STATUS, INHERITANCE PATTERN AND MECHANISM OF FIELD-EVOLVED RESISTANCE TO GEL BAIT INSECTICIDES IN THE GERMAN COCKROACH

by

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To my parents, family, and friends

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ABSTRACT

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German cockroach (*Blattella germanica* L.) is an important urban pest that poses health risks. They carry pathogenic microorganisms, and the allergens present in their feces and cast skins can trigger asthma attacks. Gel bait formulations containing insecticides (indoxacarb and fipronil) have been used for German cockroach control for more than a decade. However, historical data suggests that cockroaches can develop resistance to insecticides that are repeatedly used. Therefore, we investigated the status and inheritance patterns of fipronil and indoxacarb resistance in cockroach strains collected from the state of Indiana and Illinois. In the first objective, topical dose-response bioassays were performed to determine fipronil and indoxacarb resistance levels in adult males of three field strains (D-IL, I-IN, and S-IN) and the laboratory-susceptible Orlando strain. Comparison of LD_{50} (median lethal dose) values between the susceptible and field strains revealed that resistance to both insecticides in the D-IL and I-IN strains was <10-fold. However, fipronil and indoxacarb resistance levels in the S-IN strain were 20- and >10,000-fold, respectively.

In the second objective, choice feeding bioassays were performed to test the performance of the resistant S-IN strain (adult males) on commercial fipronil and indoxacarb baits. Complete (100%) mortality of the S-IN strain was observed on fipronil baits. However, average mortality on indoxacarb baits was ~20% at 14d. In the third objective, synergist bioassays were done with PBO and DEF to investigate the mechanism of indoxacarb resistance. PBO did not significantly increase mortality in the S-IN strain at LD_{50}, but DEF did, suggesting increased hydrolase activity as a potential mechanism of indoxacarb resistance. In the last objective, reciprocal crosses were performed between the resistant S-IN strain and the susceptible Orlando strain to determine patterns of insecticide resistance. Topical bioassays and associated LD_{50} values for the F1 generation adult males indicated that fipronil resistance was inherited as an incompletely dominant

trait with sex-linkage. In contrast, indoxacarb resistance was inherited as a codominant trait and was not sex-linked. Our results indicate that resistance can evolve independently in different field strains. High-level indoxacarb resistance observed in the S-IN strain warrants additional research on the indoxacarb target-site as a possible resistance mechanism.

Keywords: German cockroach, insecticide resistance, indoxacarb, fipronil, inheritance patterns

CHAPTER 1. LITERATURE REVIEW

1.1 Biology

The German cockroach *Blattella germanica* (L.) (Blattodea: Ectobiidae) is a hemimetabolous insect that goes through life in three stages: egg, nymph, and adult (Ross & Mullins, 1995). German cockroach infestations occur in all seasons and under ideal growth conditions such as high temperature and humidity during the summer, they can complete their life-cycle in less than 90 days (Gould & Deay, 1940). The life cycle begins as fertilized eggs that hatch into fully developed small-sized nymphs. The nymphs undergo several molts (5-6) before emerging as winged adults. Newly emerged nymphs or adults appear white before their bodies are fully sclerotized (Roth, 1968).

The egg stage starts with fertilization inside the ovules and ends with hatching. Fertilized eggs enter the vestibulum, where they are covered by secretions produced by collateral glands. As more eggs are produced, they are protected by the hard outer shell and begin protruding and are visible from the outside, referred to as ootheca (Roth, 1968). The females continue to carry ootheca and drop it shortly before the egg hatch. The number of eggs hatched from ootheca depends on water content in the eggs. Higher number of eggs hatch from egg cases that have higher water content (Barson & Renn, 1983).

As the egg hatches, the nymphal stage begins. The nymphs molt 5-6 times before becoming adults. The number of molts which the nymphs undergo before molting to the adult stage is determined by the 3rd instar stage. Smaller-sized nymphs will molt a total of 6 times, and larger nymphs will molt five times before emerging as adults. This results in the emergence or formation of adults that are relatively similar in size (Tanaka, 1981). The adult stage starts after the final molt of the nymphal stage. Adults are reproductively mature and possess vestigial wings. Adult males are distinguishable from females with their slender bodies. The males that are at least 7 days old will begin to court receptive females that release the sex pheromone (Rust et al. 1995). After mating, the females will begin producing fertilized eggs, and the new life cycle begins.

Males and females of German cockroach have a different lifespan, with females living longer than males. Males can live up to 140 days, while females can survive up to 280 days (Ogata 1976). The German cockroach population consists of males and females in the various reproductive stages, along with nymphs of each six instars. The sex ratio of the population usually 1:1, although in some cases females outnumber males (Ross and Mullins, 1995). Under favorable conditions, German cockroach populations grow exponentially, such as those reared in the laboratory (Ross et al. 1984). In the field, however, there are environmental factors that affect whether German cockroach will establish a new area or not. For example, the lack of central heating in northern region may account to the absence of German cockroach infestations, suggesting that temperature plays an important role (Ogata 1976). The availability of food, water and harborage also influence the population growth of the German cockroach (Ross and Mullins, 1995).

1.2 German cockroach as pest

Although it may not be obvious, German cockroaches interact with humans in every aspect of life. From the grains that are harvested and transferred to storage facilities, processed and made into bread, and finally purchased by homeowners, there is always a risk of contamination and infestation from German cockroach (Brenner, 1995). German cockroach has evolved in such a way that has become a significant domestic pest. Part of their successful adaptation is the ability to survive on a wide range of food materials present in human domiciles and other human-made structures. Cockroaches are perceived as a constant nuisance to humans and are considered as important household pests in the United States along with ants and bed bugs (Brenner 1995, Gangloff-Kauffman et al. 2006). German cockroaches also produce allergens that cause allergic asthma. Currently, there are at least 11 groups of German cockroach proteins that cause allergic reactions in humans (Sohn and Kim 2012). The case of allergic asthma is apparent in children as they frequently become sick due to the high-level exposure to cockroach allergens in their homes (Rosenstreich et al. 1997).

1.3 German cockroach control and the problem of insecticide resistance

There are different methods to control German cockroaches. However, insecticides remain a popular and an effective choice to control German cockroaches in many cases. Control can be done

as surface treatments, with the use of spraying or dusting. Other techniques include space treatments with the use of ultra-low volume (ULV) (Wickham, 1995). The newer technique that has been adapted by many pest management professionals is the use of bait. Baits are made by mixing an insecticide with food or water that purportedly attract German cockroach and eventually kill them after they eat the bait (Reierson, 1981). This particular method has been continually used to control German cockroaches in the field.

However, resistance can occur as a result of continuous insecticide use for German cockroach control (Atkinson et al., 1991). Insecticide resistance is a decreased susceptibility to an insecticide and has a genetic basis (Tabashnik et al. 2014). Insecticide resistance in German cockroaches was first reported against chlordane in the early 1950s (Grayson,1953). As new insecticides were invented and used for cockroach control, more cases of insecticide resistance in the German cockroach became apparent. As of 2018, the German cockroach has developed resistance to at least 44 active ingredients that have been used previously for their control (Ko et al. 2016, Whalon et al. 2016).

Insecticide resistance can be caused by physiological or behavioral mechanisms (Wang et al. 2006). Elevated activities of detoxifying enzymes (such as Cytochrome P450 monooxygenases and hydrolases) lead to insecticide resistance in the German cockroach (Scharf et al. 1998). Other mechanisms include a mutation in the target site of insecticides preventing the molecules to bind properly. For example, a mutation in the sodium channel gene of diamondback moth was correlated with resistance to indoxacarb (Wang et al.2015). Increasing cuticle thickness can also result in insecticide resistance primarily by reduced insecticide penetration rate (Lilly et al., 2016). Finally, by avoiding certain ingredients present in a bait formulation, insects such as the German cockroach can become behaviorally resistant to insecticides (Silverman and Bieman 1993, Wang et al. 2004).

Indoxacarb acts by binding at a specific site in a sodium channel, preventing sodium to move through the channel and thus through the cell membrane (Wing et al. 2000). It can be hypothesized that resistance to indoxacarb may occur because of mutation in the sodium channel gene(s) of German cockroach. A study by Wang et al. (2016) revealed that mutation in diamondback moth

sodium channel gene, specifically F1845Y (F4i15Y) and V1848I (V4i18I) had indeed caused resistance to indoxacarb. Furthermore, when these mutations were integrated into German cockroach sodium channel gene expressed in frog (*Xenopus*) eggs, it showed reduced sodium channel's sensitivity to indoxacarb (Jiang et al. 2015). Consequently, investigating the German cockroach sodium channel gene sequence is important to understand how resistance to indoxacarb may develop. Similar to the mutations in the sodium channel that have been shown to confer indoxacarb resistance in the diamondback moth, an Alanine to Serine mutation in the resistance to dieldrin (RDL) subunit of the chloride channel gene has been known to cause fipronil resistance in the German cockroach (Gondhalekar and Scharf 2012).

1.4 Objectives and rationale

Due to its status as a pest, German cockroaches have been the target of control treatments over a long period. Continually exposed to various insecticides that are used for their control caused German cockroach populations in the field to become prone to resistance development. There have been many reports of insecticide resistance to different kinds of active ingredients (AIs). However, some of these reported cases of resistance were achieved through laboratory selections. Resistance may evolve differently under field vs. laboratory conditions. However, from the perspective of cockroach control and insecticide resistance management it may be more important to investigate field-evolved resistance.

The decrease in the susceptibility to an insecticide leads to evolution of resistance traits that have a genetic basis. Since the traits or genes that confer resistance are inherited, resistant individuals will have a selective survival advantage in the presence of insecticides, thus leading to proliferation of resistant traits in a population, which makes the insect become more difficult to control. Furthermore, if the gene(s) that confer resistance to insecticides have dominant inheritance, the trait can be passed down to the next generation more quickly, leading to increased allele frequency in the population. Therefore, to understand how the resistance can occur in a field population of the German cockroaches, it is important to study the inheritance patterns for resistance traits.

Either physiological or behavioral mechanisms can cause insecticide resistance in cockroaches. Resistance to insecticides in the German cockroach can be caused by elevated activities of

detoxifying enzymes (such as cytochrome P450 monooxygenases and hydrolases). Increased cuticle thickness can also lead to insecticide resistance primarily due to reduced insecticide penetration. Other mechanisms include a mutation in the target site of insecticides preventing the molecules to bind properly and/or modify target site function. In this study, we tested three the field strains of German cockroach and discovered that one from South Bend, IN showed high resistance to fipronil and indoxacarb. Therefore, the **main objective** of our study was to investigate fipronil and indoxacarb resistance status, potential resistance mechanisms and the inheritance patterns of insecticide resistance in the German cockroach.

CHAPTER 2. STATUS, INHERITANCE PATTERNS AND MECHANISMS OF FIELD-EVOLVED RESISTANCE TO INSECTICIDES IN THE GERMAN COCKROACH

2.1 Introduction

German cockroach (*Blattella germanica* L.) is an important urban pest in the United States. In addition to food-handling facilities, they are commonly found in low-income housing, where sanitation may also be a contributing factor to their infestation. The German cockroach has been known to produce proteins that can cause allergic reactions such as asthma in sensitized individuals, especially children (Pomes et al. 2001, Sohn and Kim 2012). People also express dislike and aversion to cockroaches, probably due to their association with unhygienic conditions (Lorenz et al. 2014). Therefore, extensive efforts are devoted, and multiple methods are deployed to control German cockroach infestations.

Out of many ways to control the German cockroach, the use of chemical agents is the most popular and inarguably the most effective control method. Chemical control agents consist of insecticides such as pyrethroids, macrocyclic lactones, phenylpyrazoles, oxadiazines, neonicotinoids, insect growth regulators, inorganic insecticides such as boric acid, etc. in different forms (spray, dust, aerosol, bait) (Wang & Bennett 2006). However, the continuous use of insecticides leads to heavy selection pressure on cockroach populations and can result in resistance development (Atkinson et al.1991). German cockroaches have been shown to develop resistance to many insecticides, including but not limited to organochlorines (Bath 1977), organophosphates (Bennett & Spink 1968), pyrethroids (Atkins et al. 1991), neonicotinoids (Wen and Scott 1997, Fardisi et al. 2017), and avermectins (Wang et al. 2004). Overall, German cockroaches have developed resistance to as many as 44 active ingredients (Ko et al. 2016, Whalon et al. 2016).

Fipronil and indoxacarb are two of the relatively newer insecticides that are widely used to control the German cockroach (Curl 2011). Fipronil is an insecticide that belongs to the phenylpyrazole class. It binds on the GABA receptor and blocks the chloride channel (Gant et al. 1998). It is used in the form of gel and granular baits to control German cockroaches. Fipronil gets oxidized by cytochrome P450 monooxygenases (and possibly other oxidases) into fipronil-sulfone, and dehalogenation of fipronil can be mediated by glutathione-S-transferase (Scharf et al. 2000).

These metabolism steps appear as the only two general metabolic conversions to fipronil that can occur in insects.

Indoxacarb is an oxadiazine class of insecticide that works by blocking the pore of neuronal sodium channel proteins (Wing et al. 2000). Within the insect body, indoxacarb gets bioactivated into a highly potent decarbomethoxylated (DCJW) metabolite and into another 8 distinct but related compounds. Part of this biotransformation process is NADPH/cytochrome P450 dependent, and part is dependent on hydrolase activity. Indoxacarb has been used as an active ingredient in gel baits, granular and spray formulations to control the German cockroach (Gondhalekar et al. 2011).

To understand the metabolic pathway of an insecticide, researchers often use synergist agents, such as PBO (piperonyl butoxide) and DEF (S,S,S-tributylphoshorotrithioate). A synergist can be defined as a compound that by itself does not cause mortality but when combined with a toxicant like an insecticide it can increase toxicity. PBO works by inhibiting oxidative detoxifying enzymes such as cytochrome P450s, while DEF inhibits hydrolytic activity of esterases (Cetin et al. 2010, Lorini and Galley, 2010). Therefore, it can be hypothesized that the co-application of synergists would increase the toxicity of fipronil and indoxacarb.

Resistance to insecticides can be defined as a genetically-based decrease in susceptibility to an insecticide (Tabashnik et al. 2014). Similar to the process of natural selection, continuously or over-used active ingredients serve as selective agents that can cause a change in resistant gene frequencies over a few generations. The increase in the frequency of individuals that carry resistance genes that allow them to survive in the presence of insecticides leads to resistance build-up within a population and eventual control failures. The mechanism of resistance to insecticides in the German cockroach can be mediated by target site insensitivity, increased expression of detoxification enzymes, reduced insecticide penetration (Scharf et al. 1998, Wu et al. 1998, Wang et al. 2015), or other factors not presently well understood.

Insecticide resistance has a genetic basis and can be inherited in different ways (Cochran and Ross, 1965). Resistance that is inherited as a recessive trait will develop slower within a population but

will be difficult to manage once the population becomes homogenous. Such an inheritance mechanism can be observed in diamond back moth that developed resistance to indoxacarb as incompletely dominant trait (Nehare et al. 2010). Conversely, insecticide resistance that is inherited as a dominant trait will quickly increase its allele frequency once it is present at a detectable level in a population. Resistance to fipronil in the German cockroach can be inherited by this mechanism (Wang et al. 2006). In some cases, insecticide resistance can also be influenced by sex-linkage traits. Resistance to fipronil in diamondback moth was partly caused by a mutation on one of their sex chromosomes specifically in the gene that encodes sodium channel proteins (Wang et al. 2015). With different possibilities of inheritance of resistance to fipronil and indoxacarb, it is important to study their respective inheritance patterns in German cockroach populations.

Since both indoxacarb and fipronil have now been in use for more than 10 and 15 years, for cockroach control, some studies have already documented resistance to these active ingredients. For example, indoxacarb resistance in German cockroaches has been reported by Gondhalekar et al. (2013) and Ko et al. (2016). Similarly, field-evolved resistance to fipronil and its mechanisms were reported by Gondhalekar and Scharf (2012). In a recent study, diagnostic assays showed that the field-collected German cockroach populations from Danville, IL and Indianapolis, IN (D-IL and I-IN) have significant resistance to indoxacarb and fipronil (Fardisi et al. 2017). Another field-collected strain from South Bend, IN (S-IN) is suspected to have very high resistance to different bait insecticides based on the information provided by a pest management professional (PMP). However, the fold resistance levels to indoxacarb and fipronil, the patterns in which resistance-associated genes are inherited, and the mechanisms of resistance are unknown in these strains, especially for indoxacarb. Understanding the status, inheritance patterns and mechanisms of field-evolved resistance in a pest population are important for formulating management strategies to both delay and manage resistance (Georghiou 1990). Therefore, the overall goal of this research was to perform a detailed investigation of different aspects of field-evolved indoxacarb and fipronil resistance in the German cockroach.

2.2 Materials and Methods

2.2.1 Insect

We used the Orlando “normal” strain (O) as a susceptible strain. This strain has been kept in the laboratory without insecticide exposure for over 80 years. The insects were kept in a controlled environment inside incubators, which maintain the temperature at 25°C with 12:12 h dark: light photoperiod. They were also given laboratory diet (#8604 rodent diet, Envigo, Madison, WI), water and cardboard harborage. We also used the field strains that were collected from Danville, IL, Indianapolis, IN, and South Bend, IN (D-IL, I-IN, and S-IN). All field strains have a history of exposure to fipronil and indoxacarb in the field. The D-IL and I-IN strains have a history of reduced susceptibility to indoxacarb, fipronil and other insecticides based on discriminating/diagnostic concentration bioassays (Fardisi et al. 2017). The S-IN strain was collected from the field after control failures were reported by pest management professionals (PMPs). All field strains were kept under laboratory condition as mentioned above without further selection with indoxacarb or fipronil.

2.2.2 Chemicals

Indoxacarb (98% AI), fipronil (99.9% AI), and Piperonyl butoxide (PBO,95.8% pure) were purchased from Chem Service (Westchester, PA). S,S,S-tributylphoshorotrithioate (DEF, 95%) was obtained from Mobay Chemical Corporation (Kansas City, MO). Maxforce gel bait (0.1 % fipronil) Advion gel bait (0.6% indoxacarb) was obtained from Univar (Indianapolis, IN). Insecticide solutions used for topical bioassay were prepared in technical grade acetone, purchased from Fisher Scientific (Pittsburgh, PA).

2.2.3 Topical Bioassay

To determine the topical toxicity of indoxacarb and fipronil, various concentrations of these insecticides prepared in acetone were applied to the first abdominal sternite of adult males (1 µl per insect) using a micro applicator (Gondhalekar et al. 2011). Control insects were treated with 1 µl acetone. Each concentration was tested against ten insects and replicated three times. Treated cockroaches were held in disposable petri dishes containing a cardboard tent, water source, and rodent food, and maintained in a controlled environmental chamber (26°C temperature and 12:12 h dark: light photoperiod). Mortality was evaluated every 24 h up to 72 h. Individuals were

considered dead if they showed no movement or were unable to walk or right themselves when they were lying on their backs (i.e., moribund). Concentrations were converted doses by dividing by the average body weight of each respective strain. Mortality data were plotted against the doses to calculate the lethal dose. The LD_{50} and LD_{99} values were determined using probit analysis in SAS 9.4.

2.2.4 Choice Bait Bioassay

We determined the effects of indoxacarb and fipronil resistance on the performance of the gel bait formulations that possess these insecticides as active ingredients against all four strains. Ten adult males (1- to 3-wk old) starved for 24 h were used per replicate in a free food choice bioassay. Bioassay arenas were provisioned with 0.5 g of either 0.6% indoxacarb (Advion®) or 0.01% fipronil (Maxforce FC Select®) gel bait, rat food (Harlan-Teklad Rodent Diet #8604) as an alternative food source, water source, and cardboard harborage. Bait consumed only within the first 24h of the bioassay was measured, as the effect of the active ingredients may affect the ability of cockroaches to feed normally. Mortality was recorded daily up to 14d. These bioassays were replicated five times for each bait and strain.

2.2.5 Reciprocal crosses and toxicity bioassay with F1 adult males

Reciprocal crosses were performed by mass crossing virgin females of the S-IN strain with virgin males of the susceptible Orlando strain (S-IN ♀ x O ♂), and virgin females of the Orlando strain with virgin males of the S-IN strain (O♀ x S-IN ♂). At least 50 males and females were used for each cross. The toxicological response to fipronil and indoxacarb in F1-generation adult males from each cross was determined using topical bioassays as explained above.

2.2.6 Synergist bioassay

Highest sub-lethal concentrations of PBO (piperonyl butoxide; 100 µg /µl or insect) or DEF (S,S,S-tributyl phosphorotrithioate; 30 µg /µl or insect) were prepared in acetone. Both Orlando and S-IN strain adults males were topically pre-treated with 1 µl of either PBO or DEF or acetone solutions (Gondhalekar and Scharf, 2012). Two hours after synergist treatment, cockroaches were treated with an LD_{50} dose of indoxacarb (2.5 $\mu g g^{-1}$ for the Orlando strain and 25642 $\mu g g^{-1}$ for the S-IN strain). Control insects were treated with acetone only. Topical synergist or insecticide application procedures were similar to those explained under the topical bioassay section. Treated

cockroaches were held in disposable petri dishes containing corrugated cardboard, water, and rodent food, and maintained in a controlled environmental chamber (26°C temperature and 12:12 h dark: light photoperiod). Mortality was recorded every 24 h up to 72h using procedures described above for topical bioassays.

2.2.7 Data Analysis

Topical bioassay data were analyzed using SAS 9.4 to estimate LD_{50} and LD_{99} values and its associated parameters. The resistance ratio for each strain was estimated by dividing the LD_{50} value for the resistant strain by the LD_{50} value for the susceptible strain. Significance of LD values was determined based on the non-overlap of 95% fiducial limits. Mean mortality data from choice bait bioassay and synergist bioassay were analyzed using t tests ($p<0.05$). Synergist bioassay data were analyzed using ANOVA and further analyzed with t-tests (Prism 7,2016). The mechanism of inheritance (including sex-linkage and maternal effect) were determined by comparing the LD_{50} between F1 progeny and their respective parents. The degree of dominance for genetic inheritance data was evaluated using Stone's method (Stone, 1963), which can be summarized as follows: $D = (2X_2-X_1-X_3)/(X_1-X_3)$, with X_1, = log (LD_{50} S-IN), X_2 = log (LD_{50} O♀ x S-IN ♂) or log (LD_{50} S-IN ♀ X O ♂), and X_3 = log (LD_{50} O). The degree of dominance (D) value was in a range between -1 and +1. A -1 value reflects a complete recessive trait and a value of +1 reflects complete dominance.

2.3 Results

2.3.1 Topical Toxicity of Fipronil and Indoxacarb

Three field strains collected from D-IL, I-IN and S-IN, when subjected to topical bioassays showed variable susceptibility levels to fipronil and indoxacarb. The Orlando (O) strain had an LD_{50} value of 2.5 $\mu g g^{-1}$ for indoxacarb and 0.05 $\mu g g^{-1}$ for fipronil. The D-IL and I-IN strains showed a low level of resistance to both indoxacarb and fipronil (Table 2.1), but not significantly different based on overlap of 95% FL. The S-IN strain, however, showed significantly higher indoxacarb and fipronil LD_{50} values in comparison to the Orlando strain. The S-IN had an LD_{50} with fipronil of 1.11 $\mu g g^{-1}$ with RR_{50} of 22. Toxicity to indoxacarb was the highest of all three field strains, with an LD_{50} at 25642 $\mu g g^{-1}$. The LD_{50} resistance ratio (RR_{50}) for indoxacarb in the S-IN strain is 10257.

The Orlando strain is the most homogeneous with respect to susceptibility to indoxacarb and fipronil with a slope at least 4 time greater than any of the field strains. On the contrary, the field strains show heterozygosity to fipronil and indoxacarb, indicating that the field populations are comprised of individuals with various susceptibility. However, p-values shown in Table 2.1, did not indicate a heterogenous mortality response in the lab or field strains. Finally, based on the data presented in Table 2.1, results show that: 1) D-IN and I-IN strains did not have high-level resistance to both fipronil and indoxacarb, while 2) S-IN strain had more substantial resistance to fipronil and indoxacarb, and 3) the S-IN strain has developed a high level of resistance to indoxacarb.

Table 2.1 Toxicity of Indoxacarb and Fipronil to laboratory (Orlando) and field *Blattell germanica* strains (D-IN, I-IN, S-IN) using a topical bioassay. Mortality was recorded at 72h.

Strain	**Insecticide**	**n**	**Slope (±SE)**	**LD_{50} (95% FL)[a]**	**χ2(d.f.)**	**p-value[b]**	**RR_{50}[c]**
Orlando (S)	Indoxacarb	240	2.24 (±0.26)	2.5 (1.89-3.01)	2.906 (6)	0.82	-
D-IN		210	0.48 (±0.12)	10.3 (7.2-13.9)	6.359 (5)	0.27	4
I-IN		240	0.29 (±0.15)	8.3 (1.3-22.9)	13.64 (6)	0.064	3
S-IN		240	0.59 (±0.16)	25642 (7282-$10.3x10^4$)*	4.99 (6)	0.54	10257
Orlando (S)	Fipronil	180	11.33 (1.69)	0.05 (0.04-0.05)	1.47 (4)	0.83	-
D-IN		210	2 (0.31)	0.06 (0.04-0.08)	8.39 (5)	0.14	1
I-IN		300	2.65 (0.35)	0.26 (0.2-0.31)	8.27 (8)	0.4	5
S-IN		360	2.66 (0.33)	1.11 (0.9-1.4)*	7.65 (10)	0.66	22

[a]Lethal dose LD_{50} values with 95% fiducial limits, all values are expressed in $\mu g g^{-1}$ insect.
[b] P-values of >0.05 indicate homogeneous population response at different insecticide doses
[c]Resistance ratios at LD_{50} were determined by comparing the LD_{50} of field strain to susceptible strain Orlando (O).
(*) indicates significant LD_{50} value in comparison to the susceptible strain based on non-overlap of 95% fiducial limits.

2.3.2 Choice bait bioassay

S-IN strain adult males consumed a similar amount of fipronil bait (101.98 mg/ 10 adult males) as the Orlando strain (93.29 mg/ 10 adult males) ($p=0.6905$). Although the S-IN strain survived longer than the Orlando strain on fipronil bait ($p<0.0308$ $\chi 2= 4.667$), 100% mortality of the S-IN strain was still achieved. The S-IN strain also consumed a significantly higher amount of indoxacarb bait (211.3 mg/10 adult males) compared to the Orlando strain (58.3 mg/10 adult males). However, not only did S-IN males consumed a higher quantity of bait, but also mortality in this strain was only 20% (80% survivorship) in 14 d. Complete (100%) mortality was observed in the Orlando strain at 1 d ($p<0.0023$ $\chi 2 = 9.333$). These results were determined based on survival analysis by comparing mortality on each day of observation between S-IN and Orlando strain.

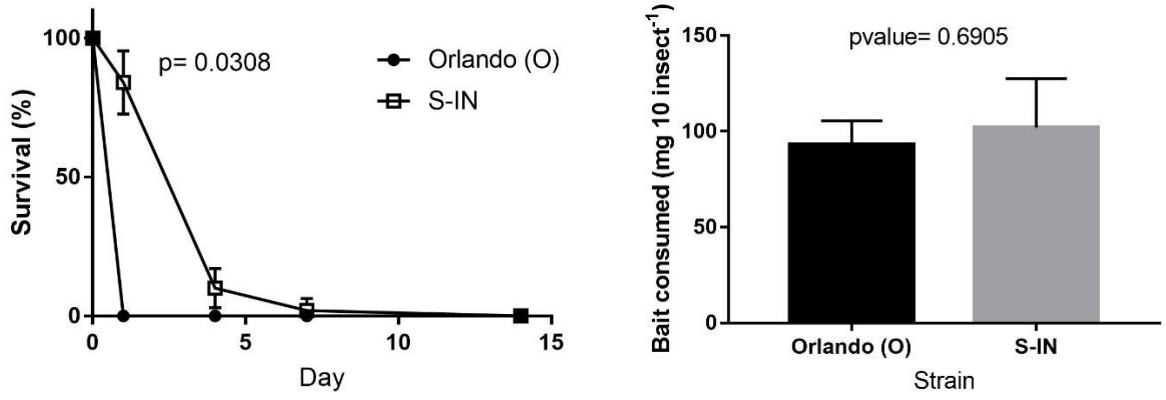

Figure 1 Survival of adult male *Blattella germanica* on commercial bait product the Maxforce select containing fipronil. P-values were determined with survival analysis and represent the differeence between Orlando strain and S-IN strain (left). Bait consumption within 24h for Orlando and S-IN strain (right). P-values were determined with t-tests.

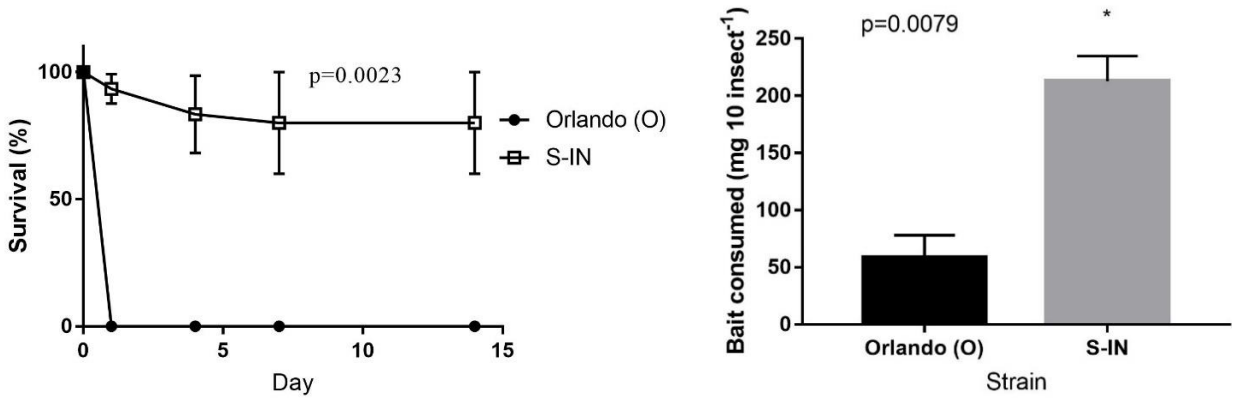

Figure 2 Survival of adult male *Blattella germanica* on the commercial bait product Advion containing indoxacarb. P-values were determined with survival analysis and represent the difference between Orlando strain and S-IN strain (left). Bait consumption within 24h for Orlando and S-IN strain (right). P-values were determined with t-tests.

2.3.3 Synergist bioassay

The Orlando (O) strain showed increased mortality (87%±7) when exposed to the combination of indoxacarb (at its LD_{50} dose) and PBO. However, mortality of the Orlando strain wasn't increased with the addition of DEF to indoxacarb (50%±7 mortality). In contrast, the S-IN strain showed increased mortality with the application of LD_{50} indoxacarb and DEF (76%±2 mortality), but the addition of PBO to indoxacarb did not significantly increase mortality (50%±5).

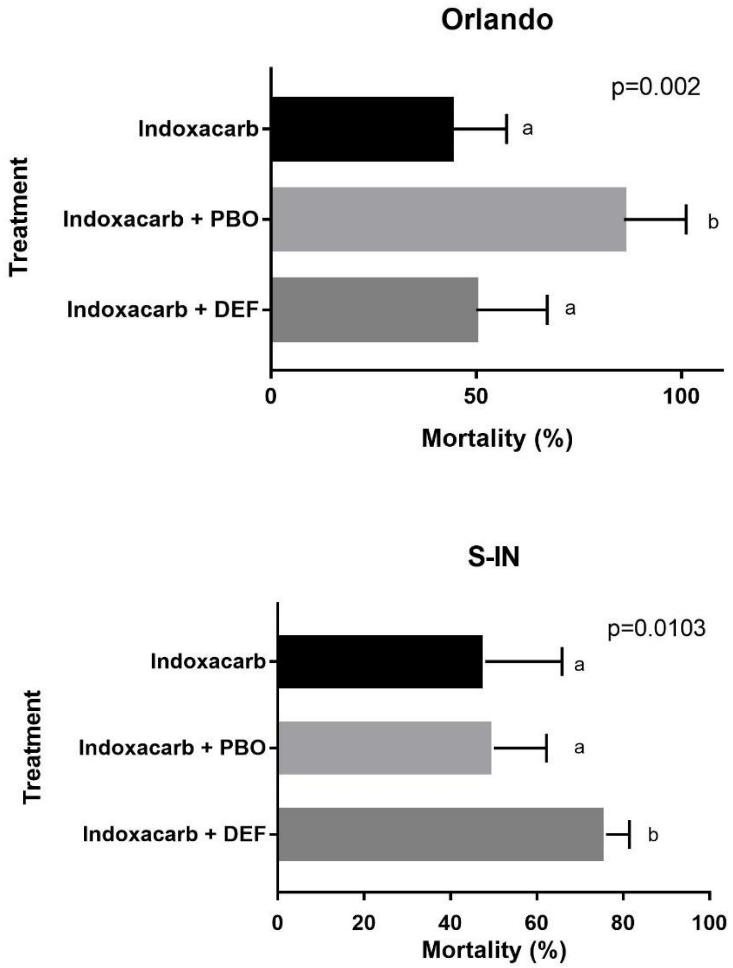

Figure 3 Mortality of the Orlando and S-IN strains at their LD_{50} values of indoxacarb (2.5 and 25642 $\mu g g^{-1}$ insect weight, respectively) alone, or when co-treated with the synergists PBO and DEF (see text for details). P-values were determined by ANOVA. Independent t-tests were used to determine treatment differences.

2.3.4 Inheritance patterns

The resistance ratio (RR) to indoxacarb in the F1 progeny O♀ X S-IN ♂ and S-IN ♀ X O♂ were 101.3 and 100.9, respectively (Table 2.2). The D values for both F1 progenies were approximately equal to 0, indicating that resistance to indoxacarb was inherited as a codominant trait. The resistance ratio (RR) to fipronil in the F1 progeny O ♀ X S-IN ♂ and S-IN ♀ X O ♂ were 9.4 fold and 17.8 fold, respectively. The D values for each of the F1 progeny were 0.44 and 0.85, indicating that the resistance to fipronil was inherited as an incompletely dominant trait. Furthermore, analysis of the dose-response relationship between the offspring showed that their fipronil LD_{50} values were significantly different because their 95% fiducial limits did not overlap. The small (1.89 fold) but significant differences indicate that resistance to fipronil may partly be inherited as a sex-linked trait (Table 3.6 in appendix). On the contrary, there was no significant difference in the LD_{50} values between the offspring, indicating that there was no sex-linkage in indoxacarb resistance in the S-IN strain (Table 3.5 in appendix).

Table 2.2 Toxicity of Indoxacarb and Fipronil to *Blattella germanica* laboratory (Orlando) and field (S-IN) strains and reciprocal crosses using topical bioassay. Mortality was recorded at 72h.

Strain	**Insecticide**	**n**	**Slope (±SE)**	**LD_{50} (95% FL)[a]**	**χ2(d.f.)**	**p-value**	**RR_{50}[c]**
Orlando (S)	Indoxacarb	240	2.24 (±0.26)	2.5 (1.89-3.01)	2.906 (6)	0.82	-
S-IN		240	0.59 (±0.16)	25642 (7282-$10.3x10^4$)*	4.99 (6)	0.54	10257
O♀ X S-IN ♂		240	1.02 (±0.17)	253.2 (117-549)*	0.898 (6)	0.61	101.3
S-IN ♀ X O ♂		240	1.026(±0.17)	252.18 (116-546)*	0.843 (6)	0.42	100.9
Orlando (S)	Fipronil	180	11.33 (1.69)	0.05 (0.04-0.05)	1.47 (4)	0.83	-
S-IN		360	2.66 (0.33)	1.11 (0.9-1.4)*	7.65 (10)	0.66	22
O♀ X S-IN ♂		270	1.237 (0.15)	0.47 (0.24-0.9)*	7.045 (7)	0.32	9.4
S-IN ♀ X O♂		210	3.5 (0.28)	0.89 (0.68-1.16)*	7.21 (5)		17.8

[a]Lethal dose LD_{50} values with 95% fiducial limits, all values are expressed in $\mu g g^{-1}$ insect.
[b]P-values of >0.05 indicate homogeneous population response at different insecticide doses
[c]Resistance ratios at LD_{50} were determined by comparing the LD_{50} of field strain to susceptible strain Orlando (O).
(*) indicates significant LD_{50} value based on non-overlap of 95% fiducial limits with those of the susceptible strain.

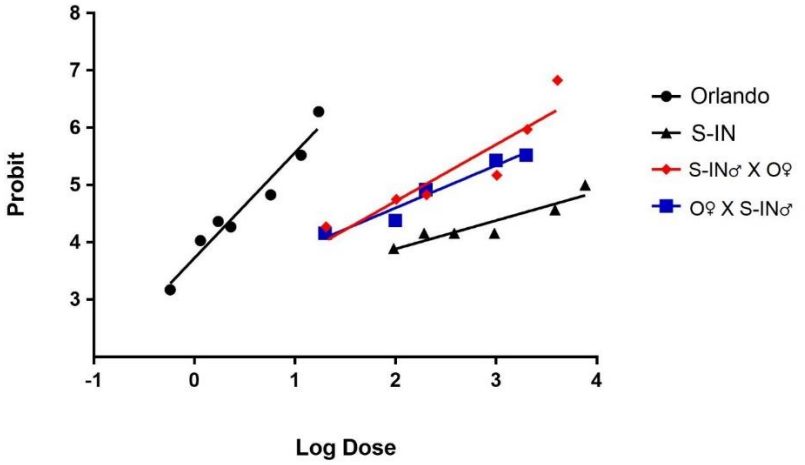

Figure 4 Toxicological responses to indoxacarb of *B. germanica* adult males from the susceptible strain Orlando, field strain S-IN and their reciprocal cross offspring (see text for details). The 95% FL overlap-analysis method was used to determine the difference in LD_{50} values between the offspring.

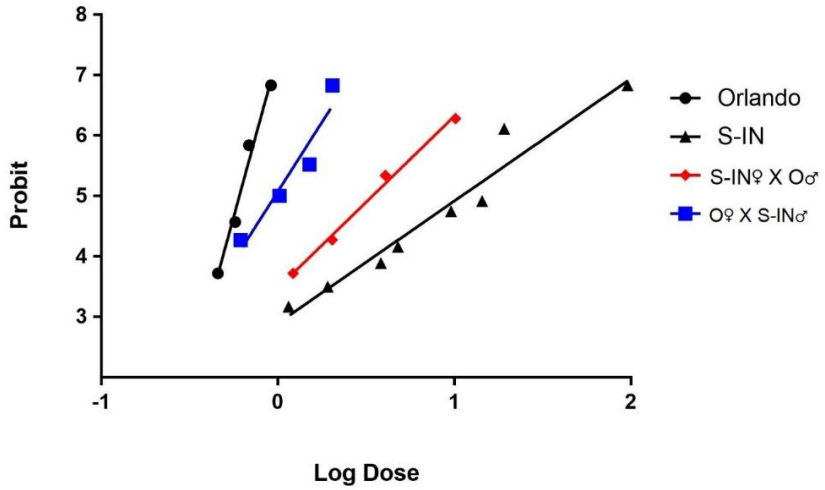

Figure 5 Toxicological responses to fipronil of *B. germanica* adult males from the susceptible strain Orlando, field strain S-IN and their reciprocal crossoffspring (see text for details). The 95% FL overlap-analysis method was used to determine the difference in LD_{50} values between the offspring.

2.4 Discussion

2.4.1 Resistance to fipronil and Indoxacarb

Insecticide resistance, often to several active ingredients (AIs), can lead to field control failures. German cockroaches have developed resistance to many organic insecticides within several years of intense usage, from the old insecticides such as chlordane and organophosphates (Rust et al. 1995) to newer ones like fipronil (Wang et al. 2006, Gondhalekar and Scharf, 2012) and indoxacarb (Chai and Lee 2010, Ko et al. 2016). We investigated the status of insecticide resistance in German cockroach populations collected from the field. We reported that the field strain D-IL and I-IN collected from Danville, IL and Indianapolis, IN respectively, showed only low-level resistance level to both fipronil and indoxacarb. In previous studies using the D-IL and I-IN strains, glass vial bioassays with a diagnostic concentration of 0.02 µg fipronil caused mortality ranging from 0-40% to both strains (Fardisi et al. 2017). Furthermore, exposure to 2.7 µg indoxacarb in the same vial assays resulted in 20-60% mortality in both strains. Our results showed less dosage was needed to achieve the same mortality rate in these strains after 1-2 years of additional lab rearing without selection.

The difference of sensitivity between the two assays likely results in different resistant values. In the vial bioassay, insects accumulate a greater amount of insecticide due to being constantly exposed to the treated surface during the assay period (Scharf et al. 1995). Whereas, with topical bioassay, each individual of German cockroach receives a known dose, thus resulting in increased sensitivity (based on RR values). However, our results indicated that both I-IN and D-IL exhibited low resistance to both indoxacarb and fipronil. This is probably due to the fact that they were first collected in 2015 and have been kept in the lab ever since. In the absence of selection pressure from the insecticides, the populations likely began reverting to a more susceptible state.

The S-IN strain collected from South Bend, IN was reported to have reduced susceptibility to insecticide treatments done by PMPs. Our results showed that the S-IN strain had developed resistance to both fipronil and indoxacarb. We found specifically that S-IN strain of German cockroach directly collected from the field had developed resistance to fipronil with a RR_{50} value of 22-fold. Previous studies of fipronil resistance in field strains of German cockroach found RR values to be 8.6-fold (Wang et al. 2006) and 36-fold (Gondhalekar and Scharf, 2012). Topical

bioassays with the S-IN strain were performed without any prior laboratory selection. This indicates that insecticide resistance can develop in the field, presumably due to prolonged exposure to the active ingredient, although the historical information on the use of fipronil for the control of the S-IN strain is not completely available.

The field strain S-IN has also developed resistance to indoxacarb, with an RR_{50} value >10000-fold. Ko et al. (2016) also reported a similar finding where German cockroaches collected from Puerto Rico exhibited high-level indoxacarb resistance. However, as per the report by Ko. et al. (2016), the >10000-fold resistance to indoxacarb resulted from ~ 2 years of laboratory selection. In this study, I report here the development of high level of indoxacarb resistance in the field without artificial selection done in the laboratory. Furthermore, the resistance level was so large that we couldn't achieve 100% mortality with the highest soluble concentration of indoxacarb in acetone. This high level of resistance may hinder the efforts of pest control using this particular AI.

2.4.2 Resistance to gel bait

According to topical bioassay results, S-IN exhibited resistance to fipronil. However, choice bait bioassay results with formulated bait product indicated that 100% mortality was still achievable. The variation between the results of topical bioassay vs choice bait bioassay may be caused by the fact that ingesting bait containing fipronil can deliver larger dose than its $LD_{50.}$ The LD_{50} of fipronil for S-IN is 57.4 ng per insect. The consumption of 10 mg of 0.01% fipronil bait within the first 24 h would deliver 1 µg fipronil, which was almost 17-fold higher than the $LD_{50.}$ This provided enough toxicant to achieve 100% mortality after 14 d of bait application. Therefore, bait application may still be efficacious in rather low or moderately resistant populations.

Another factor that may contribute to the difference between the results of topical bioassay versus ingestion was that some of the newer AIs become more toxic as they get ingested. Indoxacarb can be more toxic after ingestion due to bioactivation that produces the more toxic compound DJCW (Gondhalekar et al. 2011, 2016). However, in this case, our choice bait bioassay results were similar to topical bioassay outcomes. Even by consuming 21.1 mg of 0.6% indoxacarb bait within 24 h, which would deliver 126,600 µg indoxacarb, it was less than the amount needed to achieve 50% mortality, which would be at least 1,300,000 µg. The 20% mortality due to consumption of

indoxacarb bait was also similar to what we would expect when S-IN exposed to LD_{20} of indoxacarb. Our result strongly suggests that the application of indoxacarb bait for S-IN will no longer be effective.

2.4.3 The effect of synergists

PBO and DEF are often used as synergists to study the enzymes that metabolize insecticides and mechanisms of insecticide resistance. PBO acts as an inhibitor of cytochrome P450 enzymes, which is a group of oxidative enzymes important in detoxifying insecticides. DEF works by inhibiting esterases and hydrolases present in the insect body. PBO increased mortality in the Orlando strain but not in S-IN. In the Orlando strain, PBO effectively increased mortality most likely by inhibiting cytochrome P450 enzymes. However, a similar effect was not observed with DEF, which has been reported previously to act as a non-specific inhibitor of hydrolases. Furthermore, DEF can act as an antagonist at micromolar levels (Valles et al. 1997). A similar result was also observed with a susceptible strain of German cockroaches (Gondhalekar et al. 2016). In the S-IN strain, treatment with indoxacarb at its LD_{50} following pre-treatment with PBO did not increased its mortality compared to the application of indoxacarb alone. This finding indicates that PBO had no effect on S-IN strain. The result of the synergist bioassay for S-IN is thus inconclusive.

2.4.4 Inheritance pattern of insecticide resistance

Understanding the heritability of insecticide resistance is important for managing resistance in pest populations. Our results showed that the S-IN developed resistance to fipronil as an incompletely dominant and partially sex-linked trait. A similar finding has also been reported by Wang et al. (2006). However, in their report, Wang et al. reported the resistance to fipronil was most also apparently due in part to behavioral resistance, e.g. bait avoidance. Based on our choice bait bioassay result, we found no evidence of behavioral resistance.

Resistance to fipronil can be inherited as incompletely dominant trait controlled by an autosomal locus and a sex-linked gene, possibly point mutations in the Rdl-homologous GABA receptor gene (Wang et al. 2015). Resistance to an insecticide that is inherited as a sex-linked trait can be challenging, especially in the German cockroach. Female German cockroach has XX as its sex

chromosomes, whereas a male only has one X chromosome (Meisel and Wexler, 2018). Resistant females thus can carry the resistant gene(s) at twice the level as the male. Additionally, the sex proportion of German cockroach is 1:1, and in some cases, some populations have more females than males, and females live longer than males (Ross and Mullins, 1995). From an evolutionary perspective, the combination of these factors may increase the level of resistance to fipronil faster in the field, because a homozygous female will always pass down the resistant allele to its offspring, leading to faster resistance buildup than with autosomal resistance.

Several studies have reported the inheritance patterns for indoxacarb resistance. In diamondback moth, resistance to indoxacarb is consistently reported to be inherited as an incompletely recessive trait (Sayyed and Wright, 2006, Nehare et al. 2010). In house fly, resistance to indoxacarb was inherited as a completely recessive trait. Our finding here in the German cockroach showed that the codominant trait responsible for resistance to indoxacarb might have a different inheritance mechanism. Regarding the high level of resistance, we predicted that increased metabolic activities may not play an important role. Jiang et al. (2015) and Wang et al. (2016) reported that resistance to indoxacarb in the diamondback moth could be caused by mutations in the S6 region of sodium channel protein, the target site of indoxacarb. Therefore, further study to investigate the possible mutation is also present in German cockroach is needed.

In conclusion, insecticide resistance management is important for sustainable pest control in an urban environment. Understanding how resistance develops and is inherited in the field is crucial to provide better insights for managing resistance, for example, by implementing integrative pest management methods and by using a bait rotation strategy.

CHAPTER 3. SUMMARY

The German cockroach is a significant urban pest due to its ability to produce allergens and carry pathogenic microorganisms. They are highly adaptable in the urban environment, which makes their control very challenging. One of the key methods used to control German cockroaches is the use of insecticides, which has been heavily implemented. However, German cockroaches have developed resistance to many insecticides, including fipronil and indoxacarb. Resistance to fipronil and indoxacarb in field populations of German cockroaches ranged from low (RR_{50} value of 2-fold) to very high (RR_{50} value of >10000-fold). This might be due to the differences in their history of insecticide exposure in the field, which in most cases is unknown. One field strain (S-IN) showed resistance to both insecticides, with resistance to indoxacarb being extremely high. This warrants further investigation into target-site and other indoxacarb resistance mechanisms in the S-IN strain.

Further investigation into the toxicity of fipronil gel bait revealed that even though the S-IN strain showed resistance to the fipronil active ingredient, 100% mortality was still achieved with formulated bait product. This was expected because many gel baits contain enough active ingredient(s) to overcome a low level of resistance. On the contrary, indoxacarb bait did not perform well against the S-IN strain and only caused 20% mortality among cockroaches that were tested. Such a high level of resistance might be responsible for the control failure observed with indoxacarb bait. No behavioral resistance was observed, as the S-IN strain consumed twice as much bait compared to the susceptible strain. Therefore, we looked into the possible mechanism of elevated detoxifying enzymes with synergist bioassays.

The use of PBO and DEF as synergists resulted in different mortality trends in the susceptible and S-IN strains. PBO increased mortality in the Orlando strain but not in S-IN. On the contrary, DEF increased mortality in S-IN, but not in the Orlando strain. This might be due to the different profiles of detoxification enzyme expression between the susceptible and S-IN strains, in which the latter may have developed enzymatic resistance in response to indoxacarb. Overall, however, the outcomes of synergist bioassays were non-conclusive and did not clearly indicate the presence of cytochrome P450 or esterase/hydrolase-based resistance mechanisms in the S-IN strain.

The resistance to fipronil in the S-IN strain was inherited as an incompletely dominant trait with a partially sex-linked allele. Sex-linked insecticide resistance in German cockroaches can pose a challenge to control efforts due to the female having a greater chance of carrying the resistance allele and passing it down to the next generation. This may cause resistance to fipronil to increase quickly in field populations. On the other hand, resistance to indoxacarb in the same field strain is inherited as a codominant trait with no sex linkage. The high level of resistance may suggest that mechanisms such as target-site insensitivity may be present in this strain. Nonetheless, acquiring information regarding insecticide resistance that evolved in the field is important for sustainable pest control in an urban environment. Understanding how resistance develops and is inherited in the field is crucial to provide better insights in managing resistance, and by leading pest managers to implement integrative pest management and use bait rotation strategies. In conclusion, this research suggests that control strategies for German cockroaches should be developed based on the history of insecticide resistance of individual field populations.

APPENDIX

Table 3.1 Toxicity of Indoxacarb to laboratory strain (Orlando), field strains (D-IN and I-IN) and its reciprocal crosses using topical bioassay. Mortality recorded at 72h.

Strain	n	Slope (±SE)	LD50 (95% FL)	LD99 (95% FL)	χ2 (d.f.)	p-value	RR50	RR99
Orlando (S)	240	2.24 (±0.26)	2.5 (1.89-3.01)	20.4 (13.6-36.6)	2.906 (6)		-	-
D-IL	210	0.48 (±0.12)	10.3 (7.2-13.9)	260.3 (135.6-783.1)	6.359 (5)	0.21	4	13
F1 (♀Ox♂F)	180	1.97 (±0.25)	42 (32.14-55.38)	639.09 (342.2-1732.3)	6.72(4)	0.15	16	31
F1 (♀Fx♂O)	210	1.03 (±0.15)	5.95 (4.56-7.54)	89.9 (51.6-222.1)	3.48(5)	0.62	2	4
I-IN	240	0.29 (±0.15)	8.3 (1.3-22.9)	5611 (8089-990699)	13.64 (6)	0.17	3	275
F1 (♀Ox♂L)	180	2.08(±0.25)	33.8 (25.7-44.4)	441.9 (254.7-1031.6)	5.51(4)	0.236	14	22
F1 (♀Lx♂O)	210	1.27 (±0.19)	0.33 (0.26-0.40)	2.53 (1.62-5.21)	5.72 (5)	0.33	3	2

Table 3.2 Toxicity of fipronil to laboratory strain (Orlando), field strains (D-IN and I-IN) and its reciprocal crosses using topical bioassay. Mortality recorded at 72h.

Strain	n	Slope (±SE)	LD50 (95% FL)	LD99 (95% FL)	χ2 (d.f.)	p-value	RR50	RR99
Orlando (S)	180	11.33 (1.69)	0.05 (0.047-0.052)	0.08 (0.07-0.098)	1.47 (4)	0.83	-	-
D-IL	210	2 (0.31)	0.06 (0.04-0.08)	0.86 (0.4-2.4)	8.39 (5)	0.14	1	11
F1 (♀Ox♂F)	180	5.5 (1.08)	0.051 (0.035-0.064)	0.134 (0.095-0.365)	8.44 (4)	0.21	1	1.5
F1 (♀Fx♂O)	150	6.16 (1.42)	0.07 (0.012-0.015)	0.168 (0.104-0.35)	4.74 (2)	0.31	1	2
I-IN	300	2.65 (0.35)	0.26 (0.2-0.31)	1.94 (1.28-3.8)	8.27 (8)	0.4	5	24
F1 (♀Ox♂L)	180	5.64(0.666)	0.05 (0.048-0.059)	0.13 (0.11-0.18)	1.97 (4)	0.16	1	2
F1 (♀Lx♂O)	180	5.97 (2.11)	0.07(0.005-0.16)	0.17(0.11-0.3)	17.79 (4)	0.21	2	2.5

Table 3.3 Relative median potency estimates of indoxacarb between strains. The 95% confidence limits indicate significant difference if the value does not include 1 between the lower and upper bound

Relative Median Potency Estimates

Indoxacarb	(I) VAR00004	(J) VAR00004	95% Confidence Limits			95% Confidence Limits with LOG Transform[a]		
			Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
PROBIT	1 Orlando	2 Danville	.307	.054	1.082	-.513	-1.268	.034
		3 Indianapolis	.228	.035	1.073	-.642	-1.461	.059
		4 South Bend	.000	2.699E-6	.011	-3.308	-5.569	-1.974
	2 Danville	1 Orlando	3.257	.924	18.535	.513	-.034	1.268
		3 Indianapolis	.743	.197	2.620	-.129	-.705	.418
		4 South Bend	.002	2.550E-5	.019	-2.795	-4.593	-1.715
	3 Indianapolis	2 Danville	1.346	.382	5.066	.129	-.418	.705
		1 Orlando	4.384	0.846	28.888	.642	.059	1.461
		4 South Bend	.002	3.885E-5	.024	-2.666	-4.411	-1.612
	4 South Bend	2 Danville	624.328	51.931	39215.352	2.795	1.715	4.593
		1 Orlando	2033.495	94.127	370474.942	3.308	1.974	5.569
		3 Indianapolis	463.834	40.916	25742.283	2.666	1.612	4.411

Table 3.4 Relative median potency estimates of fipronil between strains. The 95% confidence limits indicate significant difference if the value does not include 1 between the lower and upper bound

Relative Median Potency Estimates

Fipronil	(I) VAR00004	(J) VAR00004	95% Confidence Limits			95% Confidence Limits with LOG Transform[a]		
			Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
PROBIT	1 Orlando	2 Danville	.666	.347	1.174	-.177	-.460	.070
		3 Indianapolis	.175	.056	1.071	-.758	-1.249	.430
		4 South Bend	.034	.005	.120	-1.465	-2.330	-.919
	2 Danville	1 Orlando	1.502	.852	2.884	.177	-.070	.460
		3 Indianapolis	.262	.099	.521	-.581	-1.005	-.283
		4 South Bend	.052	.009	.163	-1.288	-2.070	-.789
	3 Indianapolis	2 Danville	3.811	1.919	10.123	.581	.283	1.005
		1 Orlando	5.726	0.992	17.736	.758	.430	1.249
		4 South Bend	.196	.066	.409	-.707	-1.182	-.389
	4 South Bend	2 Danville	19.413	6.149	117.496	1.288	.789	2.070
		1 Orlando	29.166	8.302	213.865	1.465	.919	2.330
		3 Danville	5.094	2.448	15.193	.707	.389	1.182

Table 3.5 Relative median potency estimates of indoxacarb between offspring. The 95% confidence limits indicate significant difference if the value does not include 1 between the lower and upper bound

Relative Median Potency Estimates

Indoxacarb	(I) VAR00004	(J) VAR00004	Estimate	95% Confidence Limits		Estimate	95% Confidence Limits with LOG Transform[a]	
				Lower Bound	Upper Bound		Lower Bound	Upper Bound
PROBIT	1 O♀ X S-IN ♂	2 S-IN ♀ X O ♂	1.146	.862	1.800	.059	-.065	.255
	2 S-IN ♀ X O ♂	1 O♀ X S-IN ♂	.873	.556	1.161	-.059	-.255	.065

Table 3.6 Relative median potency estimates of fipronil between offspring. The 95% confidence limits indicate significant difference if the value does not include 1 between the lower and upper bound

Relative Median Potency Estimates

Fipronil	(I) VAR00004	(J) VAR00004	Estimate	95% Confidence Limits		Estimate	95% Confidence Limits with LOG Transform[a]	
				Lower Bound	Upper Bound		Lower Bound	Upper Bound
PROBIT	1 O♀ X S-IN ♂	2 S-IN ♀ X O ♂	3.290	1.651	10.869	.517	.218	1.036
	2 S-IN ♀ X O ♂	1 O♀ X S-IN ♂	.304	.092	.606	-.517	-1.036	-.218

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