a point mutation also has been found in permethrin resistant *R. microplus* (Hernandez et al. 2000).

Glutathione-S-transferases have been found to detoxify numerous toxicants in arthropods by conjugating glutathione with electrophilic substrates (Yu 1996, 2008). As reviewed by Sun et al. (2001), GST confers organophosphate resistance in several arthropods. Pesticide resistance conferred by increased GST activity may be the result of different physiological activities. Glutathione-*S*-transferase may bind to pesticides, which reduces pesticide toxicity by sequestration (Kostaropoulus et al. 2001). Sequestration was found in a deltamethrin resistant strain of *Tenebrio monitor* L., the yellow mealworm. Additionally, increased GST gene amplification was found in organophosphate resistant *M. domestica* (Syvanen et al. 1996). As reviewed by Li et al. (2007), gene over-expression also elevated GST levels in DDT- and pyrethroid-resistant *Aedes aegypti* L.

The suppression of esterase, P450 and GST activity have been found in *Rhipicephalus bursa* (Canestrini and Fanzago) in Iran (Enayati et al. 2010). In addition to enhanced enzyme activity, resistance to pyrethroids in arthropods has been

documented from target site mutations occurring in the sodium channel. Pyrethroid resistance due to target site insensitivity and carboxylesterase-mediated metabolism was documented in *R. microplus* in Mexico (Jamroz et al. 2000).

Characterization of the target site mutations that confer resistance in *R. microplus* have been investigated in several studies. Guerrero et al. (2002a) found that resistant cattle ticks possess sodium channels having either a single mutation in populations that were resistant to up to 30% permethrin or two mutations in ticks that demonstrated even higher levels of resistance. Sodium channel mutations were found in both populations at the S6 transmembrane segment of domain III that lead to a phenylalanine to isoleucine amino acid substitution. The more highly resistant strain also had increased esterase activity (Guerrero et al. 2002b). In another *R. microplus* strain, a sodium channel mutation in the domain II S4-5 linker gene caused an amino acid substitution from leucine to isoleucine in resistant strains (Morgan et al. 2009).

Target site mutations have been found to confer resistance in various insect species, but have not been documented in brown dog ticks. Two genetic mutations were found in the GABA receptor of a laboratory-selected, fipronil-resistant strain of *Drosophila simulans* Sturtevant (Le Goff et al. 2005). Both mutations occurred in the *Rdl* (resistance to dieldrin) GABA subunit, the first being an alanine to glycine substitution in the M2 domain, and the second a theonine to methionine mutation, which occurred in the C-terminal end of the M3 segment. In the M2 domain, ffrench-Constant et al. (1993) found a point mutation in the same location of *Drosophila melanogaster* Meigen that converted alanine to serine and conferred high levels of resistance. Nakao

et al. (2010) reported that fipronil-resistant *Sogatella furcifera* (Horváth) had an A2'N mutation in the GABA receptor.

As demonstrated in Chapters 2 and 3, various levels of permethrin resistance and fipronil tolerance were found in *R. sanguineus*. In this chapter, the metabolic mechanisms of resistance and target site mutations that previously have been found to confer resistance to permethrin and fipronil in other species were evaluated in the brown dog tick using synergist bioassays, protein gel electrophoresis, and polymerase chain reaction (PCR).

Materials and Methods

Ticks

The larvae used were obtained either from field-collected, engorged adult females or flat adults that were reared at the University of Florida (Appendix A, tick strain information; Appendix B, UF IACUC Protocol 200802142; 201102142, Appendix C, tick rearing protocol). Susceptible ticks were obtained from Ecto Services, INC (Henderson, NC) for bioassay screening and gel electrophoresis. Ticks were held in an incubator at 25 ± 1°C, 85% RH, with a photoperiod of 12:12.

Five permethrin-resistant field-collected brown dog tick strains were utilized in permethrin synergist assays and three strains in fipronil synergist assays. Initially, a high dose acaricide selection process was to be used to select for a homozygous resistant genotype. The selection process included rearing ticks from strains that survived the highest treatment doses in toxicity bioassays. However, this selection process was not feasible due to poor tick survival in the laboratory.

A portion of field-collected larval ticks that were not previously exposed to acaricides in the laboratory and susceptible ticks were placed in a vial and preserved at

-80°C for gel electrophoresis. In all, nine strains of ticks were preserved for gel electrophoresis evaluation. Protein extracted from a negative control strain of *R. microplus,* that did not exhibit increase esterase activity, was obtained from USDA-ARS Cattle Fever Tick Research Laboratory, Edinburg, TX for gel electrophoresis comparison.

Larval ticks that survived acaricide doses exceeding 7% permethrin or 0.3% fipronil were placed in vials, labeled as high dose survivors, and held at -80°C for PCR testing. Twelve strains were preserved. Additionally, ticks that were untested with acaricides were preserved for comparison.

Synergist Bioassays

Metabolic resistance mechanisms were examined using synergists that block detoxification mechanisms. The following synergists were used to evaluate metabolic resistance and have been modeled after Miller et al. (2007b). Piperonyl butoxide (PBO) (Sigma-Aldrich, St. Louis, MO) was used to examine cytochrome P450 inhibition. Diethyl maleate (DEM) (ChemService Inc. West Chester, PA) was used to inhibit glutathione-*S*- transferase (GST). Triphenyl phosphate (TPP) (ChemService Inc., West Chester, PA) was used to determine the presence of esterase activity.

The synergists were added at a constant rate during serial dilution procedure for the larval packet test previously described in Chapter 2 (FAO 2004). The concentration of synergist used was determined by the results of an initial experiment that involved the serial dilution of the synergist into a base solution of two parts trichloroethylene (TCE) and one part olive oil. A top dose of 5% for a synergist was used and serially diluted the lowest percentage at 0.14%. This range included levels above and below 1%, which is

used in synergist assays for *R. microplus* (R. J. Miller *unpublished data*). A dilution rate of 30% and 40% was used during two experiments with each synergist. After testing various synergist levels, a constant rate of 2% for each synergist was determined to cause sufficiently low mortality in *R. sanguineus* that it was used in further testing. An experiment was conducted using all three synergists in one solution to standardize the method. However, when all three synergists were combined high mortality was observed at all concentrations.

The synergist screening assays consisted of a small scale experiments for individual *R. sanguineus* strains. Screening experiments for permethrin (98.8%, *cis*, *trans*, 40.1:58.7) (ChemService Inc., West Chester, PA) consisted of the concentrations 10, 3, 0.9, and 0.18% active ingredient. If insufficient tick numbers were present, the 10% concentration was removed from the experimental run. For fipronil (ChemService Inc., West Chester, PA) testing, 0.3, 0.15, 0.07, and 0.04% active ingredient concentrations were used. For both acaricides three to four replicates were completed for each concentration, based on available tick numbers.

Separate stock solutions were made (w/v), using permethrin or fipronil. Each acaricide was paired with a synergist that was added at a constant rate to all dilutions. There were six treatment combinations of acaricide (permethrin and fipronil) and synergist, PBO (v/v), TPP (w/v), DEM (v/v) plus four controls. The four control treatments were used with each run, one contained only TCE and olive oil, termed the solvent, while the remaining three contained the solvent with one respective synergist.

Serially diluted solutions were applied to filter papers (7.5 x 8.5 cm) (Whatman® No. 1, Maidstone, England) at 1 mL of solution per paper. The filter papers were dried

for two hours under a fume hood allowing the TCE to evaporate. Dried filter papers were folded in half and the sides were clipped together with bulldog clips (Chapter 2, Fig. 2-2). Larval ticks, 14 to 16 days post-eclosion, were placed in each packet. A third clip was used to secure the open end. If tick numbers were available, ~100 ticks were used per packet, but in some cases fewer larval ticks were available. After tick placement, packets were held in an environmental chamber for 24 hours at 25 ± 1°C, 92% RH, with a photoperiod of 12:12 (L:D) (Chapter 2, Fig. 2-3). The packets were removed and mortality was assessed after 24 hours. Each packet was opened and the number of live and dead ticks were counted and recorded (Chapter 2, Fig. 2-4).

Tick mortality was compared between each synergist exposed treatment and ticks exposed to acaricide alone to determine if metabolic resistance was present. If a decrease in mortality was observed in synergist-containing packets, when compared to packets without a synergist, a replicated experiment using up to eight serial dilutions of these treatments was conducted to calculate a dose response curve. If results from the bioassays indicated esterase derived resistance, native gel electrophoresis was used for confirmation. Susceptible *R. microplus* and *R. sanguineus* tick protein was used as a control.

Gel Electrophoresis

The gel electrophoresis technique used to determine increased esterase activity in permethrin resistant *R. sanguineus* strains was adapted from Miller et al. (2007b). From each of the nine tick strains tested, 40 larvae that had not been exposed to acaricides since collection and were held at -80°C were pulverized in a 1.5 mL microcentrifuge tube with a pre-chilled pestle over dry ice. The susceptible strain of *R.*

sanguineus (Ecto Services, INC.) and susceptible *R. microplus* (Deutch strain) that did not exhibit increase levels of esterase production were used for comparison.

Pulverized larvae were homogenized with 100 µL of a protein extraction buffer that was held at 4°C. The buffer consisted of 100 µL 0.1 M sodium phosphate buffer (pH 7.5), 25 µL 20% TritonX-100 (Sigma-Aldrich®, St. Louis, MO), 286 µL 70% w/v sucrose, 2 µL 0.5 M ethylenediaminetetraacetic acid (EDTA) pH 8 (Thermo Fischer Scientific Inc., Waltham, MA) solution, and 588 µL water. The EDTA solution was made by combining 18.6 g EDTA, 2.2 g sodium hydroxide pellets, and 80 µL water. Nanopure water was used to make all solutions. The homogenate was centrifuged for 2 minutes at 14,000 rpm to separate tick protein from the rest of the homogenate. An aliquot of the supernatant (10 µL) was extracted from the microcentrifuge tube, above the pellet. The supernatant aliquot was combined with 10 µL Novex® Tris-Glycine Native 2x Sample Buffer (Life Technologies™, Carlsbad, CA) into a 2 mL microcentrifuge tube and vortexed until a uniform purplish color was achieved. The remaining supernatant was divided into 10 µL aliquots and placed into multiple microcentrifuge tubes and stored at -20°C to be used in later experiments, as needed.

Samples were loaded into a 4% acrylamide stacking gel with a 10% acrylamide separating gel (Bio-Rad, Hercules, CA). The gel was placed into a clamp system and secured with glass plates to hold the running buffer in place as described below. Prior to initiating the experiment, the running buffer was placed in between the glass plates to assure the clamps were secure. A pipette was used to remove air bubbles and to ensure running buffer was present in all of the wells. Ten µL of Kaleidoscope™ pre-stained standard (Bio-Rad, Hercules, CA) was loaded into the far left lane to serve as a

size reference. Ten µL of the supernatant containing the loading buffer was then dispensed with a pipette into each well. The gel was run with a 1.0 molar (M) Tris-glycine running buffer prechilled to 4°C, on a Bio-Rad Sub-Cell® GT Agrarose Gel (Bio-Rad, Hercules, CA). The running buffer was made from100 mL of Bio-Rad 10x Tris-glycine buffer (Bio-Rad, Hercules, CA), 200 mL methanol (Thermo Fisher Scientific, Waltham, MA, >99.8%) and 700 mL nanopure water. The gel was run at 200 v, 40 mA at 4°C.

After running the gel, a gel knife was used to remove it from the casing. The gel was then placed into a glass container for staining. To visualize esterase activity, the gel was incubated for 1 hour at 37°C in 50 mL of the staining solution. The staining solution described below consisted of Fast Blue BB salt (Sigma-Aldrich®, St. Louis, MO) and β-naphthyl acetate (Sigma-Aldrich®, St. Louis, MO). Staining solutions were made with a sodium phosphate buffer that was prepared first. Sodium phosphate monobasic (Thermo Fisher Scientific, Waltham, MA) and sodium phosphate dibasic (Thermo Fisher Scientific, Waltham, MA) were made into a 1 M solution with 120 g/liter of nanopure water (LabStar, Siemens, Munich, Germany) and 142 g/liter of nanopure water, respectively. One liter of sodium phosphate buffer (pH 7.5) was made by adding 16 mL of sodium phosphate monobasic to 84 mL of sodium phosphate dibasic. A pH meter was used to confirm that the pH was 7.5. Adjustments were made as needed by adding 1 M hydrochloric acid to increase the acidity or 1 M sodium hydroxide to produce a more basic solution.

A gel-staining solution was produced by adding 200 mg of Fast Blue BB salt to 200 mL of 0.1 M sodium phosphate buffer (pH 7.5). The solution was vortexed for one

minute, then filtered using a Corning® 250mL Vacuum Filter (Corning Incorporated, Corning, NY). ß-naphthyl acetate (60 mg) was made into solution by the addition of 1 mL of acetone. The Fast Blue BB salt and ß-naphthyl acetate solutions are light and temperature sensitive and their holding containers were wrapped in aluminum foil and held at 4°C. Solutions for the protein assays were prepared in advance and held separately until they were ready for use. Before use, 50 mL blue fast salt was combined with 500 µL of ß-naphthyl acetate. The concentration of the final solution was 0.1 M sodium phosphate buffer, which contained 100 mg/mL Fast Blue BB salt and 15 mg of β-naphthyl acetate. Fast blue BB salt and ß-naphthyl acetate are highly toxic therefore, full PPE (lab coat, gloves, goggles, N95 mask) were worn and chemicals were mixed under the fume hood to prevent inhalation.

In order to confirm esterase activity and its inhibition with triphenyl phosphate (TPP), a second inhibition gel assay was run with the same protein samples used in the standard staining procedure. An inhibitory staining solution containing TPP was made. One gram of TPP was added to 10 mL of acetone, then vortexed to produce the stock solution (3.3×10^{-4} M). Thereafter, 16.5 µL of the TPP inhibitory staining solution was added to 50 mL Fast Blue BB salt with 500 µL of ß-naphthyl acetate. The gel was incubated at 37°C on a rotator table (50 rpm) (Barnstead/LabLine MaxQ™ 5000, Thermo Scientific, Waltham, MA) for 15 minutes in the inhibitory staining solution containing 50 mL of 0.1 M sodium phosphate buffer and 0.33 mM TPP to test for esterase activity inhibition.

After a 15 minute holding period the inhibitory staining solution was removed and the standard staining solution (0.1 M sodium phosphate buffer, 100 mg/mL Fast Blue

BB salt, 15 mg of β-naphthyl acetate) was added to the container with the gel. The gel box container was incubated on a rotator table (50 rpm) at 37°C for 1 hour.

The gel staining solution and the gel were added to an airtight glass container under a fume hood. The glass container was covered by aluminum foil to prevent inactivation of the light sensitive solution. The inhibitory and non-inhibitory gels were incubated at 37°C for 1 hour on a rotator table (50 rpm) in order to keep the solution continuously moving over the gel. After 1 hour the staining solution was poured into a hazardous waste container for disposal. Thereafter, 50 mL of a destaining solution was added to the glass container. The staining solution consisting of 400 mL of methanol, 100 mL of acetic acid, and 500 mL of nanopure water. The gel with the destaining solution was placed on the rotator table (50 rpm) for 1 hour at room temperature. After the gel was removed it was photographed on disposable plastic film and the gel was disposed of in the hazardous waste bin, using good laboratory practice standards.

Polymerase Chain Reaction

Tick preparation

When possible, ticks that survived the top two doses of acaricide from Chapter 2 larval packet test experiments were used for PCR testing. In all cases, the larval and adult ticks were preserved at -80 °C. Degradation of tick DNA to detect target site mutations occurs rapidly, so dead ticks were not held off dry ice for more than two to three minutes during PCR preparation.

DNA isolation

Methods from Guerrero et al. (2001) or the QIAamp® DNA Mini Kit (Venlo, Limburg) kit were used for DNA extraction. Extraction of DNA was completed on single larval ticks, pools of 10 and 20 larval ticks from the same strain, and single adult ticks.

These latter extractions were utilized to obtain larger concentrations of DNA. Microcentrifuge tubes containing larval or adult ticks described earlier were placed on dry ice and the following procedures were conducted on dry ice. Liquid nitrogen prechilled pestles were used to pulverize larval ticks by grinding for 15 to 20 seconds. Tubes were briefly removed from the dry ice, and while keeping the pestle inside the tube, 25 µL of Perkin-Elmer PCR reaction buffer II (DNA isolation buffer) was added. The DNA isolation buffer was made in 10 mL batches by adding 1,667 µL of 3 M KCl, 600 µL of 1 M Tris-Cl (pH 8.5), 400 µL of 1 M Tris-Cl (pH 8.0) and 7,333 µL of nanopure water. Following the addition of the DNA isolation buffer, tick grinding was continued for an additional 15 seconds or until the ticks were broken apart. The pestle was removed and discarded and tubes were returned to the dry ice. When each tick specimen preparation was completed contents were concentrated to the bottom of the tube using a table top centrifuge for five seconds. Tubes were placed in a water bath, at 100°C, for five minutes then removed and cooled on ice for one minute. Tubes were centrifuged at 14,000 rpm at 4°C for five minutes to prepare samples for DNA amplification.

DNA amplification

Amplification methods developed by Guerrero et al. (2001) were used and optimized as needed. Using this approach, 1 µL of DNA from a single tick, 20 pmol of each primer (1 µL), 12.5 µL PCR Master mix (PCR Master, Roche Applied Science, Mannheim, Germany), and 9.5 µL PCR grade water, to achieve a 1x concentration, were added to a 0.5 mL microcentrifuge tube. The tubes were then placed into a Applied Biosystems Veriti™ Thermal Cycler (Applied Biosystems, Foster City, CA) PCR conditions were optimized based on each set of primers used. After DNA was

processed, 1 μL of a gel loading dye (DNA Gel Loading Buffer Loading Buffer (10x), 5 Prime, Gaitherburg, MD), 4 μL, 1x Tris/Borate/EDTA (TBE), and 5 μL of amplified DNA were mixed then dispensed onto an agarose gel. The gel was made by mixing 1x TBE with agarose, to achieve a 1 to 2.5% concentration, depending on PCR product size, then heated until agarose dissolved. GelRed (Phenix Research Products, Candler, NC) was added at rate of 1μL per 10 mL of agarose and TBE solution, which varied based on the size of the gel box. Once agarose solution cooled to 55°C, it was poured into the gel box to set for 30 minutes. Once the DNA product was loaded into the gel wells, the gel was run at 120 volts and photographed under UV light.

DNA primers

The *Ixodes scapularis* Say genome has been fully sequenced, while several genes significant to this work have been sequenced for *R. microplus*. Highly conserved sequenced portions of the two tick genomes served as a starting point to create PCR primers for the *R. sanguineus* sodium channel. All genetic sequences were obtained from GenBank. For sodium channel degenerate base pair primer design the following sequences were used, *Ix. scapularis* (XM_002407075), *R. microplus* (AF134216.2), and *Varroa destructor* Anderson and Trueman (AY259834.1). The chloride channel portion of the glutamate receptor for *R. sanguineus* has been sequenced (GV659520.1) (GQ215235.1), and served as a positive control.

Three regions that were targeted for sodium channel mutation detection were in Domain III in segment S6 and Domain II in the S4-5 linker region, which have been determined to confer resistance in *R. microplus* (He et al. 1999b, Morgan et al. 2009, Jonsson et al. 2010). Two programs were used to create the primers for the brown dog

tick sodium channel locations that were predicted to have target site mutations. The first approach used degenerate base pairs, wherein the sequences were aligned for *Ix. scapularis, R. microplus,* and *V. destructor* using Clustal Omega Multiple Sequence Alignment (EMBL-EBI, Cambridgeshire, UK). Primers were selected based on highly conserved regions. The primers were designed using IDT® OligoAnalyzer 3.1 (Integrated DNA Technologies, Coralville, IA).

The second primer design method used either *Ix. scapularis* or *R. microplus* sequences. Primers were selected around Domain II and III of the sodium channel using Primer3 (Whitehead Institute for Biomedical Research, Cambridge, MA) after the degenerate primers were unsuccessful at producing a PCR product (Rozen and Skaletsky 1998).

For a positive control, susceptible (Fernandez) and resistant (San Felipe) *R. microplus* were obtained from the USDA-ARS Cattle Fever Tick Research Laboratory, Edinburg, TX. Water was used for the negative control. For the resistant control ticks, killed *R. microplus* samples were obtained, while extracted DNA was provided for the susceptible control, both shipped on dry ice. The susceptible and resistant control *R. microplus* primers at the mutation site in Domain III of the sodium channel can be found in Guerrero et al. (2001). Primers were designed to discriminate and replicate PCR products based the presence or absence of a target site mutation. Each PCR assay was conducted with a resistant and susceptible strain for comparative purposes with the *R. sanguineus* samples. An additional primer was used that amplified the gene sequence that overlapped the mutation that should be present for both strains.

Given the fipronil tolerance levels observed earlier, target site resistance was not evaluated in the chloride channel (Chapter 2 and 3), but should still be evaluated in further research. However *R. sanguineus* putative glutamate-gated chloride channel mRNA previously had been sequenced (GV659520.1) (GQ215235.1). Primers using both IDT® OligoAnalyzer 3.1 and Primer3 were used to standardize the PCR protocol and develop a *R. sanguineus* positive control.

In the GABA-gated chloride channel, several mutations confer fipronil resistance. The *Rdl* (resistance to dieldrin) GABA subunit in *D. melanogaster, D. simulans* and the M2 subunit in *Sogatella furcifera* Horváth have been identified (ffrench-Constant et al. 1993, Le Goff et al. 2005, Nakao et al. 2010). Susceptible (Oregon-R) and resistant (*Rdl* 1675) *D. melanogaster* obtained from the University of Florida, Emerging Pathogens Institute were used to serve as a model for detecting a point mutation that confers resistance to channel blocking convulsants and insecticides. Primers used to cover the point mutation region are published in Le Goff et al. (2005). *Rhipicephalus sanguineus* has not been yet evaluated using PCR in the GABA subunit.

Optimization of PCR protocol was done for each set of primers. A list of primers can be found in Appendix D (Table D-1). The troubleshooting technique that was employed included using DNA extracted from eight tick strains using two extraction methods, from a single larva, pools of 10 and 20 larvae and single adults. Two different master mix products were used with three primer concentrations. A 10 temperature gradient test was completed to determine the best annealing conditions using five different ticks, to account for variations in PCR products that were detected in early experiments. The PCR products were run on agarose gels of varying agarose

concentrations depending on targeted product size. If bands were present that corresponded to the expected product size, DNA extraction was conducted using QIAquick® Gel Extraction Kit (Qiagen®, Venlo, Limburg). The extracted DNA concentration was measured using the Qubit® 2.0 Fluorometer (Invitrogen™, Carlsbad, CA). When proper DNA concentrations were not present for direct sequencing, the sample DNA was cloned using TOPO® TA Cloning® Kit with pCR™ 2.1 Vector and One Shot® TOP19F' Chemically Competent *E. coli* (Invitrogen™, Carlsbad,CA). After DNA was cloned, it was isolated using a Qiagen® QuickLyse Miniprep Kit (Qiagen®, Venlo, Limburg). The isolated product was stored at -20 °C. A gel was run with 5 μL of isolated DNA to determine if the cloning process amplified the target DNA. If the gel determined the proper product size and a DNA concentration of 20 ng/μl was available, the PCR product was sent for Sanger sequencing at University of Florida Interdisciplinary Center for Biotechnology Research. Isolated DNA (15 μL) was placed in a 1.5 mL microcentrifuge tube and transported on ice.

Statistics

The LC_{50} was generated for the synergist and non-synergist acaricide using Probit analysis, (PoloPlus©, LeOra Software, Petaluma, CA) (LeOra 1997). Synergist ratios were calculated (LC_{50} of strain not containing synergist / LC_{50} of strain containing synergist) to determine the change in mortality.

Results

The greatest mortality increase for the Broward strain occurred following the addition of TPP to the permethrin-exposed ticks (Fig. 4-1). Mortality was increased at all doses in which TPP was added; however, the greatest mortality increases occurred

at the highest permethrin concentrations of 0.9 and 3%, where 100% mortality was recorded. In the Gilchrist F2 strain, the individual inclusion of each synergist to permethrin resulted in increased mortality (Fig. 4-2), but TPP had the greatest impact on increasing mortality (89% at 0.9% permethrin). A synergist ratio could not be calculated for this tick strain because data did not fit the Probit model due to low mortality at high concentrations for the permethrin treated ticks. Permethrin plus TPP increased mortality in the Palm Beach-2 strain at concentrations exceeding 0.9% but no difference was detected at lower concentrations (Fig. 4-3). Sarasota-2 strain permethrin plus TPP results were similar to the Broward and Gilchrist F2 strain, wherein mortality increased from 50 to 97% at all permethrin concentrations tested (Fig. 4-4).

A concentration response experiment was conducted with the Gilchrist, FL and Webb, TX tick strains to assess the effects of adding TPP and PBO to permethrin. Although the data did not fit the Probit model, mortality was impacted by synergist type, albeit quite differently between the two strains (Fig. 4-5). In the Webb, TX tick strain, mortality increased from 8% in the 0.15% permethrin alone treatment to 100% in the permethrin plus TPP treatment (Fig. 4-5), indicting esterase is an important mechanism of resistance.

Mortality in the Broward strain increased two- to three-fold at the higher permethrin concentrations with the addition of PBO (Fig. 4-1). In the Gilchrist F2 strain the synergist ratio could not be calculated due to low mortality at high concentrations with permethrin alone; however, mortality data indicate an increased in mortality in all doses with the addition of PBO (Fig. 4-2). The Palm Beach-2 F2 strain demonstrated an increase in tick mortality at all permethrin concentrations in which PBO was added

(Fig. 4-3). There also was an increase mortality in the Sarsota-2 with >15% in the 0.9 and 3% permethrin plus PBO concentrations (Fig. 4-4). The Webb, TX F2 strain had a PBO synergist resistance ratio of 6.6 (Table 4-1).

The effects of DEM slightly decreased mortality for the Broward strain from 2.5 and 4.5% at concentrations of 0.9 and 3% permethrin, respectively (Fig. 4-1). Diethyl maleate resulted in the smallest increase mortality (7 to 18%) among the three synergists in Gilchrist F2 ticks (Fig. 4-2). The mortality increase was observed in the Palm Beach-2 F2 strain DEM in concentrations exceeding 0.18%, but less markedly than the effect of TPP and PBO (Fig. 4-3).

Similar results in the effect of synergists were observed in both the Broward and Gilchrist F2 strains, where the addition of synergists did not result in increased in tick mortality at 0.04 and 0.07% fipronil concentrations (Figs. 4-6 and 4-7). However, at 0.15% fipronil, increased tick mortality was observed. In the Sarasota-2 tick strain, TPP and PBO similarly increased mortality at 0.03% fipronil to 18 and 8% respectively. The addition of DEM resulted in decreased fipronil toxicity at the 0.03 and 0.07% concentrations, when compared to fipronil alone (Fig. 4-8). A concentration response curve could not be calculated for most fipronil and synergist assays. The Gilchrist F2 strain fipronil and DEM resistance ratio was 0.57 at the LC_{50} (Table 4-1). When mortality in the Sarasota-2 strain tick was compared between fipronil alone and 2% DEM with 0.07% fipronil there was a decrease in mortality from 100% to 55% (Fig. 4-7). The use of DEM also decreased mortality at 0.03% fipronil, although not to full susceptibility.

Further evidence for increased esterase activity was examined in eight field-collected strains and compared to the acaricide-susceptible tick strain using gel electrophoresis methods that exhibit esterase activity to β-naphthyl acetate. Five of the eight strains evaluated with gel electrophoresis also were tested for esterase activity using the synergist assays described previously. The three additional strains had high levels of resistance documented in previous experiments, with survival at 10% permethrin. Two bands of increased esterase activity were demonstrated in all field-collected strains, with the exception of the Marion strain (Figs. 4-9 and 4-10). As indicated by stained proteins on the gel, the Broward and St. Johns strains demonstrated the greatest expression of increased esterase, followed by the Palm Beach-2 F2 strain. Elevated esterase activity also was present in Sarasota-2 and Gilchrist F2 strains and the least activity was observed in Collier-2 and Marion strains. The susceptible strain that served as the negative control, demonstrated low levels of esterase activity.

The addition of TPP greatly inhibited esterase activity in the top bands of all strains (Fig. 4-10). With the addition of TPP, a middle band was able to be visualized during the inhibition assay but appeared inhibited in all strains except St. Johns and Broward. The bottom band, which was less prominent in the gel stained without TPP, remained active in the Palm Beach-2 F2, Broward, and Collier-2 strains with the addition of TPP.

The *R. microplus* acaricide resistance PCR assay that was used to standardize the DNA extraction, demonstrated amplification of the predicted product size. Additionally, primers designed to discriminate susceptible and resistant genotypes

presented bands accordingly (Fig. 4-11). The primer set designed to cover the entire gene, regardless of a target site mutation, was present in DNA from both strains. Sequencing was not completed due to a low yield of DNA after gel extraction. The original DNA concentration in the positive control ticks, which were extracted at the USDA-ARS Cattle Fever Tick Research Laboratory, contained 19.6 ng/μL with an A260/A280 ratio of 2.294. The DNA that was extracted at University of Florida from larval ticks contained 55.0 ng/μL with an A260/A280 ratio of 1.264. After DNA was amplified using select primers and extracted, concentrations were <5 ng/μL.

The primers designed to discriminate target site mutation locations proved to be ineffective at amplifying *R. sanguineus* DNA for the targeted region. In the glutamate-gated chloride channel that was used as a positive control, *R. sanguineus* primers did not amplify DNA of correct product size. The sodium channel experiments that used degenerate primers and primers based individually off *I. scapularis* and *R. microplus* sequences also were unsuccessful at amplifying target DNA. In total, 41 primer sets were evaluated. Bands were present in several experiments; however, the size of the band did not correspond with the expected product size. When bands that corresponded to the desired product size were observed, they were extracted and sent for Sanger sequencing (Fig. 4-12). The returned sequence was tick DNA but did not correspond with the desired replication sites.

Primers designed to detect the *Rdl* mutation in the GABA-gated chloride channel in *D. melanogaster* were effective at replicating the target DNA (Fig. 4-13) and the DNA was sent for Sanger sequencing. The first two products sequenced were from a single wild-type and a single *Rdl*-resistant fly. The mutation was not detected in the resistant

strain during the first sequencing attempt. The following two samples that were sequences contain pools of five resistant flies. Three mutations were detected. The first mutation location corresponded to the M2 region that was found in and ffrench-Constant et al. (1993) (Fig. 4-14). Analysis using CLC Genomics Workbench 4.9 (CLC bio A QIAGEN® company, Cambridge, MA) determined two prominent peaks are presented in the sequencing data, indicating two different base pairs in the populations. One base pair from the pooled sequences was a guanine, which is found in the susceptible strain, while the others was a thymine that confers an amino acid change from an alanine to a serine (A302S). The second mutation was in the first base pair of the amino acid sequence and resulted in the replacement of valine with isoleucine (V283I) (Fig. 4-15). The third mutation altered the third base pair in the amino acid sequence, but did not result in an amino acid change (Fig. 4-16).

Discussion

Determining resistance mechanisms is important to help formulate brown dog tick control treatment plans. For example, many, but not all, pyrethroid products are formulated with the synergist PBO. Knowing if cytochrome P450 activity confers resistance is, therefore, important to anticipate the effectiveness of the PBO additive. Metabolic resistance mechanisms were found in several of the permethrin-resistant and fipronil-tolerant brown dog ticks evaluated in this study. Increased esterase, cytochrome P450 and glutathione-*S*-transferase metabolic mechanisms evaluated in the current study may be simultaneously up-regulated, as has been found to occur in *Sarcoptes scabiei* (L.) (Pasay et al. 2009). In several tick strains, two or more mechanisms were present. In the Gilchrist F2 strain, inhibition of all three resistance mechanisms increased mortality at all concentrations of permethrin tested (Fig. 4-2).

Inhibition of both esterase and cytochrome P450 activity increased mortality at all permethrin concentrations in the Broward strain and at 3 and 10% permethrin in the Palm Beach-2 F2 strain.

The results demonstrated that in all tested permethrin resistant strains of brown dog ticks cytochrome P450 was implicated as a resistance mechanism; however susceptibility was not completely restored by the additional of synergists. Additional studies should be completed that implement other synergists in order to evaluate other possible isozymes that could be conferring resistance. Pimprikar and Georghiou (1979) found variations in cytochrome P450 inhibition with the use of sesamex versus PBO. Resistant results in this chapter also could indicate other resistance mechanisms are present because the inhibition of one metabolic enzyme seldom restored full acaricide susceptibility. Scott and Georgiou (1986) determined that in pyrethroid-resistant *M. domestica,* cytochrome P450 provided 32-fold increase in resistance but the resistance mechanisms that conferred >6,000-fold resistance included a target site mutation and reduced cuticular penetration. In that strain of resistant *M. domestica,* high levels of P450 reductase and cytochrome b_5 were present.

The greatest increase in permethrin synergist-induced mortality was found in the *R. sanguineus* bioassays that included TPP, which inhibits esterase activity. An increase in mortality following the addition of a synergist indicates that the mechanism blocked was conferring resistance. Gel electrophoresis methods also confirmed the presence of increased esterase activity in the Broward, Gilchrist F2, Palm Beach-2 F2, Sarasota-2, and Webb TX F2 strains. In the synergist screening assays the Webb, TX strain demonstrated the greatest increase in mortality with the inhibition of esterase, but

expressed lower levels of esterase with gel electrophoresis than the other resistant strains in this study. The Collier-2 strain, which was unable to be screened with synergists due to insufficient tick numbers, demonstrated slightly less activity in the top band (Fig 4-9); however in previous studies (Chapter 3), presented 20% survival at 10% permethrin. Our assays did not detect esterase activity in the Marion tick strain. However, this tick sample may have been compromised due to tick samples thawing during processing. Permethrin resistance due to increased esterase activity also was found in various strains of the closely related cattle tick, *R. microplus*, using similar TPP synergist bioassay and gel electrophoresis experiments (Miller et al. 1999). High esterase activity has been demonstrated as the sole source of permethrin resistance in *R. microplus* (Pruett et al. 2002), but also has been found in conjunction with target site mutations (Rosario-Cruz et al. 2009). Increased esterase activity also has been demonstrated to be absent in the presence of high levels of resistance where only target site mutations conferred resistance (Miller et al. 2007b). It is possible that only target site mutations may be present in the Collier-2 tick strain.

Mortality for the Webb, TX F2 strain was near 100% for all concentrations of permethrin when TPP was added, negating my ability to calculate a synergist ratio and indicating that upregulation of esterase activity was an important mechanism for conferring permethrin resistance. Changes in mortality from the Sarasota-2 strain were not observed at 0.18% permethrin, while the other strains tested demonstrated up to 50% increased mortality at this concentration. These widely divergent results suggest that TPP mechanisms are not widely distributed among tick populations. There are no commercially-available esterase inhibitors on the market, making field comparisons

impractical. The development of esterase inhibitors would be advantageous for brown dog tick management.

In the present study, GST activity was not a major cause of resistance in comparison to cytochrome P450 and esterase activity based upon, the lack of increased mortality when it was inhibited with a synergist. However, in the Gilchrist F2 and Palm Beach-2 F2 permethrin-resistant strains, inhibited GST activity restored some level of susceptibility. Pyrethroid resistance via GST binding, resulting in sequestration has been documented in *T. molitor* (Kostaropoulos et al. 2001). Sequestration may be occurring in those brown dog tick strains demonstrating increased mortality with GST inhibition.

In the Sarasota-2 strain, which was determined to be susceptible to fipronil, the inhibition of GST activity resulted in decreased mortality. Fipronil has been documented to be metabolized by GST and cytochrome P450 in the western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Scharf et al. 2000). Three additional fipronil metabolites were detected in GST *in vitro* metabolic studies that are believed to be the result of dehalogenization. Li et al. (2007) did not find a significant difference in GST levels in fipronil-susceptible and resistant strains of *Chilo suppressalis* Walker, suggesting an alternate fipronil resistance mechanism. It is unclear why a decrease in fipronil mortality was found in the Sarasota-2 strain when GST activity was inhibited. The increase in mortality in the Broward and Gilchrist F2 could be a result of increased acaricide penetration with the addition of a synergist.

In brown dog ticks there was an increase in mortality with the inhibition of cytochrome P450 in permethrin-resistant strains, which indicates P450's as a

detoxification method. However, cytochrome P450's only increased fipronil mortality in the Broward and Gilchrist F2 strains at 0.15% fipronil. Cytochrome P450 synergist inhibition assays resulted in variation between fipronil-tolerant and -susceptible *C. suppressalis* (Huang et al. 2010). Tolerant strains demonstrated increased mortality with cytochrome P450 inhibition, while no difference was demonstrated in the susceptible strain. The exposure to fipronil and PBO decreased susceptible strain mortality, which authors suggested was due to decreased fipronil to fipronil-sulfone conversion, whereas the increase in mortality in the susceptible strain was hypothesized to be related to increased cuticle penetration. More rapid penetration may decrease the time required for efficient detoxification. Sun and Johnson (1972) found evidence of increased cuticular transport of pesticides, with the use of certain synergists. In *M. domesica* organochlorine, organophosphorus, and carbamate susceptibility were evaluated using four synergists in both injection and topical applications. Results determined that Thanite and kerosene increased carbamate mortality due to increased penetration. Engorged ticks could be used to conduct injection studies, to evaluate the effects of the synergist penetration. Additional synergist assays with brown dog ticks should be completed using both flat and engorged ticks to determine if cuticle expansion may impact susceptibility.

Fipronil metabolized cytochrome P450 into fipronil sulfone in *D. virgifera* (Scharf et al. 2000) and in the Eastern corn borer, *Ostrinia nubilalis* Hübner (Durham et al. 2002). Fipronil sulfone was documented to have increased toxicity compared to fipronil, indicating cytochrome P450 detoxification is unlikely to play a role in fipronil resistance (Zhao et al. 2005, Fang et al. 2008). The increase in mortality could be the result of the

toxicity from the original compound and metabolite. However, increased mortality was demonstrated with the addition of all three synergists at 0.15% fipronil, which could suggest dehalogenization or increased cuticle penetration.

A decrease in *B. germanica* mortality for six strains, including susceptible and resistant strains, was documented when fipronil was synergized by PBO and *S,S,S*-tributylphosphorotrithioate (DEF), an esterase inhibitor (Ang et al. 2013). However, Gondhalekar and Scharf (2012) found that PBO increased mortality in a fipronil resistant *B. germanica* strain, while decreasing mortality in the susceptible strain. These variations indicate the evolution of different resistance mechanisms in independent populations.

Tang et al. (2010) suggested that increased GST and esterase activity may be implicated in laboratory-selected fipronil resistant strains of the planthopper, *Sogatella furcifera (*Horváth) using synergist studies. No change in mortality was documented with cytochrome P450 inhibition synergist assays; however increased P450 levels were detected in the fipronil resistant population with enzyme assays. Although all three metabolic mechanisms were present, it was suggested that they may not be the cause of fipronil-resistance and that target site mutation studies should be completed (Tang et al. 2010). The physiological mechanisms in *S. furcifera* that would result in decreased mortality with the presence of the fipronil metabolite, fipronil sulfone, were unclear.

My permethrin resistance synergist studies suggest that commercially-available treatments such as Sentry® Pro Flea & Tick Shampoo for dogs (Sentry Pet Care Products, Inc., Omaha, NE) that is formulated with AI 0.10% permethrin, 0.50% piperonyl butoxide, 0.01% pyriproxyfen and Sector™ (MGK®, McLaughlin Gormley King

Company, Minneapolis, MN) label rate 0.5% AI permethrin and PBO, would be ineffective in controlling resistant tick populations. The AI rates of the formulated products fall below the levels required to significantly increase mortality in the tested tick strains. Experiments in the present study used 2% PBO with the closest concentration to compare to this product at 0.18% permethrin. Among the tested strains, all except the Webb, TX strain (10%), had <1% mortality with 0.18% permethrin alone (Figs. 4-1 to 4-5). The addition of PBO increased mortality to levels between 4% and 25%. With these resistant tick strains, Sentry® Pro Flea & Tick Shampoo would not be effective at killing many ticks. Comparisons using topical applications of permethrin and PBO to dogs is challenging as the surface area of dogs, including fur, is quite variable. Although many formulations include permethrin concentrations as high as 44%, the volume applied is small and the dispersed concentration becomes smaller on a per cm^2 basis.

In addition to metabolic resistance, target site mutations have been found to confer resistance in numerous arthropod species, including *R. microplus* (Jamroz et al. 2000). The results presented for the PCR work completed in the current study for *R. microplus* that detected a sodium channel mutation in domain III (S6 subunit) are consistent with Guerrero et al. (2001), who developed the resistance diagnostic method. Susceptible *Rhipicephalus microplus* positive control DNA extracted at the USDA-ARS Cattle Fever Tick Research Laboratory, and the resistant *R. microplus* positive control DNA that was extracted at University of Florida both resulted in faint bands (Fig. 4-11). After gel extraction the DNA concentration from the desired band decreased to levels so low that it was undetectable. It is unclear why, with a detectable band, the DNA

extraction process that was designed to concentrate existing DNA was completely unsuccessful. This process was repeated several times with up to 50 μL of DNA run in each gel well. Regardless of variation, concentrations of DNA were consistently low.

The PCR reactions conducted during this project using *R. sanguineus* were not successfully completed. Several incorrectly sized PCR products were generated and sequenced products that were extracted from bands of the correct size did not correspond with the target sequence. It is unclear why the brown dog tick sodium channel sequences for the two domain II and one domain III sites could not be amplified considering the myriad of protocol alterations that were attempted. The degenerate primers that were designed for the sodium channel based on the *I. scapularis, R. microplus,* and *V. destructor* sequences could have been too dissimilar to the sequences in *R. sanguineus.* However, this also occurred for the glutamate-gated chloride channel, a gene that has been sequenced for *R. sanguineus*, and for that reason was targeted as a positive control. Interestingly, there are two sequences in Genbank labeled as a complete coding sequence for brown dog tick, the first containing 1,907 bp (GQ215234.1) and the second containing 2,400 bp (GP441809.1), however, these sequences do not align. Sequence GQ215235.1 has almost no similarity with any sequences, with the highest match at 2% in *I. scapularis* (Altschul et al. 1990). Sequence GP441809.1 had 40% alignment with *R. microplus* and 48% alignment with *I. scapularis*. Primers designed from both sequences did not produce products at the targeted product size.

The lack of alignment demonstrated with the glutamate-gated chloride channel could have been the reason there was a lack of amplification with degenerate base

pairs, even though they were designed around highly conserved regions. Attempts to optimize the PCR conditions included utilizing temperature variations, two different types of master mix, different concentrations of DNA (10, 20, 50, 100, 150 ng/μL), various primer concentrations (10 pmole and 100 pmole), and combinations of different amplification cycles, all of which did not result in site specific amplification. Interestingly, the DNA extraction method provided positive results for *R. microplus* and PCR amplification did occur for 33 of 41 primer sets with either extraction method, but not in the correct region. Lack of amplification is not thought to be a result of improper extraction methods but it could be a factor.

The detection of the *Rdl* mutation in *D. melanogaster* yielded the same results published in ffrench-Constant et al. (1993). The mutation was not uniformly found in the resistant population. The amino acid from the susceptible strain also was detected. The lack of current insecticide selective pressure may have resulted in the survival of both genotypes in the laboratory colony.

In future studies, PCR could be used to rapidly detect multiple point mutations, as has been done with *R. microplus*. A multiplex PCR was developed by Lovis et al. (2012) to simultaneously evaluate the known sodium channel point mutations in *R. microplus*. There are two mutations in Domain II and one in Domain III. Tick strains from Brazil, Argentina, South Africa, Mexico, and Australia were tested to evaluate the presence of resistance (Lovis et al. 2012). The resistant ticks from Mexico were found to have the sodium channel target site mutation located in Domain III on S6 segment, which was not found outside of Mexican populations. The S6 mutation was linked to higher resistance levels in comparison to ticks with the two Domain II mutations. The

L641 Domain II mutation, originally found in Australia, was not found in the Mexican strains, but was present in resistant populations collected from Brazil, Argentina, and South Africa. The G72V Domain II mutation was only found in resistant ticks from Australia (Lovis et al. 2012).

Molecular technique should be designed for rapid resistance detection assays. A high throughput *Rdl* mutation selection assay was developed for *Ctenocephalides felis* (Bouché) (Daborn et al. 2004). A PCR Restriction Endonuclease analysis produced different products sizes that were used to differentiate between genotypes and a TaqMan diagnostic assay was designed to distinguish between genotypes with fluorescent dye. Both techniques discriminated between resistant, susceptible, and heterogeneous populations (Daborn et al. 2004).

In order to successfully complete molecular research, improved methods of DNA extraction should be evaluated. Studies have evaluated extraction methods where the Qiamp® DNA extraction kit resulted in 77% of the samples being successfully amplified (Halos et al. 2004). With the use of a steel micro-bead shaker to crush ticks, 100% of the samples provided positive amplification; however, this was not used for this study because producing product was not the greatest challenge, but it could become more important in further research.

Ixodid DNA has been found to be vulnerable to degradation (Hill and Gutierrez 2003). The target genes from this study may be susceptible to degradation and their deterioration during DNA extraction could have caused the lack of amplification. Studies are needed to determine the causes of rapid degradation in order to develop improved extraction methods.

Understanding acaricide resistance mechanisms is critical for successful tick management. Increased cytochrome P450 activity can be mitigated with synergists; however, as demonstrated in this study, esterase activity was more prominent for conferring permethrin resistance. It is unclear what provided increased tolerance to fipronil, as the synergists did not greatly increase mortality. Esterase was found to inefficiently hydrolyze organophosphates and carbamates, but pesticide sequestration conferred resistance in *M. persicae* (Devonshire and Moores 1982). Acaricide sequestration may be occurring in brown dog ticks, as the inhibition of esterase activity restored some level of susceptibility to fipronil in the Broward and Gilchrist F2 strains as well as all strains permethrin resistant strains. Additionally, the evaluated synergists have been used for metabolic resistance evaluation in *R. microplus* for several years, but cuticular penetration has not been studied. In order for a synergist to block metabolic activity it must successfully penetrate the cuticle. Future studies also should include a 24-hour synergist exposure period prior to acaricide application in order to determine if mortality is increased. Synergists may aid in increased the penetration of the acaricide.

High dose selection for fipronil resistance would improve our understanding of the effect of synergists, as metabolic activity may increase along with the resistance level. Populations of ticks that demonstrate high levels of metabolic activity should be challenged with additional acaricides in the laboratory to determine if enzymatic activity confers cross-resistance. Control methods must be considered when increased detoxification confers resistance. Ticks with high esterase activity that has been induced by contact with permethrin may be able to metabolize other acaricides with

ester linkage, including other pyrethroids and organophosphates (Yu 2008). Increased metabolic activity can confer resistance to acaricides that have different target sites but contain with structures in which are able to be detoxified by increased metabolic activity. Increased cytochrome P450 activity in *D. melagaster* has been found to confer resistance to DDT, neonicotinoids, and insect growth regulators (Daborn et al. 2007). Information that identifies how best to rotate chemicals to delay resistance should be readily available to pest management professionals, so that selection of further resistance is delayed.

Table 4-1. Evaluation of synergists with permethrin or fipronil conducted on *Rhipicephalus sanguineus* (Latreille) larvae collected from four Florida and one Texas county, using the larval packet test method.

Tick strain	Treatment	Number tested (replicates)	LC_{50} (95% CI) [a]	SR_{50} [b,c]	Slope (SEM)	χ^2(df)
Broward	Permethrin[d]	1,945 (28)	ND[C]	—		
	Permethrin +TPP	1,641 (24)	0.229 (0.178-0.274)	—	3.88 (0.25)	87 (18)
Gilchrist F1	Fipronil	2,408 (24)	0.054 (0.047-0.063)	—	3.56 (27.03)	158 (22)
	Fipronil+DEM	2,655 (32)	0.094 (0.089-0.099)	0.57	10.78 (0.69)	50 (26)
Palm Beach-2 F2	Permethrin[d]	2,346 (32)	ND[C]	—		
	Permethrin +PBO	2,576 (27)	0.900 (0.796-1.021)	—	1.73 (0.06)	43 (22)
Sarasota-2	Permethrin[d]	2,419 (32)	ND[C]	—		
	Permethrin +TPP	2,579 (32)	0.172 (0.150-0.193)		3.67 (0.22)	55 (26)
Webb, TX F2	Permethrin	3,221 (36)	0.833 (0.759-0.908)	—	2.58 (0.20)	32 (30)
	Permethrin +TPP	1,522 (21)	ND[C]			
	Permethrin +PBO	1,596 (21)	0.230 (0.198-0.266)	6.60	2.38 (0.14)	42 (16)

[a] Values represent percentage of active ingredient (w/v) applied to treated filter papers 7.5 x 8.5 cm
[b] Synergist ratios (SR) at LC_{50} were calculated by dividing the LC_{50} or the not synergist assay by the synergist assay
[c] Data did not follow Probit model, but marked changes in mortality were observed
[d] ND: Lethal concentration could not be determined due to low mortality at high concentrations
[e] Unable to calculate with the Probit model due to high levels of heterogeneity

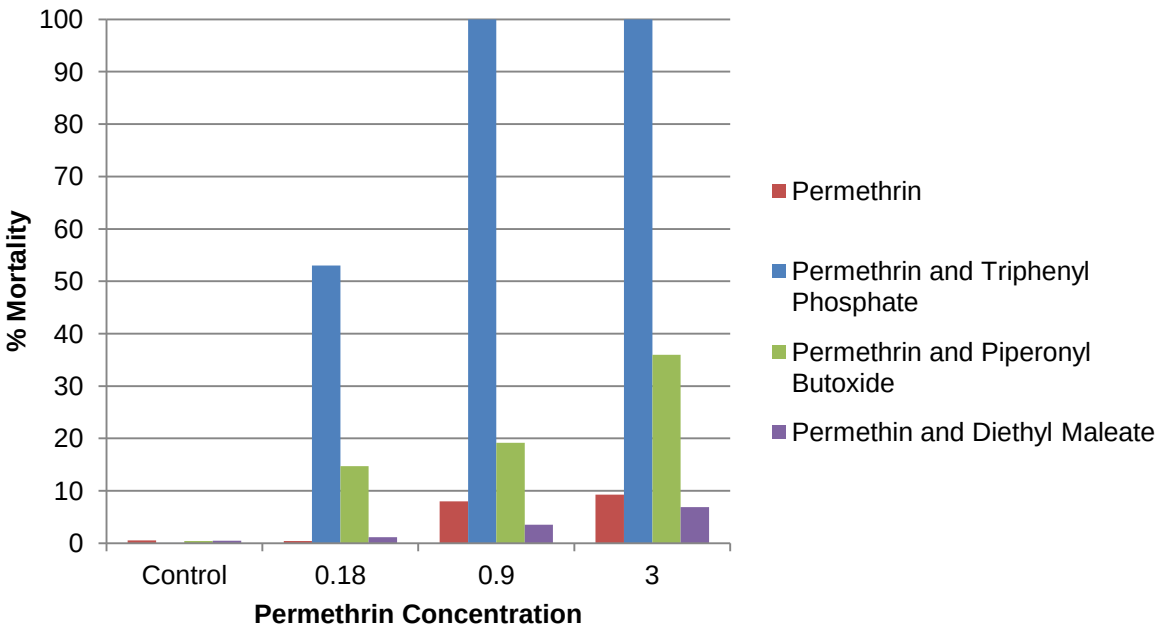


Figure 4-1. Broward strain of larval *Rhipicephalus sanguineus* (Latreille) tested with 2% synergists added at a constant rate to varied concentrations of permethrin in a Food and Agriculture Organization larval packet test to determine synergist effect on mortality. Controls were treated with trichloroethylene and olive oil alone and then combined with each synergist without the acaricide.

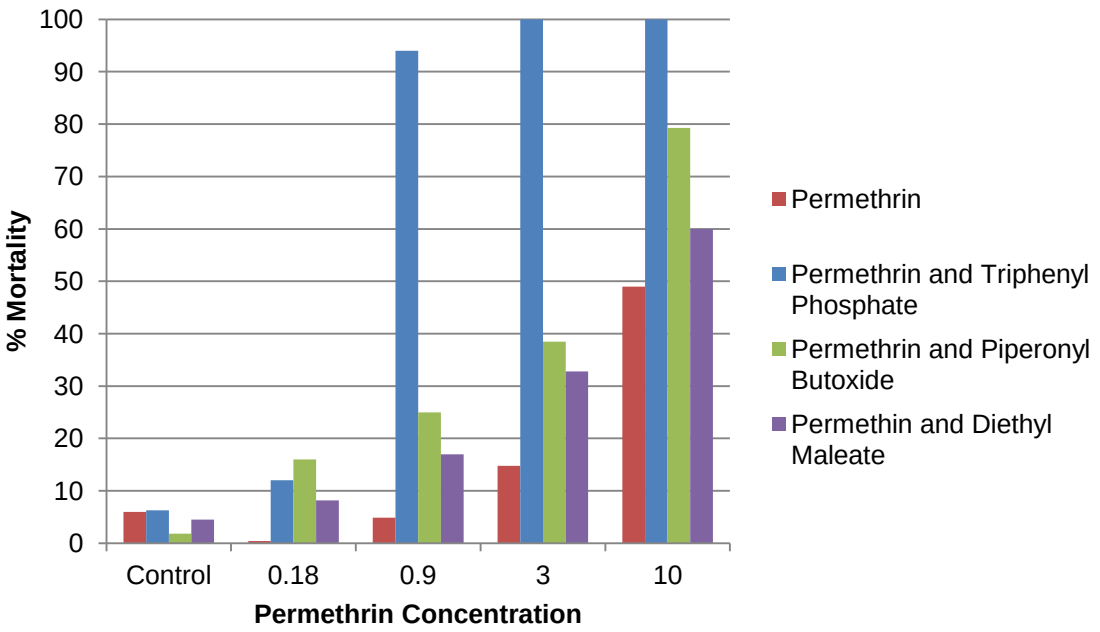


Figure 4-2. Gilchrist F2 strain of larval *Rhipicephalus sanguineus* (Latreille) tested with 2% synergists added at a constant rate to varied concentrations of permethrin in a Food and Agriculture Organization larval packet test to determine synergist effect on mortality. Controls were treated with trichloroethylene and olive oil alone and then combined with each synergist without the acaricide.

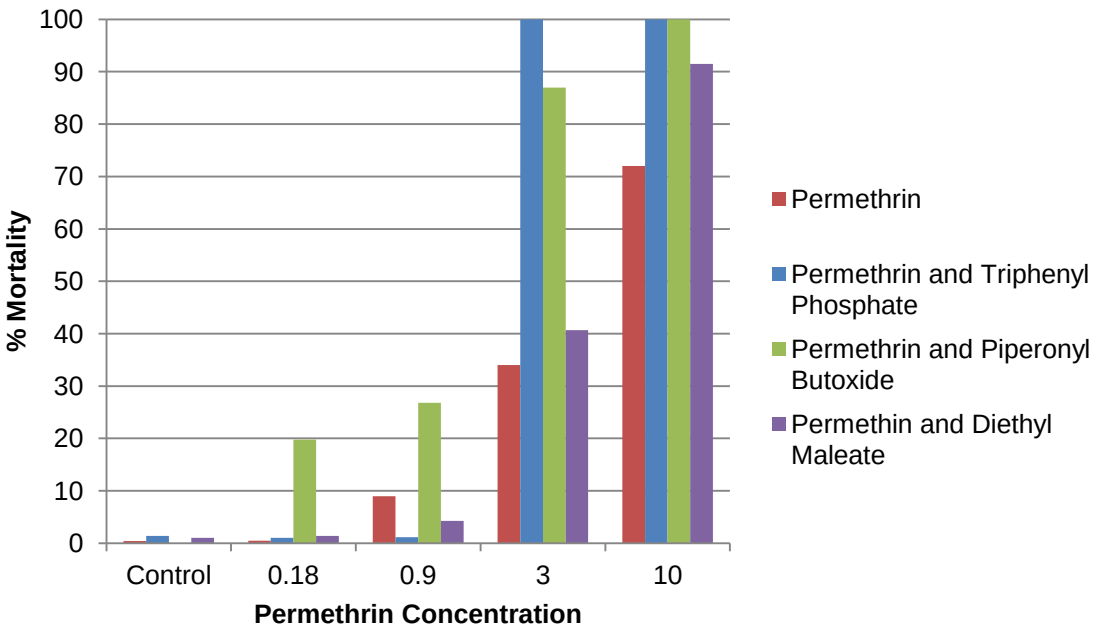


Figure 4-3. Palm Beach-2 F2 strain of larval *Rhipicephalus sanguineus* (Latreille) tested with 2% synergists added at a constant rate to varied concentrations of permethrin in a Food and Agriculture Organization larval packet test to determine synergist effect on mortality. Controls were treated with trichloroethylene and olive oil alone and then combined with each synergist without the acaricide.

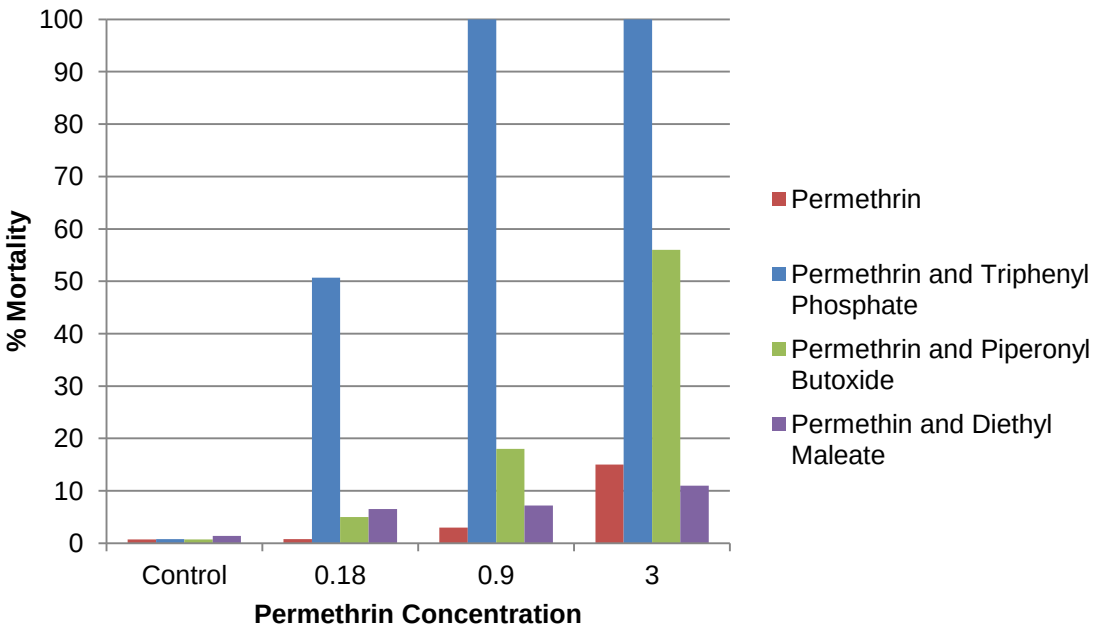


Figure 4-4. Sarasota-2 strain of larval *Rhipicephalus sanguineus* (Latreille) tested with 2% synergists added at a constant rate to varied concentrations of permethrin in a Food and Agriculture Organization larval packet test to determine synergist effect on mortality. Controls were treated with trichloroethylene and olive oil alone and then combined with each synergist without the acaricide.

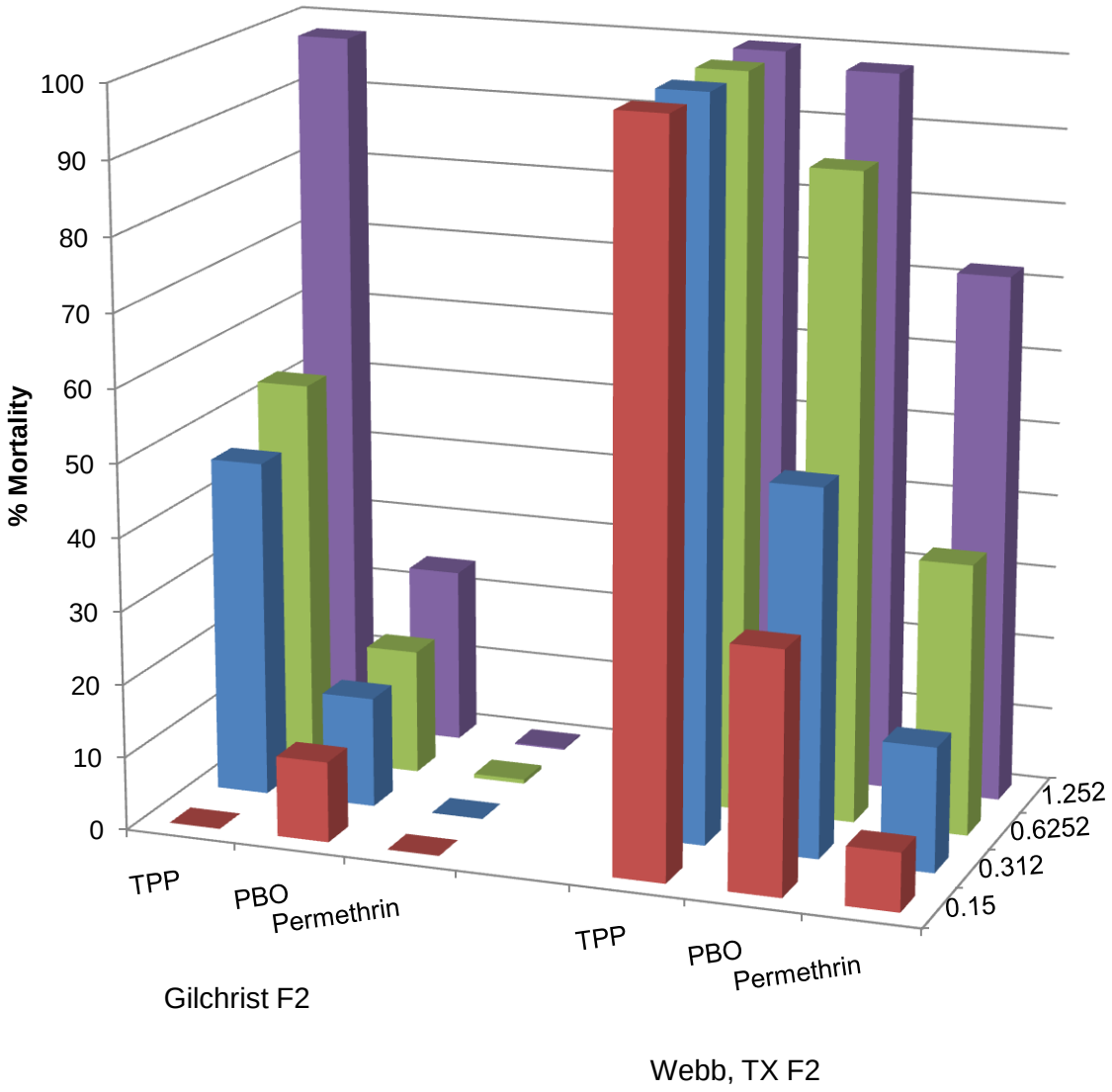


Figure 4-5. Tick mortality responses for the Gilchrist, FL F2 and Webb, TX F2 *Rhipicephalus sanguineus* (Latreille) strains tested using the Food and Agriculture Organization larval packet test evaluating permethrin alone or in combination with 2% triphenyl phosphate (TPP) or 2% piperonyl butoxide (PBO).

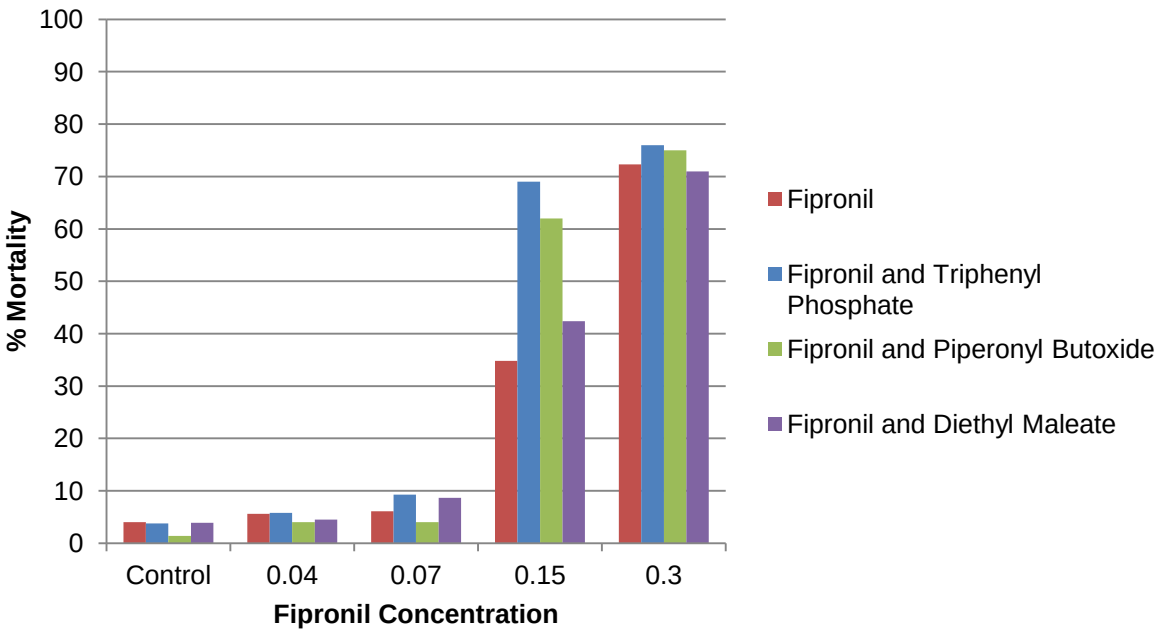


Figure 4-6. Broward strain of larval *Rhipicephalus sanguineus* (Latreille) tested with 2% synergists added at a constant rate to varied concentrations of fipronil in a Food and Agriculture Organization larval packet test to determine synergist effect on mortality. Controls were treated with trichloroethylene and olive oil alone and then combined with each synergist without the acaricide.

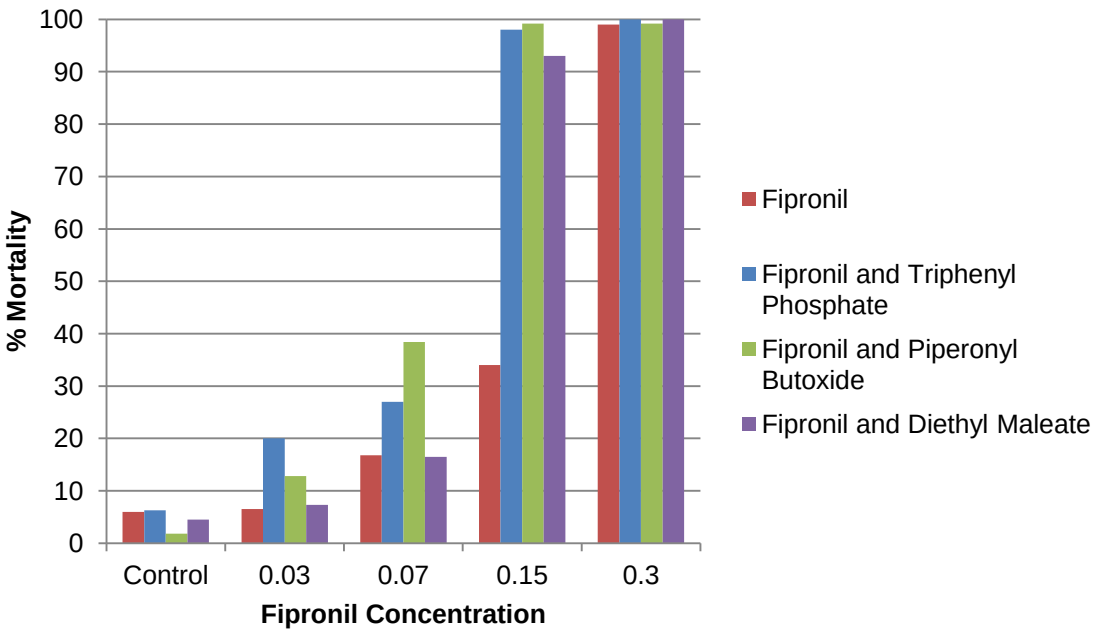


Figure 4-7. Gilchrist F2 strain of larval *Rhipicephalus sanguineus* (Latreille) tested with 2% synergists added at a constant rate to varied concentrations of fipronil in a Food and Agriculture Organization larval packet test to determine synergist effect on mortality. Controls were treated with trichloroethylene and olive oil alone and then combined with each synergist without the acaricide.

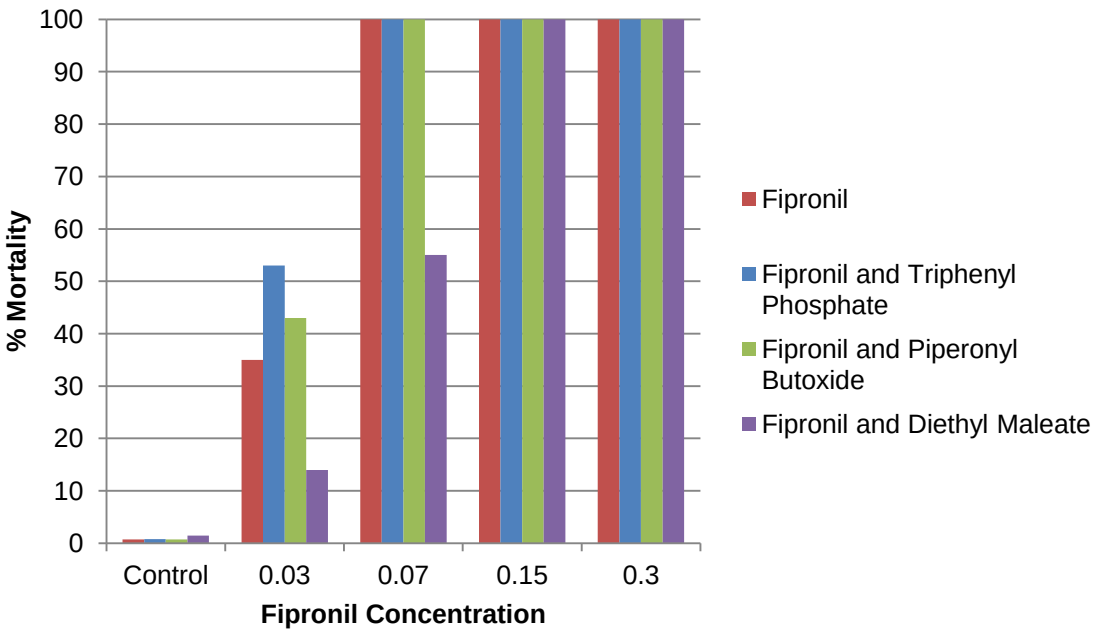


Figure 4-8. Sarasota-2 strain of larval *Rhipicephalus sanguineus* (Latreille) tested with 2% synergists added at a constant rate to varied concentrations of fipronil in a Food and Agriculture Organization larval packet test to determine synergist effect on mortality. Controls were treated with trichloroethylene and olive oil alone and then combined with each synergist without the acaricide.

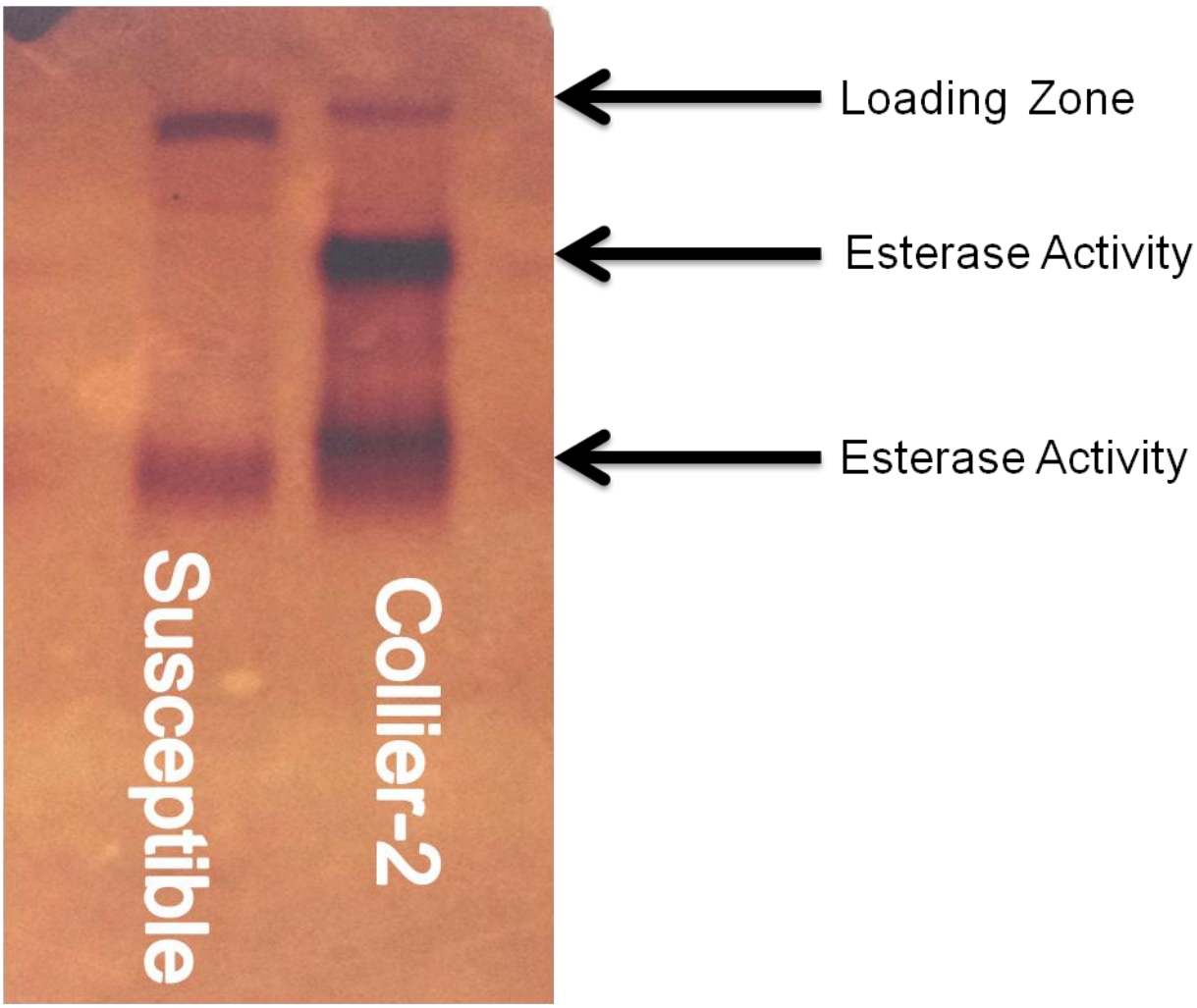


Figure 4-9. β-naphtyl acetate activity, esterase staining gel comparing esterase activity of a *Rhipicephalus sanguineus* (Latreille) susceptible and resistant strain (Collier-2). Two areas of increase activity are found in the resistant strain. Gel electrophoresis esterase staining comparing esterase bands in the susceptible versus the Collier-2 permethrin resistant strain. Photo courtesy of Amanda L. Eiden.

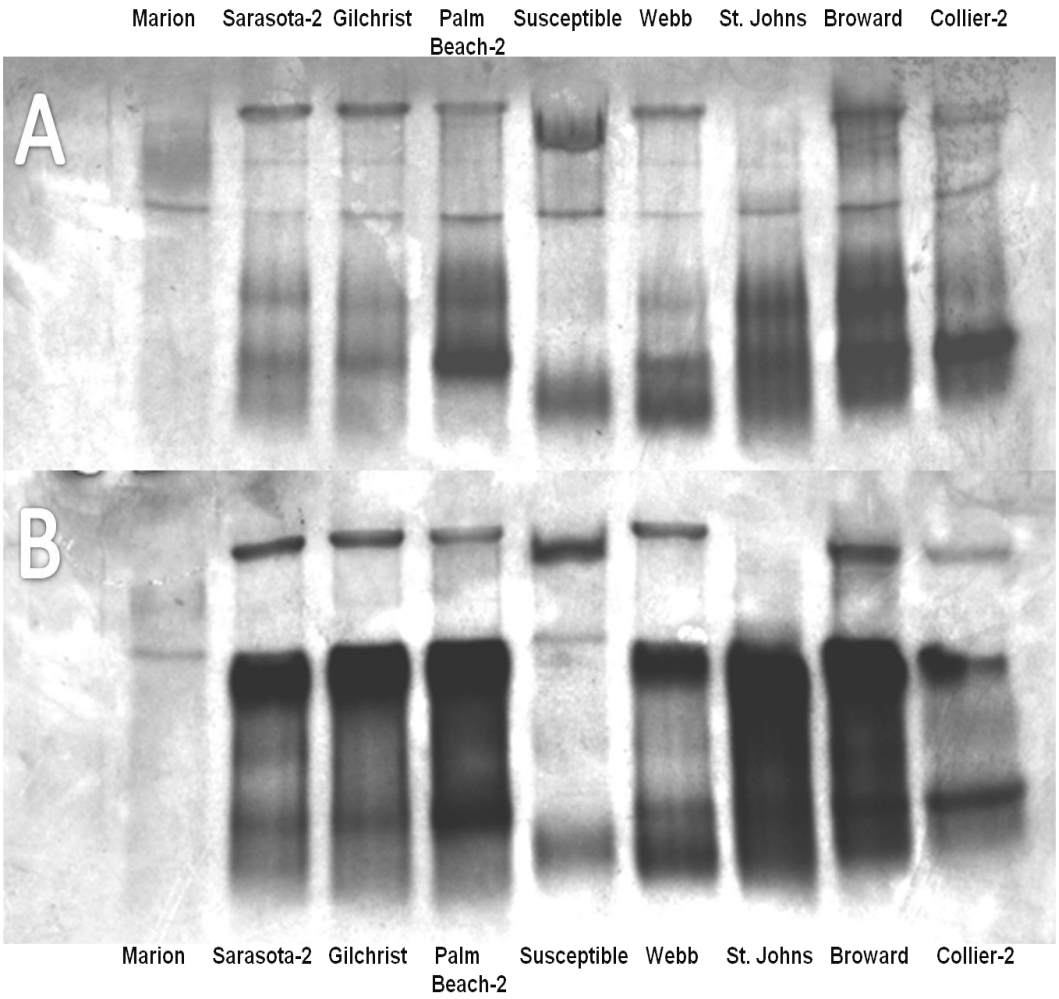


Figure 4-10. Eight permethrin-resistant *Rhipicephalus sanguineus* (Latreille) strains and the permethrin-susceptible *R. sanguineus* strain evaluated for esterase activity using an esterase staining gel to demonstrating activity to β-naphthyl acetate in *R. sanguineus*. A) Esterase activity is inhibited by the addition of triphenyl phosphate where bands are light. B) Esterase activity is detected by indication of thick bands. Photo courtesy of Amanda L. Eiden.

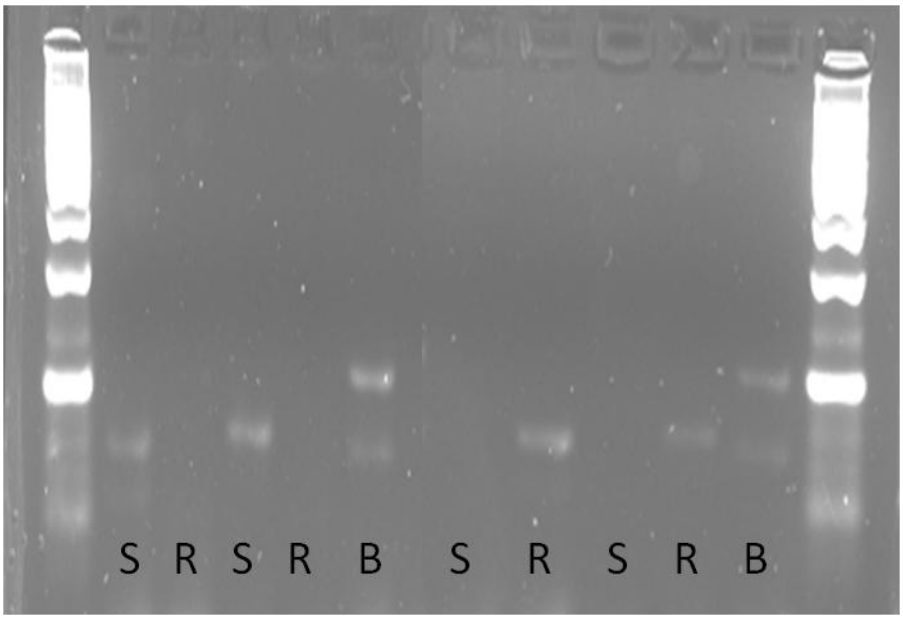


Figure 4-11. Polymerase chain reaction assay to detect a point mutations in the sodium channel S6 transmembrane segment of domain III comparing two strains of *Rhipicephalus microplus* (Canestrini). Letters on the bottom row indicate genotype: susceptible (S), resistant (R), or both (B). Lanes 2-6 from the left were run with product of a single DNA extraction from a susceptible tick and lanes 7-11 were run with product of a single DNA extraction from a resistant tick. Lanes labeled S used primers that amplify DNA when a tick lacks a target site mutation that confers resistance to permethrin. Lanes labeled R used primers that amplify DNA in ticks that have the mutation present. Letter B was used for the two lanes that contained amplification products that used primers that amplify the entire target site and should be present regardless of resistance status. The 100 bp plus ladder was used. Photo courtesy of Amanda L. Eiden.

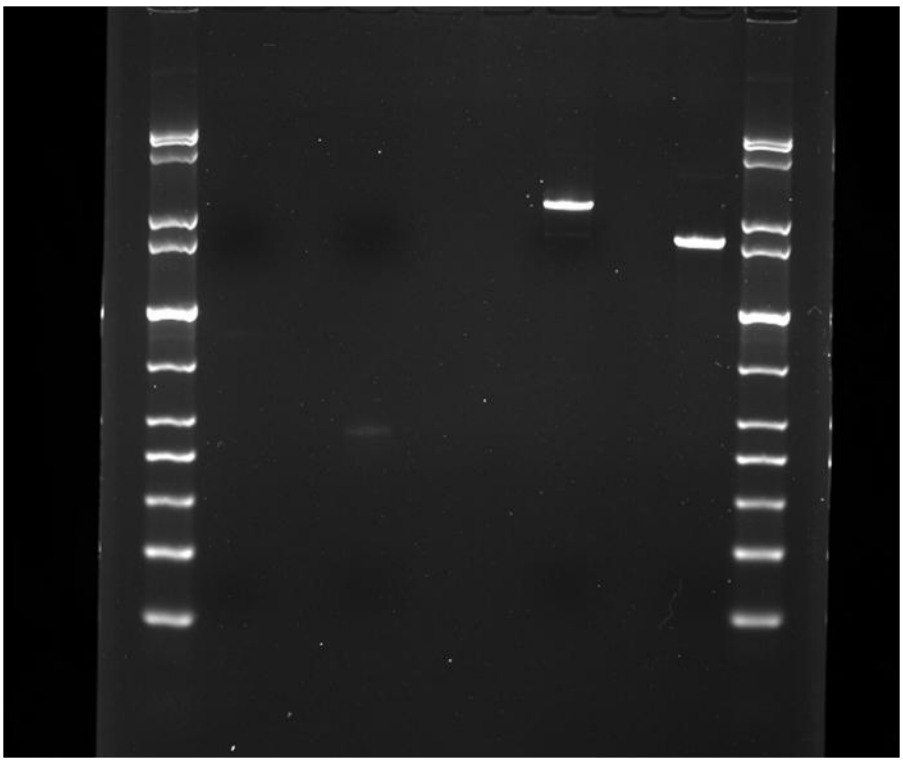


Figure 4-12. Cloned sequences of three amplification sites in the sodium channel gene from *Rhipicephalus sanguineus* (Latreille). The product in lane three did not replicate well and is barely detectable. The remaining two bands had sufficient amplification, however, Sanger sequencing did not reveal amplification at the desired site. The 1 kb plus ladder was used. Photo courtesy of Amanda L. Eiden.

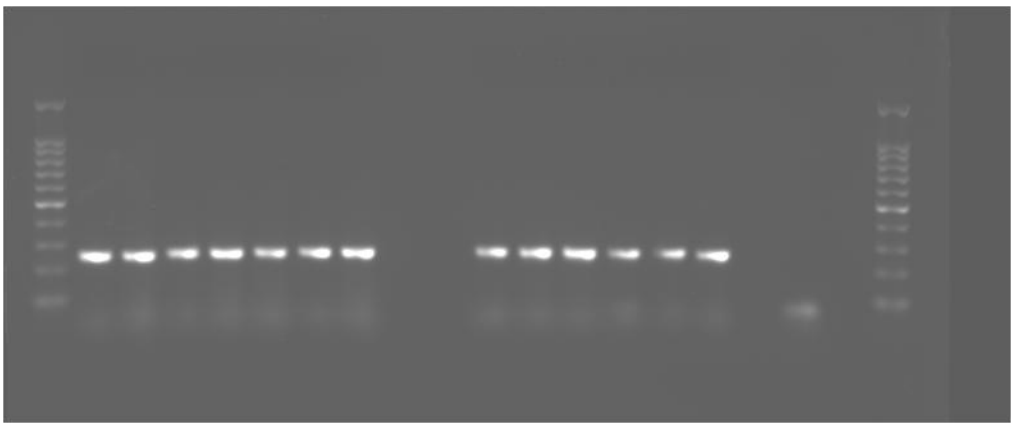


Figure 4 -13. Amplification of the *Rdl* GABA receptor subunit in the chloride channel for *Drosophila melanogaster* Meigen. Desired product size was 268 base pairs. This product was sent for Sanger sequencing. The 100 bp plus ladder was used. Photo courtesy of Amanda L. Eiden.

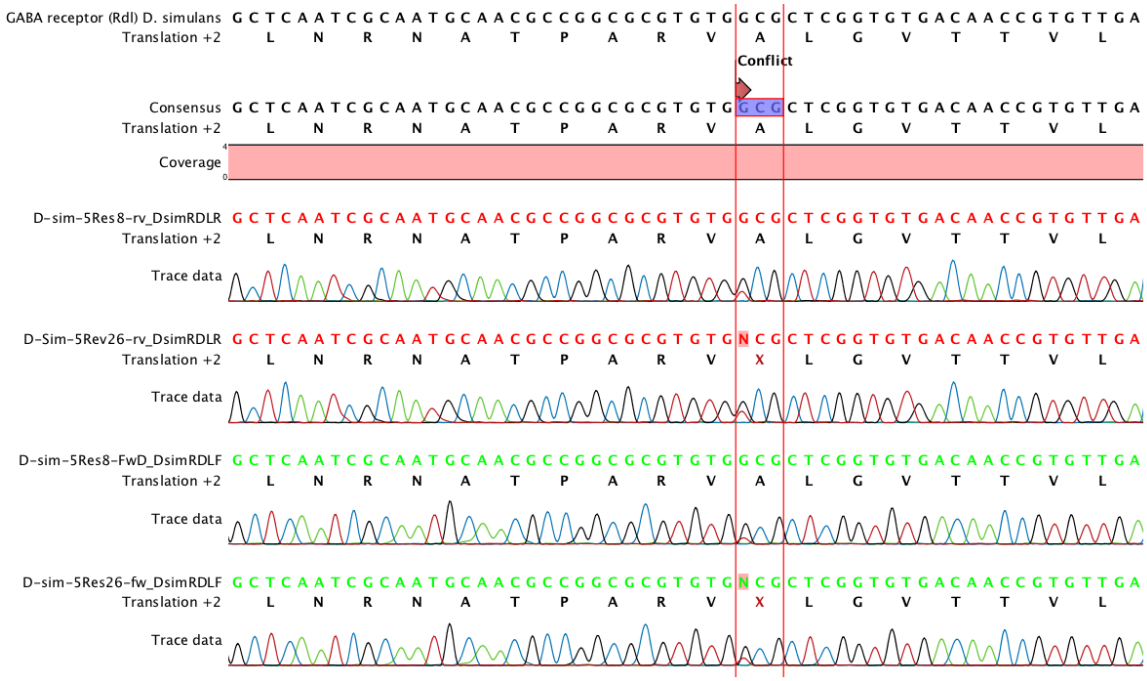


Figure 4-14. Sanger sequence of the *Rdl* GABA receptor subunit in the chloride channel for *Drosophila melanogaster* Meigen. The top row is the DNA-susceptible or wild-type sequence. The following rows contain the sequence for two groups of five pooled flies. The top line in each row contains the nucleic acid present and the bottom row indicated the amino acid. This graph shows a site mutation that was found to conflict between the susceptible and resistant strain that resulted in an amino acid change. Part of the population has the same amino acid as the susceptible strain while some of the population the alaline was substituted by serine (A302S).

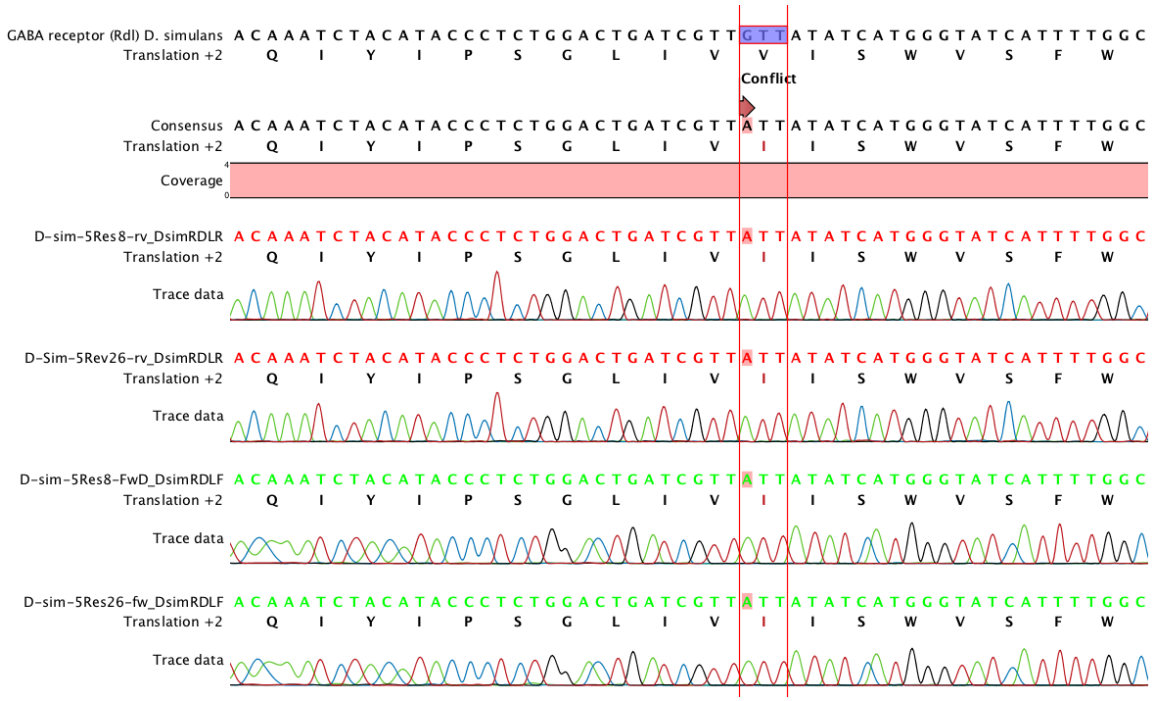


Figure 4-15. Sanger sequence of the *Rdl* GABA receptor subunit in the chloride channel for *Drosophila melanogaster* Meigen. The top row is the DNA-susceptible or wild-type sequence. The following rows contain the sequence for two groups of five pooled flies. The top line in each row contains the nucleic acid present and the bottom row indicated the amino acid. This graph shows a site mutation that was found to conflict between the susceptible and resistant strain that resulted in an amino acid change from a valine to isoleucine (V283I).

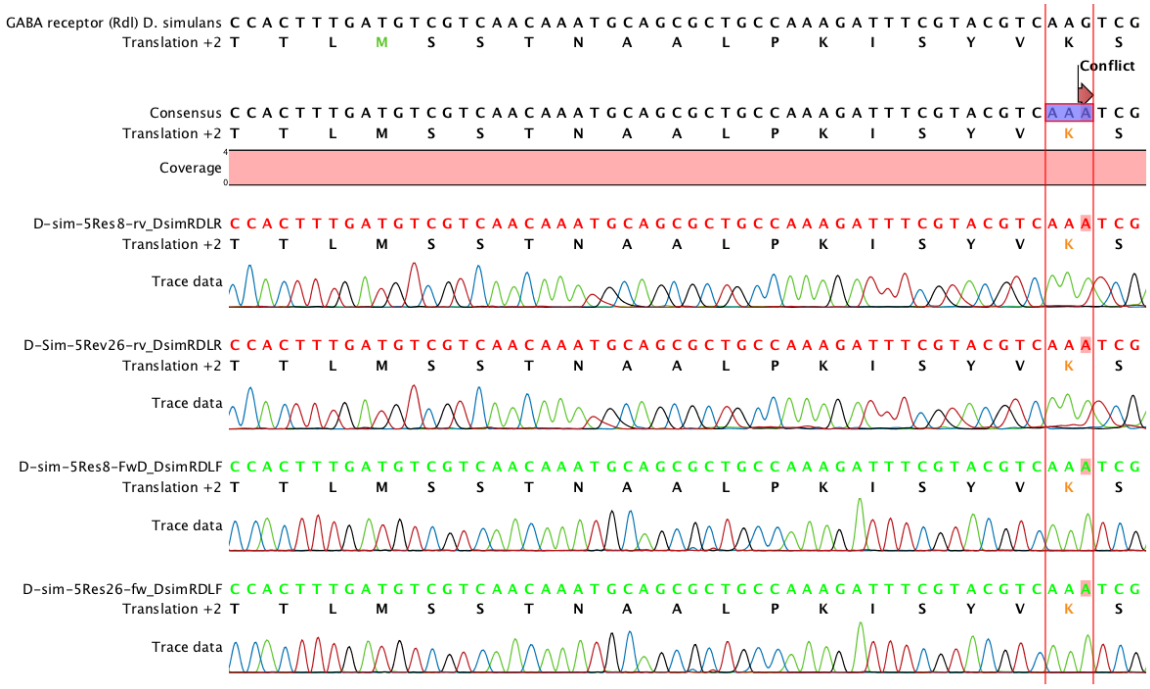


Figure 4-16. Sanger sequence of the *Rdl* GABA receptor subunit in the chloride channel for *Drosophila melanogaster* Meigen. The top row is the DNA susceptible or wild-type sequence. The following rows contain the sequence for two groups of five pooled flies. The top line in each row contains the nucleic acid present and the bottom row indicated the amino acid. This graph shows a site mutation that was found to conflict between the susceptible and resistant strain that did not result in an amino acid change.

CHAPTER 5
IMPLICATIONS AND FUTURE DIRECTIONS FOR *RHIPICEPHALUS SANGUINEUS* (LATREILLE) CONTROL AND RESEARCH

Rhipicephalus sanguineus (Latreille), the brown dog tick, is found worldwide as a veterinary pest that also inhabits and infests indoor areas (Demma et al. 2005). Chemical control methods are heavily relied upon to control brown dog ticks. Reports of control failures directed this research to determine if resistance was occurring in tick populations across Florida. In order to confirm field reports of resistance, tick samples were requested from any resident or dog kennel that contacted our program. Typically, when tick samples are submitted, ticks must be reared on a mammal prior to resistance determination, a costly and time consuming process. As such, rapid resistance diagnostic methods needed to be developed to determine the presence of resistance in order to help guide control recommendations. For several types of pesticide resistance, if mechanisms conferring resistance can be determined, it is possible to restore some efficacy of the acaricide using synergists. For this reason, it is important to understand the mechanisms developed by ticks to survive acaricide exposure.

In this study, permethrin resistant populations were documented in several locations across Florida and in one Texas site. However, only tolerance to fipronil was found in these tick populations. Prior to this study, the only published case of resistance in the brown dog tick was found in Panama (Miller et al. 2001).

After determining the presence of resistance, the discriminating concentration was set for permethrin and fipronil. Characterization of these concentrations will be used for rapid and inexpensive resistance screening. Understanding prevalence of resistant populations is important, as it can be used to direct control purposes. Permethrin, a pyrethroid, is a commonly used acaricide for tick control on dogs as an

on-animal application as well used to treat tick habitats. In addition to permethrin, several other pyrethroids, such as bifenthrin, sumithrin, and esfenvalerate, are used in dog tick control. Although they have different chemical structures, all are pyrethroids that act on the sodium channel (Narahashi 1986, Bloomquist 1996). Target site mutations are a resistance mechanism that changes the molecule binding site and can render all pyrethroids ineffective, due to a common mode of action (Byford et al. 1985).

Fipronil also is used widely as an on-animal treatment, but does not have a label that allows for indoor applications. Some of the tick-submitting homeowners experiencing brown dog tick infestations claimed that using fipronil on-animal treatments, per manufactures label, were ineffective in controlling ticks on their dog. However, upon laboratory testing in this study, these tick populations demonstrated only tolerance or susceptibility. Fipronil tolerance, which was defined in this study as a resistance ratio (RR_{50}) <10, was documented in the populations of ticks that were tested in this study. One of the fipronil-susceptible populations was collected from a home that claimed to have been using monthly fipronil spot-on treatments during the previous year. The result that this cohort was susceptible to fipronil, despite being exposed per a year to on-animal spot-on treatments, may reflect the difference in use patterns between products that do not allow for indoor applications with those that do. In comparison permethrin products, which have had broad indoor, outdoor and on-animal label site applications for decades brown dog tick exposure has been considerably greater than fipronil given that the ticks spend greater than 90% of their life off the host (Needham and Teel 1991).

The technical grade acaricides that were evaluated and presented in this dissertation were based on the most frequently used active ingredients sold in veterinary formulations. In future studies, experiments utilizing commercial products could address differences in acaricides formulated for on-animal use versus technical grade active ingredients. Testing commercial formulations can pose some challenges, such as achieving high concentrations to obtain a dose response curve, developing a proper control treatment because many products contain proprietary ingredients, and standardizing testing methods to account for the variation in the product components.

Future studies should focus on the evaluation of additional acaricides, particularly new chemistries. It would be important in order to gather baseline data on susceptibility and resistance potential prior to broad spectrum use. One such acaricide, amitraz, is marketed in multiple veterinary use products including Certifect® (Merial, Duluth, GA), a spot-on animal treatment, and in the tick collar, Preventic® (Virbac, Carros, France). Amitraz resistance has been demonstrated in *Rhipicephalus microplus* (Canestrini) (Li et al. 2004); therefore, it is possible amitraz resistance could be selected for in the closely related brown dog ticks. Amitraz is an example of one acaricide that was challenging to test using the standard the larval packet test and required modifications from paper to nylon substrates and the use of formulated rather than technical grade acaricide. Miller et al. (2002) modified the larval packet test to evaluate *R. microplus* resistance to amitraz because the standardized method was unable to discriminate between the susceptible or resistant ticks because high variability. It was suggested that the nonporous nylon surface and stabilized amitraz improved the bioassay outcome.

Additionally, evaluations of other pyrethroids against permethrin-resistant *R. sanguineus* populations should be done to determine levels of resistance for active ingredients other than permethrin. *Rhipicephalus microplus* was tested for resistance to flumethrin, deltamethrin, and cypermethrin susceptibility, which are synthetic pyrethroids commonly used for tick control on cattle (Rosario-Cruz et al. 2009). Out of 28 tested populations, researchers reported that one-half of the populations were resistant to all three pyrethroids, however, resistance to one chemical did not guarantee resistance to all three chemicals (Rosario-Cruz et al. 2009). The different chemical structures could result in the resistance variation. This is important to understand because of the wide range of pyrethroids used for residential treatment. Brown dog ticks spend only around 5% of their time on the host (Needham and Teel 1991); therefore, greater exposure comes from pest control treatments to the environment and products other than permethrin may have been used. The high levels of permethrin resistance that were documented in this study could be related to a combination of pyrethroid exposure from on-animal treatments and environmental pest control applications.

Evaluation of products formulated into shampoos and acaricidal on-animal sprays could use adult and larval immersion tests. Permethrin is formulated into on-animal sprays, spot-on topicals, dips, and shampoos. For example, Sentry® Pro Flea & Tick Shampoo (Sentry Pet Care Products, Inc., Omaha, NE) contains 0.10% permethrin. After the shampoo is diluted in water, it would be interesting to determine efficacy against susceptible and tolerant tick populations and then compare permethrin shampoo to the detergent alone. Alternatively, some kennels have a protocol based on anecdotal evidence, that shampooing dogs with detergent alone kill ticks. Studies

should be conducted to measure the efficacy of permethrin-based shampoos after they have been diluted in water.

The residents and facilities that tick populations were collected from often were unsure of their infestation source. Pioneer ticks were either already under resistance selection pressure, or selection could have occurred at the collection site. Each home can serve as a different tick population, as ticks remain relatively isolated due to limited mobility. The lack of migration and immigration can lead to increased selection pressure for each population of ticks. There are no currently published brown dog tick research papers that evaluated the rate at which resistance evolves or how high resistance levels can reach with different resistance mechanisms. As reviewed by Scott (1990), resistance mechanisms include increased metabolic activity leading to detoxification, target site mutations, or reduced cuticular penetration. In addition to these mechanisms, behavioral resistance also has been documented, in which the arthropod avoids pesticide exposure. For example, *Blattella germanica* (L.) has demonstrated behavioral resistance by avoiding insecticide baited traps (Silverman and Bieman 1993, Wang et al. 2004).

Obtaining the complete genomic sequence of the brown dog tick will aid in evaluating resistance mechanisms. During this project, several attempts to evaluate genetic mutations in the sodium and chloride channels that confer permethrin and fipronil resistance, respectively, were unsuccessful. In this study, the genetic sequences of *Ixodes scapularis* (Say) and *R. microplus* were used as proxies, but proved to be too dissimilar to the brown dog tick sequence to yield positive results.

After sequencing, genetic variations between resistant and susceptible ticks could be compared to identify sites that confer resistance. Upon determining resistance sites, a polymerase chain reaction (PCR) method could be constructed to differentiate susceptible and resistant genotypes similar to studies with *R. microplus* (Lovis et al. 2012). There have been three confirmed mutations in the sodium channel that confer pyrethroid resistance in *R. microplus* (He et al. 1999c, Morgan et al. 2009, Jonsson et al. 2010). These three mutation sites can be evaluated using one multiplex-PCR (Lovis et al. 2012. Results of these studies documented that the three mutations conferring resistance occurred in ticks from different regions of the world. Resistant populations of *R. microplus* that were screened from North America were unique in having a mutation in Domain III. Whereas, the L641 Domain II mutation was found in resistant populations collected from Brazil, Australia, Argentina, and South Africa. A third mutation site, G72V in Domain II, was found to confer pyrethroid resistance only in one Australian population (Lovis et al. 2012). In brown dog ticks, several mutations may be present but their distribution could vary worldwide.

In addition to the molecular work that was completed to evaluate esterase activity in Chapter 4, cytochrome P450 levels could be estimated by using quantitative-PCR methods. In permethrin resistant *Helicoverpa armigera* (Hübner), the Australian cotton bollworm, one out of eight cytochrome P450 genes was found to have double expression levels compared to the susceptible strain (Pittendrigh et al. 1997). Increased levels of cytochrome P450 transcription also were found in DDT-resistant *Drosophila melanogaster* Meigen using quantitative-PCR methods (Daborn et al. 2002).

Cuticular penetration experiments might determine changes in structure that lead to increased levels of resistance. In *H. armigera*, a delay in cuticular penetration of deltamethrin conferred resistance (Ahmad et al. 2006). Increased concentrations of protein and lipids in the cuticle have been correlated with reduced DDT penetration in *Heliothis virescens* (F.) (Vinson and Law 1971).

In addition to understanding physiological mechanisms of the brown dog tick, knowledge on ecology can be important for their management. Population dynamics, such as annual reproduction cycles and population fluctuations, can be helpful for implementing control measures and determining how many developmental stages ticks will pass through annually. Areas that have high populations all year will require continued monitoring. There was a slight peak in calls and ticks submissions from community stakeholders between April to June and a lower rate of contacts from November to March. A seasonality study conducted to improve our understanding of brown dog tick population dynamics in Florida is needed.

A study conducted on outdoor dogs at homes in rural and suburban areas by Koch (1982) in Oklahoma and Arkansas determined brown dog tick population peaks for different life stages, with larvae and nymphs peaking in July and September and adult populations peaking in April, July, and September. No ticks were found in Oklahoma during December. The study area covered large portions of Oklahoma and Arkansas, with variations in temperature and rainfall.

Comparatively, brown dog tick populations were collected from dogs that were brought to veterinary facilities in Cuernavaca City, Mexico (Cruz-Vazquez and Garcia-Vazquez 1999). Adult populations peaked from December to February and again in

May to September. The highest numbers of nymphs were collected from March to July and again in November, which was slightly different than larvae, which peaked in March, June and October (Cruz-Vazquez and Garcia-Vazquez 1999). The average year round daily average temperatures ranged from 18°C in January to 23°C in May. August experienced the highest levels of rainfall with around 260 mm while December received around 5 mm of rain. In Mexico, population fluctuations were attributed more to rainfall than temperature, with higher populations collected during the wet season (Cruz-Vazquez and Garcia-Vazquez 1999). However, in Belo Horizonte, Brazil, the dry season was associated with higher brown dog tick infestation rates (Silveira et al. 2009). Florida has a wet and dry season and climate variation from temperate in the north, to subtropical in the south. These factors most certainly impact brown dog tick life cycles, and should be studied to understand population dynamics. Because brown dog ticks can inhabit indoor and outdoor regions, populations can be maintained during less optimal climate conditions. It is important to understand the impact of the indoor life cycle in maintaining populations during cooler and dry conditions.

Many people experiencing brown dog tick control problems are unaware of the ticks' ability to infest homes until they realize that they have an infestation. Florida residents are often unaware that brown dog ticks are more likely to be introduced after exposure to kennel facilities, veterinarians, groomers, or other dogs, rather than exposure in the outdoor environment. Animals should be inspected for ticks to prevent introduction into homes after they are exposed to areas where multiple dogs are present. A few of the homeowners that contacted the University of Florida for tick control advice denied that the ticks were residing indoors and repeatedly treated

outdoor areas, despite *R. sanguineus* being positively identified. Suggested monitoring methods, such as lining doors with duct tape so homeowners can help identify the location of pockets of congregating ticks and lead to focused eradication control efforts in the home, were explained to residents with many using this technique to protect dogs held in indoor crates.

Rotating the acaricides used for tick control can minimize resistance selection. This is particularly difficult for pet owners because spot-on treatments are typically veterinarian prescribed and come packaged with sufficient applications for six months. The production of multi-chemical products might increase the longevity of currently approved compounds if they have different modes of action (Curtis 1985). Although superficially, it appears that several products used for veterinary control currently have multiple ingredients, typically only one active ingredient provides protection against ticks. As demonstrated in Chapter 4, synergists could increase product efficacy, especially with the inhibition of esterase activity. However, there are currently no esterase inhibitors approved for sale or use of dogs. If chemical companies work towards esterase inhibitor development that is approved for commercial use, synergized permethrin may be used to effectively control residential tick populations again. Challenges with adding synergists to pesticides include cost effectiveness, product registration, and producing a functional formulation that is stable (Raffa and Priester 1985). Additionally, the use of synergists must not cause increased risks to humans, companion animals, wildlife, or the environment.

Using cultural controls, such as steam cleaning, washing dog beds, and physical removal of ticks can help reduce tick burdens. Developing updated brown dog tick

protocols for pest control operators to incorporate treating resistant populations can aid in reducing the duration of tick infestations. Homeowners receiving pest control treatment have claimed treatments were ineffective for tick control. In order to decrease the burden of brown dog tick infestations and prolong the effectiveness of market acaricides, integrated pest management education must be provided by pest control operators and extension agents for homeowners and kennel facilities.

As a result of this research educational information has been shared with the community. We have provided consultations for clientele that contacted the University of Florida and educational materials have been made available for brown dog tick control: https://pmu.ifas.ufl.edu/. In addition to using acaricides treatments, homeowners and kennel owners need to be responsible for preventing and controlling infestations. Diligent inspections for ticks, using steam cleaning, washing fabrics that may be harboring ticks, and using the appropriate acaricidal treatment can help eliminate tick populations and prevent the development of resistance.

APPENDIX A
TICK STRAIN COLLECTION AND HISTORY

Table A-1. Colony history of susceptible *Rhipicephalus sanguineus* (Latreille) obtained from Ecto Services, INC, North Carolina.

Source	Date Introduced
Professional Laboratory and Research Service	March 2008
Professional Laboratory and Research Service	November 2008
Professional Laboratory and Research Service	October 2009
Professional Laboratory and Research Service	May 2010
Center for Disease Control	April 2011
Sierra Research Labs	November 2011

Table A-2. Acaricide treatment history of brown dog ticks submitted to the University of Florida for acaricide resistance evaluation.

Strain	Submission date	Animal treatment	Indoor treatment	Ticks submitted	Notes
Broward	5/9/2012	Sporadic Advantix® use during summer months. No treatment used 8 months prior to submission.	None	226 engorged nymphs	Five dogs
Charlotte	10/19/2010	Advantix®: 3 years with monthly treatments. Preventic® collar 3 times	None	>20 engorged females	Three dogs. Finding engorged females and larvae. Gained control with Promeris™ and professional pest control
Collier-1	6/24/2011	Frontline® for 1 year with monthly treatments	None	17 engorged females 8 flat adult females 12 flat adult males	
Collier-2	2/13/2012	None prior to collection	None	12 adult flat females 14 adult flat males	Yard was treated with unknown pesticide. Developed infestation 3-6 months before collecting ticks

Table A-2. Continued

Strain	Submission Date	Animal Treatment	Indoor Treatment	Ticks Submitted	Notes
Duval	10/13/2010	Cedar oil	Permethrin fogger, diatomaceous earth	70 engorged females >50 flat adult females and males	30 dogs
Gilchrist	8/9/2011	Inconsistent history of spot-on treatments containing permethrin and fipronil. Poor records kept, inconsistent use	Was not conducting proper follow up treatments but pyrethroids had been used	68 engorged females 167 flat adults	19 dogs
Marion	10/26/2010	Monthly Advantix® for 3 years Frontline® spray Hi-yield® garden spray (Voluntary Purchasing Groups, Inc) (bifenthrin) –not approved for use on companion animals	Talstar®, bifenthrin, Cynoff®, Demon®, Suspend®, permethrin, Orthene®	35 engorged females	5 to 10 engorged ticks a day in the house 10 to 50 ticks a day from the dog
Palm Beach-1	11/10/2010	Unknown	Precor 2000® (permethrin / PBO)	41 engorged females	
Palm Beach-2	6/9/2011	History of Frontline® Plus use. Unknown treatment regimen	Collected 10 days post-treatment with Onslaught®	16 engorged females 136 flat adults	3 dogs

Table A-2. Continued.

Strain	Submission Date	Animal Treatment	Indoor Treatment	Ticks Submitted	Notes
Palm Beach-3	2/21/2012	Unknown	Unknown	18 adult flat females 13 adult flat males	
Palm Beach-5	9/15/2010	Frontline® Plus, Revolution, Advantix® all used at different times during 3 years Preventic® 3 years	Unknown	>15 engorged adult females	5 year problem 1 Dog
Sarasota-1	6/19/2012	Frontline® for 1 year	Inside, Suspend® (deltamethrin), Demand® (lambda-cyhalothrin), DeltaDust®, boric acid, Diatomaceous earth, unspecified insect growth regulators Onslaught®. Outside: Tempo® (β-cyfluthrin), Talstar® (bifenthrin)	23 engorged females	Adopted a dog from an animal shelter and notices the ticks afterwards

Table A-2. Continued

Strain	Submission Date	Animal Treatment	Indoor Treatment	Ticks Submitted	Notes
Sarasota-2	4/3/2013	Advantix®, Certifect® and Frontine® use history. Other source of permethrin use such as on-animal sprays. Frontline® used several years from 2010-2012 and on and off previous to that. Advantix®: intermittent over the years.	Permethrin fogs and history of self-treating with professional grade treatment but did not recall products	32 engorged females	10 year infestation
St. Johns	9/12/2011	Advantix® for 5 years with monthly treatments	Deltamethrin®, Temprid™, Kicker®, Precor®	79 engorged females 44 adult flat males 36 adult flat females 4 nymphs	Kennel facility. Noticed infestation 1.5 months before collection
Webb, TX	3/25/2011	Prolate/Lintox-HD™ dip. Not approved for use on companion animals.	Unknown	>50 flat adults >40 engorged females	Kennel facility

APPENDIX B
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE DOCUMENTS

UF UNIVERSITY of FLORIDA
The Foundation for The Gator Nation

Notification of Initial Approval

From: William Buhi

To: Phillip Kaufman

CC:

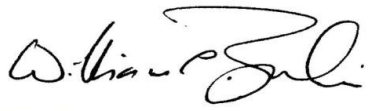
Re: IACUC Protocol #: 200802142

Title: Improving management of the brown dog tick, Rhipicephalus sanguineus, in Southeastern residential environments

The above referenced study was APPROVED on 2/3/2009 . This approval has been granted by the Institutional Animal Care and Use Committee (IACUC) at the University of Florida. Animals may be purchased and used for the above project and housed in any IACUC approved facility. Any significant change in this approved animal project must occur through a modification procedure and be approved by the IACUC before instituting the change. You are required to return to this site 60 days prior to 2/2/2010 and file a Continuation. If you fail to submit your Continuation and have it approved before this date, your study will automatically expire, and you will no longer be able to work with these animals. The study can only be reactivated with submission and approval of a Continuation.

If this IACUC protocol pertains to a sponsored research project it is the responsibility of the PI to forward a copy of IACUC approval and associated PeopleSoft Project number to the Office of Award Administration via Fax at (352)392-4522 or email at ufawards@rgp.ufl.edu

Sincerely,



William Buhi

IACUC Chair

Institutional Animal Care & Use Committee
PO Box 100142
Gainesville, Florida 32610-0142
Tele: (352) 392-9917 Fax: (352) 392-9919

UF | UNIVERSITY of FLORIDA

The Foundation for The Gator Nation

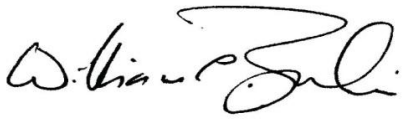
Notification of Initial Approval

From:	William Buhi
To:	Phillip Kaufman
CC:	Amanda Eiden
Re:	Continuing Review ID: IC00003099 2013 Review for 201102142
Title:	Development and delivery of an innovative, alternative pest management program for the brown dog tick Improving management of the brown dog tick, Rhipicephalus sanguineus, in Southeastern residential environments

I am pleased to inform you that the Continuation for study 201102142 was APPROVED on 11/15/2012. You are required to return to this site at least 60 days prior to the Expiration Date of 1/25/2014 and file a Continuation or a Triennial.

If this IACUC protocol pertains to a sponsored research project it is the responsibility of the PI to forward a copy of IACUC approval and associated PeopleSoft Project number to the Office of Award Administration via Fax at (352)392-4522 or email at ufawards@rgp.ufl.edu

Sincerely,



William Buhi

IACUC Chair

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APPENDIX C
TICK REARING INSTRUCTIONS

Tick Cage Construction

In order to contain the brown dog ticks and monitor tick attachment while on the host, a tick feeding cage was constructed that was adhered to the rabbit (Lysyk and Majak 2003). The cage was constructed by modifying the bottom portion of a screw-topped plastic container (120 mL) with a diameter of 6 cm. To improve the fit of the container on the rabbit, curves were formed into the bottom of the container. The edges were then smoothed with sand paper (Fig. B-1).

To create an area for secure attachment, two 13 x 13 cm squares of fiberglass mesh window screening were cut. The first piece of screen was cut into a circle resulting in a diameter that was 3.5 cm wider than the outside diameter of the plastic cage while leaving ~ 1 cm of overlap on the inside. The screen was secured to the bottom of the plastic container using a hot melt glue gun. The second piece of mesh was cut into two semi-circular pieces 13 x 6 cm (Fig. B-2). These pieces were cut in this manner to form a downward curve in the screen that created a more curved fit for attachment to the rabbit, as opposed to a flat screen surface. After the screen was secured, contact cement was applied to the mesh using a foam brush, until no open holes were visible between the two layers. The glued cages were left to dry overnight (Fig. B-3).

In order to provide ventilation to the tick-feeding cage, the cage lids were modified by cutting a 1.5 cm diameter hole in the lid using a cork borer that was heated over a Bunsen burner. A 2 x 2 cm piece of brass 80 woven wire mesh® (Paragona®, Indianapolis, IN) was used to cover the hole in the lid and was secured into place by

melting screen into the plastic container lid creating a secure seal (Lysyk and Majak 2003) (Fig. B-4). PARAFILM® (Sigma®, Saint Louis, MO) was pulled around the lid of the lid during larval feeding as an extra precautionary measure (Fig. B-5).

Tick Host Preparation

All life stages of brown dog ticks were collected at or accepted from residential homes or dog kenneling facilities. Tick strains from each location were held and reared as separate colonies only if 20 or more viable adults, with at least 10 females, were obtained. Tick rearing was conducted at the University of Florida.

Ticks were placed into glass vial containers that were secured with cotton placed into the top. Ticks were held at 25 ± 1°C, with a photoperiod of 12:12 (L:D). Relative humidity was maintained through one of two methods. Either a saturated solution of potassium nitrate salt (ScienceLab, Inc, Houston, TX) and distilled water placed in glass holding chambers that maintained 92% RH (Winston and Bates 1960) or ticks were held in a sealed chamber (Dry-Keeper™, Cole-Parmer, Vernon Hills, IL) with containers of distilled water at 85% RH.

Each active, brown dog tick life stage was fed on a ~4 kg New Zealand White rabbit to obtain a blood meal (UF IACUC Protocol 200802142, 201102142 Appendix B). In preparation of cage attachment, rabbits were anesthetized using isoflurane and their eyes were coated with petrolatum ophthalmic ointment (Puralube® Vet Ointment, Overland Park, KS) to prevent them from drying out. A circular area of approximately 6 to 8 cm in diameter was shaved with a clipper on the lateral side of the rabbit's abdominal region between the front and hind legs. After the fur was removed with a vacuum, an outline of the cage was drawn on the rabbit using a light colored non-toxic

marker, such as a highlighter or Sharpie®, in order to outline the location to place the adhesive used for attaching the cage.

The tick cages were attached to the rabbit using Helmiprene contact cement (Helmitin, Toronto, Ontario) that was applied with a foam brush. To ensure a tight seal, the cement was applied to both the tick feeding cage and the area on the rabbit where the cage was designated to be secured. For the rabbits comfort, a hairdryer was used on the cool heat setting to shorten contact cement drying time and limit the amount of time that the rabbit spent under anesthesia. Cotton balls were dabbed on the inside of the tick feeding chamber to remove excess contact cement and to prevent ticks from getting caught in the adhesive. After the contact cement dried, ticks were placed into the tick feeding cage for up to two weeks, where they remained until feeding completion. During tick feeding the rearing room temperature was held at 23 ± 1°C. A minimum of two weeks was provided between feedings on each rabbit and rabbits were used for no more than three feedings. Tick feeding chambers detached from rabbits between 14 and 21 days after attachment from degradation of the glue.

Tick Feeding

Larvae

Two methods of preparing larvae for feeding were used. When a sufficient number of eggs for acaricide testing were oviposited, 125 mg of eggs, which yielded approximately 2,500 larvae, were placed into a 1.5 mL microcentrifuge tube. The top of the microcentrifuge tube was removed and cotton was placed into the end to allow for ventilation and to prevent tick escape. Eggs were monitored for eclosion and held as larvae until they were 4 to 5 weeks post-eclosion whereafter they were introduced to rabbits for feeding.

Larvae that survived acaricide resistance bioassay exposure were prepared for feeding using an alternative handling process. Larvae held in glass vials were aspirated from larval packet tests using a disposable glass pipette attached to vacuum pump tubing. Fabric mesh prevented ticks from passing through the pipette into the vacuum pump tubing. Ticks were then anesthetized within the pipette with CO_2 gas by placing it over the disposable pipette and squeezing shut the hose that was connected to the vacuum pump. Thereafter, the pipette was removed and the ticks that were caught in the mesh were placed into a 1.5 mL microcentrifuge tube that was promptly sealed with cotton.

When introducing ticks to rabbits, sticky backed Velcro® was placed around the microcentrifuge tube and along a 1.5 cm portion on the inside of the tick feeding cage. Tick-containing microcentrifuge tubes were placed on ice for one minute, then the cotton was removed from the tube and the tube was attached to the inside of the tick feeding enclosure. The enclosure was sealed and the larvae were checked for engorgement following 7 days. The rabbit was checked twice daily to ensure it had not dislodged the cage.

Engorged larvae were aspirated from the tick-feeding chamber using a 1/6 hp vacuum pump (Gast Manufacturing Corporation, Benton Harbor, MI). All larval ticks were removed from the rabbit immediately if the cage began to detach to decrease the number of escaped ticks. To reduce the chance of tick escape into the environment the multi-rabbit hutch holding rack was always placed over a soapy water moat. In case of cage detachment, individual rabbit hutches were placed over a moat of water that had the edges coated with petroleum jelly and rabbits were removed from their cage daily to

be combed with a metal flea comb to locate ticks. Water moats were searched daily and ticks that were recovered were rinsed, dried, and placed back into the humidifying incubator with the other colony ticks of that strain. The humidifying incubator consisted of a sealed glass chamber (Sanpia), which contained four bowls of distilled water to maintain humidity. Engorged larval ticks were placed in glass vials that were fitted with cotton balls. Tick-containing vials were held in the previously described laboratory incubator and were not disturbed until molting was completed.

Nymphs

After their first molt, nymphs were held for approximately two weeks before their next feeding. To prepare nymphs for feeding they were placed into a microcentrifuge tube on ice, using either soft forceps or a water color paint brush. Up to 700 nymphs were placed on a rabbit during each feeding session, however, due to a limited number of ticks acquired for tick colonies, typically 50-100 nymphs were place on the rabbit during each feeding. A single adult male was placed in the tick feeding cage with nymphs to improve nymphal attachment (Rechav and Nuttall 2000). Nymphal ticks were allowed access to the rabbit for two weeks to allow feeding completion of ticks that failed to attach rapidly. Feeding chambers were checked daily and detached. Fed nymphs were removed from chambers using soft forceps and placed in glass vials fitted with cotton. Nymphs were held in the previously described incubator until they had completed their second molt.

Adults

Following the nymph-to-adult molt, ticks were held for 30 days prior to placement on a rabbit. A maximum of 30 adult ticks were placed into the tick feeding chamber attached to a rabbit during each feeding session. When possible, a ratio of 15 males

and 15 females was used. Males were placed on the rabbit one day prior to placing the females. If tick mortality occurred during the first three days of the feeding cycle the dead ticks were removed and replaced with live ticks. The adults were held in glass vials before feeding and chilled on ice prior to placement on the rabbit to minimize the chance of tick escape. Adult ticks were allowed host access over a period of two weeks. The females would attach to the host and take a partial blood meal. After females initiated feeding, blood fed males would mate with the females while they remained attached. After mating occurred, the female would complete her feeding cycle, becoming fully engorged whereafter she would detach from the rabbit. Engorged females were removed from the tick feeding cage using soft forceps and placed in a glass Petri dish. They completed oviposition in standard environmental holding conditions. All live males remained inside the tick feeding cage until the last engorged female tick was removed or all remaining ticks were removed after 14 days.

Free-Release Tick Feeding

In the case of early cage detachment, ticks were allowed to remain on the rabbit to feed using a free-release method. During the free-release method, a soft Elizabethan (E) collar (KONG EZ Soft™ Collar, Golden, CO) was placed on the rabbit in the reverse direction. The E-collar created an area around the rabbit's neck where the ticks could safely feed without being removed by the rabbit during grooming, while still allowing for cecotrophy (ingestion of feces) by the rabbit, a requirement of the IACUC protocol. Additionally, a 13 cm piece of tubular cotton stockinet was cut and placed around the rabbit's thoracic region. It was secured by cutting two holes for the front legs and cutting a slit at the top of the stockinet that was used to fasten the stockinet to the E-collar. The stockinet provided additional protection for the ticks to minimize tick removal

due to rabbit grooming behaviors. Unattached ticks that were found outside the stockinet area during routine checks were placed under the stockinet, near the E-collar.

A water pan, with the rim coated in petroleum jelly, was placed under the rabbit hutch in order to capture engorged ticks that detached and dropped from the rabbit. Ticks retrieved from this pan were placed back on the rabbit if they were not engorged or put back into colony if they had completed feeding. For additional biosecurity measures, the rabbit hutch holders remained over a large soapy water pan to a capture any ticks that may have escaped beyond the hutch water pan. Both pans were searched daily to retrieve ticks. This approach required sifting through fecal pellets and discarded feed to retrieve the ticks. After searching was completed, the liquid waste was removed using a siphon and the solid waste was put into a sealable plastic bag then double bagged, in order to prevent the escape of unrecovered ticks. Engorged ticks that were removed from the water pan were rinsed in a sieve (No. 120, 125 mm, Fisher, Mexico) and dried with paper towel.

In the case of tick escape during larval free-release feeding, additional measures were taken to contain and retrieve the ticks. The waste collection pan under the rabbit hutch was filled half-full with tap water to capture and hold any ticks that fell off the rabbit. To retrieve the fallen ticks, the water was removed from the pan using a siphon pump. The remaining contents were then poured through two different sieves. The first larger sieve was (No. 6, 3.35 mm, Fisher, Mexico) and the second smaller sieve was (No. 120, 125 mm, Fisher, Mexico). The larger sieve retained the large debris, such as hay and fecal pellets, while the smaller mesh sieve retained larval ticks. As the contents of the pan were poured into the sieves the pan was rinsed with clean water to

dislodge remaining ticks. Both sieves, while still stacked, were then rinsed with tap water. The sieves were separated and the debris and larger sieve was placed into a bucket with soapy water to kill any ticks that were not washed into the smaller sieve. The finer grade sieve was rinsed with tap water an additional time and the entire sieve was placed into a plastic bin that was sealed. The lid of the plastic bin that contained the small mesh sieve had an opening with a fine screening that allowed the contents to dry, while preventing tick escape. After two days, or when the contents were dry, the ticks were collected and the debris was discarded.

In comparison to the tick feeding-cage method, the free-release tick feeding technique yielded fewer successfully engorged ticks. This was likely due to the ticks being dislodged or eaten by the grooming rabbit and failure to locate ticks in the waste water pan.

Figure C-1. Partially-constructed tick feeding chamber. Edges were cut to have a contour fit on the rabbit and were smoothed with sandpaper. Photo courtesy of Amanda L. Eiden.

Figure C-2. Mesh pieces were secured to the plastic portion of the feeding chamber (Fig. B-1). A) The square piece on the left was glued along the bottom contoured surface. B) The two pieces on the right were attached above the square piece, which allowed for the curves on the square piece to fit flush against the rabbit. Photo courtesy of Amanda L. Eiden.

Figure C-3. Tick feeding chamber assembled with mesh screening that has been glued to the bottom of a 6 cm diam plastic feeding chamber creating a surface used to attach the chamber to the rabbit. Photo courtesy of Amanda L. Eiden.

Figure C-4. The lid for the plastic feeding chamber was constructed using a cork borer to create a 3 cm hole and a hot melt glue gun to secure an 80 mesh brass screen into place. Photo courtesy of Amanda L. Eiden.

Figure C-5. Assembled tick feeding chamber. The yellow mesh was adhered to the shaved side of a rabbit. Photo courtesy of Amanda L. Eiden.

APPENDIX D
PRIMERS USED IN SODIUM AND CHLORIDE CHANNEL TARGET SITE MUTATION EVALUATION

Table D-1. Primers designed to evaluate sodium and chloride channel mutations in *Rhipicepahlus sanguineus* (Latreille).

Primer ID[a]	Sequence	Orientation	Range nt#[b]	GenBank number[c]
	Sodium channel domain II			
Ix-NaD2a-Fw	5'-ATTCACGCGAGAGTCCCTG-3'	Upstream	51-69	XM002407075.1
Ix-NaD2a-Rv	5'-AAGGCTCTAAGTACGCGGAA-3'	Downstream	694-713	XM002407075.1
Ix-NaD2b-Fw	5'-GCTGTTCCGGCCATTCAC-3'	Upstream	39-56	XM002407075.1
Ix-NaD2b-Rv	5'-GCTCTAAGTACGCGGAAGGT-3'	Downstream	691-710	XM002407075.1
Rm-NaD2a-Fw	5'-CGCGCTCTCCTTAATCGAAC-3'	Upstream	42-61	AF134216.2
Rm-NaD2a-Rv	5'-CAAGGCGAGGAAAAGGTTGA-3'	Downstream	467-486	AF134216.2
Deg-NaD2-Fw	5'-ATYGTYGCRYTSTCSYTRMTC-3'	Upstream	2,535-2,556	XM002407075.1
Deg-NaD2-Rv	5'-AHGCYCCRAACGARGASAG-3'	Downstream	2,988-3,007	XM002407075.1
	Sodium channel domain III			
IX-NaD3a-Fw	5'-ACGCCATTGACTCAAGAGGA-3'	Upstream	4,468-4,488	XM002407075.1
IX-NaD3a-Rv	5'-ACACCATCGCCTGAAGTTTG-3'	Downstream	4,740-4,759	XM002407075.1
Ix-NaD3b-Fw	5'-CTGACATCATGGACAACGCC-3'	Upstream	4,454-4,473	XM002407075.1
Ix-NaD3b-Rv	5'-TCGCCTGAAGTTTGAATCGT-3'	Downstream	4,734-4,753	XM002407075.1
Rm-NaD3a-Fw	5'-GCGTGTGAAGCCAACAACTT-3'	Upstream	1,912-1,931	AF134216.2

Table D-1. Continued

Primer ID[a]	Sequence	Orientation	Range nt#[b]	GenBank number[c]
Rm-NaD3a-Rv	5’-TAGTGGTCGAGTGCCATGAC-3’	Downstream	2,389-2,408	AF134216.2
Rm-NaD3b-Fw	5’-AACAGAAAGGCGTGTGAAGC-3’	Upstream	1,903-1,922	AF134216.2
Rm-NaD3b-Rv	5’-TCGAGTGCCATGACTGTCA-3’	Downstream	2,384-2,402	AF134216.2
Deg-NaD3-Fw	5’-AACAACTTYACYTGGRAMAACCCYATG-3’	Upstream	4,359-4,386	XM002407075.1
Deg-NaD3-Rv	5’-GGGTCGAACTGCTGCCAGATC-3’	Downstream	5,567-5,588	XM002407075.1
	Texas sodium channel domain III primers from Guerrero et al. 2001			
FG-221	5’-TTATCTTCGGCTCCTTCT-3’	Wild type-specific Sense	2,117-2,134	AF134216.2
FG-222	5’-TTATCTTCGGCTCCTTCA-3’	Resistant-specific Sense	2,117-2,134	AF134216.2
FG-223	5’-GAATAGATTCAAGGTGAA-3’	Wild-type-specific antisense	2,134-2,151	AF134216.2
FG-224	5’-GAATAGATTCAAGGTGAT-3’	Resistant-specific antisense	2,134-2,151	AF134216.2
FG-227	5’-TTGTTCATTGAAATTGTCGA-3’	Downstream non-diagnostic	2,081-2,098	AF134216.2
FG-228	5’-CTAACATCTACATGTACC-3’	Upstream non-specific	2,081-2,098	AF134216.2
	Sodium channel domain II primers from Morgan et al. 2009			
DB-011	5’-GGAAAACCATCGGTGCTC-3’	Wild type-specific Sense	173-190	AF134216.2

Table D-1. Continued

Primer ID[a]	Sequence	Orientation	Range nt#[b]	GenBank number[c]
DB-012	5'-GGAAAACCATCGGTGCTA-3'	Resistant-specific Sense	173-190	AF134216.2
FG-447	5'-GAACTTGTGTTTACTTTCTTCGTAGT-3'	Downstream non-diagnostic	266-291	AF134216.2
	Entire sodium channel sequences			
Deg-Na#1-Fw	5'-TTYGATTTYMTCATYGTYGCRYTSTCSYTRMTC-3'	Upstream	2,523-2,556	XM002407075.1
Deg-Na#1-Fw	5'-GAYTTCATGCRYTCRTTSATGATYGTSTTYCG-3'	Upstream	2,823-2,855	XM002407075.1
Deg-Na#3-Rv	5'-ATCTCGTAGTACATRTCGTARTCGTCGTC-3'	Downstream	5,541-5,570	XM002407075.1
Deg-NA#4-Rv	5'-AAGATKGCGWAGATGAACATSACVAGGAAMAG-3'	Downstream	5,216-5,184	XM002407075.1
Deg-Na#2-Fw	5'-TGGTGYTGGCTMGACTTTGTCATCGTRCTSG-3'	Upstream	4,018-4,048	XM002407075.1
	Entire chloride channel primers			
Rs-Cl-#1-Fw	5'-CTCCTGCGTCTCTCCACTCGATGAAGACC-3'	Upstream	121-150	GV659520.1
Rs-Cl-#2-Fw	5'-CGCTCCCCCAATCCTGAGGTTCCTTCT-3'	Upstream	286-314	GV659520.1
Rs-Cl-#1-Rv	5'-TTTTCTGGCTGTGGCGATGACGATTCGAG-3'	Downstream	2,357-2,386	GV659520.1
	Various glutamate-gated chloride channel locations			
Rs-ClGlu-a-Fw	5'-ACCTGGTGTTCAAGCGCGAGTTCAG-3'	Upstream	467-491	GQ215234.1
Rs-ClGlu-a-Rv	5'-TTTGGATCGCGTGGGAAACCTGG-3'	Downstream	1,068-1,086	GQ215234.1

Table D-1. Continued

Primer ID[a]	Sequence	Orientation	Range nt#[b]	GenBank number[c]
Rs-ClGlu-b-Fw	5’-CATCATGCCCAACGTGCTTCTACGC-3’	Upstream	165-189	GQ215234.1
Rs-ClGlu-b-Rv	5’-GTGGCGATGACGATTCGAGCGTG-3’	Downstream	1,369-1,391	GQ215234.1
Rs-ClGlu-c-Fw	5’-CACTCCACGGGAACGAGACC-3’	Upstream	432-452	GV659520.1
Rs-ClGlu-c-Rv	5’-CACTGCTCTCTGAACGTCATTTGCACTGTG-3’	Downstream	984-1,014	GV659520.1
Rs-ClGlu-d-Fw	5’-TCTAACGAGAAGGAGGAGCC-3’	Upstream	310-330	GV659520.1
Rs-ClGlu-e-Fw	5’-TGGTGCTTTCATGTCCGATG-3’	Upstream	233-255	GQ215234.1
Rs-ClGlu-e-Rv	5’-AGCTCCCATTTCCTCTGCTT-3’	Downstream	790-809	GQ215234.1
Rs-ClGlu-f-Fw	5’-AAACTACGATCGAAGGGCCC-3’	Upstream	995-1,014	GQ215234.1
Rs-ClGlu-g-Fw	5’-GACATCGTCGACCACAATGA-3’	Upstream	465-485	GV659520.1
Rs-ClGlu-h-Fw	5’-CATCGTCGACCACAATGAACT-3’	Upstream	467-488	GV659520.1
Rs-ClGlu-h-Rv	5’-CCCATAGCTCACCATGACGATA-3’	Downstream	1,269-1,291	GV659520.1
Rs-ClGlu-i-Fw	ATCTGCTCTATCGTCATGGTG-3’	Upstream	283-303	GQ215234.1
Rs-ClGlu-i-Rv	5’-TGTAGGAAACGGGAGGCAG-3’	Downstream	649-667	GQ215234.1

Table D-1. Continued.

Primer ID[a]	Sequence	Orientation	Range nt#[b]	GenBank number[c]
Rs-ClGlu-j-Fw	5'-GCTCTATCGTCATGGTGAGC-3'	Upstream	287-306	GQ215234.1
Rs-ClGlu-j-Rv	5'-TTGGTGTAGGAAACGGGAGG-3'	Downstream	652-671	GQ215234.1
	Drosophila GABA-gated Chloride Channel from Le Goff et al. 2005			
	5'-GCCTGCGAAATTCAGTTCAGTTCGTGCG-3'	Upstream	2,421-2,438	NM_001274688.1
	5'-GGCAAAGACCATAACGAAGCATGTTCCC-3'	Downstream	2,651-2,678	NM_001274688.1

[a]Primer ID identifies organism in which the sequence was created. Ix= *Ixodes scapularis* Rm= *Rhipicephalus microplus* Rs= *Rhipicephalus sanguineus* Deg= Sequences aligned from *Ixodes scapularis, Rhipicephalus microplus* and *Varroa destructor*. Fw= Forward Rv= Reverse. Na= Sodium channel, CL= Chloride channel

[b]Nucleotide number

[c]GenBank sequence number used to determine nucleotide number

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BIOGRAPHICAL SKETCH

Amanda Eiden was born and raised in Wisconsin. She received her undergraduate degree in biology and Spanish from the University of Wisconsin-Stevens Point. During her undergraduate years she developed a passion for travel and an interest in the disproportionate levels of disease that were found in less developed parts of the world. This led her to enroll at the University of South Florida in Tampa to pursue a Master in Public Health. At this point, Amanda's concentration was on global communicable disease and infection control. During her program she was able to spend three months doing community outreach work in Panama. While completing her master's degree she also worked full time for the Department of Health, which gave her great exposure to different areas of public health. Amanda began her employment with the Department of Health as an Environmental Scientist Specialist then transferred to work as an Epidemiologist. At this time her interest had peaked within the field of vector borne disease ecology. After completing her master's degree she began the Ph.D. program in entomology at the University of Florida. Her emphasis was on medical and veterinary entomology. Amanda's research focused on evaluating the presence and mechanisms of pesticide resistance in brown dog ticks. Despite conducting research for the majority of her time at the University of Florida, Amanda developed her interest in extension. She took opportunities to interact with stakeholders and members of the public either through her project directly or during community outreaches. Towards the end of her Ph.D. studies, Amanda found her overall interest was in extension and she took a class in agricultural extension that proved to her that this was the area she desired to pursue in her post-Ph.D. career. Amanda thinks that it is important to understand science, research, and how programs in health and agriculture function in order to create a

systems approach to problem solving. Her career goal is to work in project management for an international organization focusing on vector-borne disease prevention.