CHARACTERIZATION OF IMIDACLOPRID RESISTANCE IN THE HOUSE FLY, *MUSCA DOMESTICA*.

A Thesis

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Master of Science Entomology

by

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May 2014

ABSTRACT

House flies are major pests of agriculture and public health. Globally, imidacloprid is formulated as bait for house fly control. However, there have been reports of both physiological and behavioral resistance to imidacloprid. Surveys in California and Florida in the United States determined physiological resistance to imidacloprid, yet there has been no study to characterize resistance in house flies from this region.

Three laboratory imidacloprid selections of a strain obtained from Florida increased resistance, creating a strain 2,300-fold and 130-fold resistant in females and males, respectively. Resistance was unstable, declined over time, and synergistic. Treatment with piperonyl butoxide (PBO) could not suppress imidacloprid resistance, indicating cytochrome P450s detoxification does not confer resistance. There was ≥100-fold cross-resistance to acetamiprid and dinotefuran, but none to spinosad. Resistance mapped to autosomes 3 and 4. This study discusses the significance of these results to practical imidacloprid resistance management, and the potential resistance mechanisms involved.

BIOGRAPHICAL SKETCH

Kafui was born on April 17, 1983 in the multi-cultural city of Tema in Ghana to Richard and Victoria Kavi. Her life has been marked by an interest in public health, plant and animal life, poetry as well as service to other people, due to values imparted by her parents and older siblings. She grew up on a poultry farm and spent her vacations on a vegetable farmstead owned by her parents in Takoradi. She enjoyed growing up on the farm, and participating in explorations and conservation awareness programs as a member of the Ghana Wildlife Society. She attended the all-girls' Yaa Asantewaa Girls' Senior Secondary School – a boarding school in Kumasi, Ghana – where she studied biological science. In spite of the anxiety of being away from home for the first time, she focused on her studies and excelled. She represented the school as a team member for the competitive Ghanaian National Science and Math Quiz.

She continued her next four years of academic pursuit in the biological sciences at the University of Ghana, Legon, (from 2002 to 2006) graduating with a BSc degree in Zoology (specializing in Animal Biology and Conservation). While at Legon, Kafui developed an interest in studying resistance during a school-sponsored field research exercise in 2005. On this trip, she met farmers who applied a concoction of potentially harmful pesticides on their farms. Even though these farmers seemed aware of the repercussions of such practices to their own health, ground water, their local communities, and the many consumers of their farm produce, the concoction was their only imaginable antidote to agricultural pests, which were developing resistance to

individual pesticides. This inspired her to complete her undergraduate thesis investigating insecticide resistance in *B. tabaci*, a species of whitefly, a major pest of agricultural significance. Kafui furthered public health interest, administering vaccine trials for the malaria parasite, *plasmodium falciparum*, at the Noguchi Memorial Institute for Medical Research. Kafui's interest in studying resistance deepened at a time when persisting prevalence in malaria in Ghana was suspected to be linked to the malaria parasite developing some form of resistance to anti-malarial drugs. In 2011, she joined the formidable team of scientists in the Scott Lab group of the Entomology Department at Cornell University to pursue her Master's degree, furthering this interest.

While at Cornell University, Kafui has achieved some laurels: she successfully co-authored a publication on houseflies' resistance to imidacloprid in the Pesticide Biochemistry and Physiology Journal; she also won the best student poster at the Second Annual Entomology Symposium in 2012. Kafui considers her time at Cornell University as a most momentous preparation experience for her future academic and professional pursuits.

To my husband, Kwesi, for believing in me. To my family, the Kavi's, for all the sacrifices they have made for my dreams

ACKNOWLEDGMENTS

I thank Dr. Jeffrey Scott, my advisor for his advice, guidance and support of my research. His financial provision enabled me to conduct my research and he encouraged me to strive for excellence each time. I will miss our discussions, his expertise and thoughts on my research; talking with him is always enlightening. I am grateful to Dr. Ping Wang and Dr. Brian Lazzaro who together with Jeff served on my committee for their valuable comments on my dissertation and for accommodating my urgent deadlines. Dr. Phil Kaufman, for providing the Fdm flies for this research and his assistance with my manuscript for publication. Dr. Shinji Kasai, for valuable comments on my manuscript and for his advice. Thank you to the Sarkaria Institute for Insect Physiology and Toxicology and the Hatch Projects 139412 and 139416 for providing funds for this research.

I would also like to thank Cheryl Leichter and Krista Seraydar for much needed technical assistance with my thesis research. Thanks to Keri, Leticia, Lauren, Dorte, Jessica, David, Frank and Khahn Phoung from the Scott Lab group who helped in various ways and supported me as a family would. To the friends I made here – Rakim, Derick, Kuukua, Alex, Awura-Abena, Andrew and Tele – you made the time away from home enjoyable; thank you.

Thanks to my undergraduate advisor, Dr. Ebenezer Owusu, who has inspired me all this while. My utmost gratitude goes to my parents who gave me an equal chance to education, and loved me unconditionally. Thanks to my husband, Angie, Kofi, Sachu, Korsi and my entire

family who made sacrifices so I could achieve a higher education. To my friends – Tope, Oko and Phanie – your advice always inspired me to achieve more; thanks.

Finally, I thank God for strength and health to keep going during my time at Cornell.

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LIST OF ABBREVIATIONS

α3: nicotinic acetylcholine receptor alpha 3 subunit

α4: nicotinic acetylcholine receptor alpha 4 subunit

α7: nicotinic acetylcholine receptor alpha 7 subunit

β1: nicotinic acetylcholine receptor beta 1 subunit

αBGT: α-bungarotoxin

ac: ali-curve

µg: microgram

µl: microliter

ACh: Acetylcholine

AChE: Acetylcholinesterase

ar: aristapedia

AVER: Abamectin Resistant, a strain of house fly

B. tabaci: *Bemisia tabaci*

bwb: brown body

CI: Confidence Interval

CNS: Central nervous system

D: Degree of dominance

DDT: Dichlorodiphenyltrichloroethane

DNA: Deoxyribonucleic acid

F: Female determining factor

F_1: First filial generation

GPx: Glutathione peroxidase

GRN: Gustatory Neuron Receptor

GST: Glutathione S-transferases

hr: Hour

I^M: autosome 1 bearing male determining factor

II^{IM}: autosome 2 bearing male determining factor

III^M: autosome 3 bearing male determining factor

IPM: Integrated pest management

IR3: Imidacloprid Resistant chromosome 3

IR4: Imidacloprid Resistant chromosome 4

kdr: knockdown resistance , F1014 allele of *Vssc1*

kdr-his: knockdown resistance , H1014 allele of *Vssc1*

LC_{50}: Lethal concentration that kills 50% of test animals

LPR: Learn Pyrethroid Resistant strain of house fly

Mdα2: *Musca domestica* nicotinic acetylcholine receptor alpha 2 subunit

mg: milligram

mRNA: messenger ribonucleic acid

nAChR: Nicotinic acetylcholine receptor

Nlα1: *Nilaparvata lugens* nicotinic acetylcholine receptor alpha 1 subunit

Nlα3: *Nilaparvata lugens* nicotinic acetylcholine receptor alpha 3 subunit

nt: nucleotide

OCR: Cyclodiene resistant strain of house fly

OPs: Organophosphates

P450s: P450 monooxygenases

PBO: Piperonyl butoxide

PCR: polymerase chain reaction

pen: Reduced cuticular penetration

Rdl: Resistance to dieldrin

RR: Resistance Ratio

SE: Standard Error

snp: snipped wings

SR: Synergistic Ratio

super-kdr: super knockdown resistance, T918 + F1014 allele of *Vssc1*[1]

ULV: Ultra-low-volume

Vssc: voltage sensitive sodium channel gene

Ye: yellow eyes

$Y^{M:}$ Y chromosome bearing male determining factor

LIST OF SYMBOLS

♂: Male

♀: Female

CHAPTER 1

Research goals

1.1 General research goal

The objective of this Master's thesis research was to examine imidacloprid resistance in the house fly, *M. domestica*. Imidacloprid resistance in house flies has begun to evolve since imidacloprid was first introduced for house fly control in 2004 [48, 149, 158]. The house fly has successfully evolved resistance to almost all the major classes of insecticides used for their control, characterized by control failures that have had direct and indirect impacts on humans and animals [7]. Thus, it is essential that the few insecticides that are still effective for house fly control maintain their efficacy, or at least, slow down house flies' resistance to them. Though some work has been done on imidacloprid resistance in the housefly which determined behavioral and physiological resistance [158], very little is known about imidacloprid resistance in the house fly. The factor(s) that confers imidacloprid resistance and where it (they) maps to is unknown. There is no information on the stability of resistance and the cross-resistance patterns associated with the neonicotinoid class, to which imidacloprid belongs. The mechanisms involved in resistance are also unknown. All these are all essential topics that require answers to be able to effectively manage resistance and increase the longevity of use of imidacloprid, and neonicotinoids in general.

The specific objectives of this study were to:

1. Determine the level of resistance conferred by the factor(s) responsible for imidacloprid resistance in the house fly and ascertain whether resistance was stable.
2. Determine the linkage, inheritance, dominance and cross-resistance patterns to imidacloprid.
3. Investigate the mechanisms responsible for imidacloprid resistance in the housefly.

Results from these experiments should provide insights that will lead to a better understanding of imidacloprid resistance in the house fly and generate valuable information to incorporate in a scheme to effectively manage resistance.

1.2 Specific objectives

1.2.1 Objective 1

Imidacloprid selections were carried out over three generations, starting with the KS8 strain to generate the KS8S1, KS8S2 and KS8S3 strains respectively. All these strains had varying levels of imidacloprid resistance. Bioassays were conducted over time to assess the stability of resistance; in effect to assess whether there was a fitness cost associated with resistance in the absence of insecticide pressure.

1.2.2 Objective 2

My objective was to describe the genetic basics of imidacloprid resistance in the KS8S2 strain. First, I conducted a linkage analysis to identify the chromosome(s) involved in resistance in the KS8S2 strain by crossing aabys with KS8S2 and backcrossing the F_1 males to unmated

aabys females and testing the different phenotypes at a diagnostic concentration of imidacloprid. Imidacloprid resistance mapped to chromosomes 3 and 4. I was then able to evaluate the inheritance of imidacloprid resistance by testing the F_1 progeny of reciprocal crosses between aabys and KS8 and determined the dominance using the LC_{50}'s generated. I analyzed cross-resistance patterns of this strain to other insecticides that acted at the nAChR.

1.2.3 Objective 3

From the F_1 backcross individuals of the linkage analysis, I isolated isochromosomal strains, IR3, and IR4, which have resistance factors on chromosomes 3 and 4 respectively. These strains were used to investigate the resistance mechanisms on chromosome 3 and 4 that conferred resistance in the parent strain; KS8S3. I investigated the effect of the synergist, PBO and genotyped the *rdl* gene in these strains.

CHAPTER 2

Literature review

2.1 House fly

2.1.1 Introduction

The house fly, *Musca domestica* Linnaeus, is a non-biting, syanthropic fly of the order Diptera, family Muscidae [2, 3] described in 1758. An adult is 8-12 mm in length, grey colored with a pale ventral abdomen and four characteristic dark grey longitudinal stripes on the dorsal thorax with mouthparts (proboscis) specially adapted for sucking up fluid or semifluid foods. They are holometabolous with four life stages; egg, larvae, pupae and adult [4, 5]. A female house fly lays her eggs on decomposing materials 5-10 times during her 2-4 week adult lifetime 25^{o}C , 45% RH) [5]. She deposits 75-150 eggs, either together, or in separate batches. The eggs are creamy-white, 1-1.2 mm long, and noticeably concave dorsally [4]. Development time from egg to adult is 14-16 days at 25^{o}C [5].

House flies have a worldwide distribution, from the Tropics to Palearctic regions [6], they are peri-domestic pests, and usually found where humans, animals and their related waste products are concentrated. These include landfills, farms, dairies, slaughterhouses, open-air markets and composting facilities, which provide abundant decaying organic matter such as rotting vegetation, garbage, carrion and excrement necessary for the development of larvae [4, 5]. These substrates have rich microbial communities, which support the proper development of the larvae; thus can foster enormous fly populations in a very short period.

2.1.2 Pest Status

House flies are important sanitary pests of humans and animals. They are one of the major pests at dairy, horse, sheep, and poultry facilities [7-11]. Infestations cause public health and nuisance concerns, lowered egg and milk production, reduced feed conversion efficiency and increased stress levels for young or adult animals [7, 12]. They mechanically vector >100 human and animal pathogens including species of bacteria shown to cause enteric diseases [5, 10, 11, 13, 14], and have been implicated in the transmission of pathogens that cause trachoma and epidemic conjunctivitis [5]. They can also vector *Yersinia pseudotuberculosis* leading to high avian mortality on poultry farms [15, 16].

Based on a summary report of national research efforts in 1976, losses attributed to the house fly exceeded $100 million annually [17]. This estimation was deemed to be equivalent to >$400 million in 2013 (due to inflation) with most of the losses experienced arising from the costs of control [18]. The cost of house fly outbreaks is not always easy to quantify; the disease burden due to childhood blindness caused by trachoma [19], legal fees and disruptions in production operations due to arguments with non-farm neighbors over fly outbreaks[20, 21] and the cost to military troops when activities grind to a halt due to sickness [22].

2.1.3 House fly control

Effective house fly management comprises sanitation, exclusion, chemical and non-chemical measures [3, 23, 24]. Sanitation remains the primary means of house fly control through

elimination of breeding sites for adults and food sources for larvae. This serves to effectively control fly populations as removal of these resources disrupts all life stages of the fly and limits their growth and survival [3, 23, 25]. Another cultural method to prevent house fly problems is exclusion. House flies move to human structures via temperature and odor attractants. The use of window and door screens successfully keeps them outside homes. Caulking and plugging of all openings is necessary for this approach to be effective. Exclusion can also be achieved using fans and air doors between rooms [3, 26].

Mechanical control is implemental to an effective house fly management program. The most economical and probably the oldest control measure; fly swatting falls under this category [3]. There is plethora of modern flytraps available; baited traps, ultraviolet light traps, and sticky traps which are designed using different attractants and killing methods [3, 27-30]. These are effective supplements to other control methods because they are able to trap flies with minimal effort [23]. Augmentative biological control of the housefly is advised as part of a successful IPM program. *Carcinops pumilio* (Erichson) and *Machrocheles muscadomesticae* (Scopoli) are predators of house fly eggs and are capable of reducing fly populations by 80-90 % [31-34]. Fly larval predators also exist as biological control agents. For example, the black dump fly, *Ophyra aenescens* (Wiedemann), is a predator of house fly larvae [35]. Use of entomopathogenic fungi can control fly populations adequately [36, 37]. Although all the above methods are used for house fly control, they cannot be used as the sole means to control house flies; chemical control has the advantage of offering lasting effectiveness compared to all these, quick response and the ability to be used for large-scale production facilities effectively.

Chemical control of house flies pre-dates World War II (West 1951), and caution is advised when using insecticides due to the flies' ability to develop resistance to almost every class of insecticides used against it [38-42]. Different formulations of insecticides are used for house fly management; sticky traps, sugar and pheromone based insecticidal baits and adulticides formulated as knockdown or residual sprays, aerosols and ultra-low-volume (ULV) applications are used as space treatments [23, 43]. Pyrethroids are the most common insecticidal class used for house fly control due to their relatively low mammalian toxicity, low persistence in the environment, decreased toxicity to many non-target organisms and their high efficacy [44, 45]. Relying solely on chemicals for house fly control is not optimal, as this can lead to rapid evolution of resistance. A careful implementation of all the control methods discussed is necessary for an effective management program.

2.1.4 Resistance in houseflies

Since the first reported case of insecticide resistance in house flies to DDT in Sweden in 1946 [46], this species has continued to develop resistance to several classes of insecticides. After the failure of DDT, parathion, an organophosphate, was used to control house fly populations, but resistance developed quickly in as little as two years. Following this, a mixture of pyrethrins and piperonyl butoxide (PBO) was used, and resistance developed within a year of application [47]. This pattern of insecticide use and development of resistance in fly strains has continued, reducing the options available for control of fly populations. House fly control has involved "DDT and methoxychlor, as well as other chlorinated hydrocarbons (e.g., lindane, and chlordane), organophosphates (e.g. malathion, diazinon, and dimethoate), carbamates (e.g.,

methomyl), pyrethrins (usually with piperonyl butoxide), pyrethroids (e.g., permethrin, fenvalerate, and cyfluthrin), and most recently spinosad (limited use) and neonicotinoid baits (e.g., imidacloprid)" [7]. Moderate levels of tolerance and resistance to neonicotinoids [18, 48, 49] and spinosad [50] have been detected in field collected house fly strains. Decreased target site sensitivity[51, 52] and increased metabolic detoxification [53, 54] are the major mechanisms involved in the development of insecticide resistance in house flies.

2.1.5 Genetic analysis of insecticide resistance in house flies

The house fly has a well-defined linkage map for the five autosomes and two sex chromosomes [55-57], and crossing over is rare in male house flies [58]. These attributes have been exploited to study the genetics of insecticide resistance in house flies [59]. Genetic linkage analysis maps resistance factors to their positions on chromosomes. Using this approach, major mechanisms of resistance have been mapped to their position on chromosomes. In houseflies, monooxygenase mediated resistance to pyrethroids maps to autosomes 1, 2, and 5 [60-66]. On autosome 3, *kdr* and *super-kdr*, mechanisms for knockdown resistance to pyrethroids, have been studied [63, 67-71]. Reduced cuticular penetration (*pen*), confers resistance to several classes of insecticides [62, 63, 72, 73], and is located on autosome 3. Dieldrin resistance, *rdl*, maps to chromosome 4. *Rdl* confers resistance to organochlorines and fipronil [69, 74-76]. Resistance to DDT maps to autosome 2, 3 and 5 [77].

Genetic linkage analysis is able to detect the position of a single factor responsible for resistance, as evidenced in the spinosad resistant NYSPINR [78], as well as the locations of

different factors conferring resistance in a single strain. Acetylcholinesterase (AChE) maps to autosome 2 [79]. Resistance due to increased esterase levels maps to chromosome 2 for malathion, trichlorphon and permethrin [80]. Elevated levels of glutathione *S*-transferase contribute to fenitrothion resistance and is positioned on chromosome 2 [77]. In the R_{Hokotn} strain, carbamate resistance maps to chromosome 3 and 5 [81]. Fipronil cross-resistance in LPR is due to factors on autosomes 2, 3, 4 and 5 [62]. Analysis of genetic linkage can detect the effect of each factor on overall resistance; in the diazinon resistant SKA strain, the effect of the chromosomes identified to carry factors that conferred resistance were ranked 2>5>3 [82]. A factor on autosome 2 conferred the highest pyriproxyfen resistance to the YPPF house fly strain, when compared to factors on autosome 1, 3 and 5 [83]. Knowledge of resistance factors/ mechanisms, and the autosomes they map to is instrumental when investigating the unknown factors/mechanisms that cause resistance in strains where resistance is linked to a linkage group, but the mechanism of resistance is unknown.

Mapping the position of sex determining factors in different house fly strains, is also possible and important. Sex determination affects house fly reproduction, inheritance of resistance and ultimately control. Males of the SDF strain from Fiji were shown to possess I^M, II^M and II^{IM} males, with females bearing III^M and the female determining factor, F^D on chromosome 4 [84]. In the US, house fly populations from Florida possessed the M factor on chromosome 3 only, males from New York and North Carolina possessed both Y^M and III^M and males from Maine were only Y^M [58]. House flies collected from a dairy in Ipswich, Australia possessed M on Y and autosomes 2, 3 and 4 [85]. Studies on sex determination have been

extensive [58, 84, 86-93], the ability to use linkage analysis as a tool to identify the position of a sex determining factor has been instrumental in this endeavor.

2.2 Insecticide resistance

2.2.1 Introduction

Insecticide resistance is "the inherited ability of a strain of an organism to tolerate doses of a toxicant which would prove lethal to the majority of individuals in a normal population of the same species" [94]. The first report of resistance to an insecticide was in the San Jose scale (*Quadraspidiotus perniciosus)*, in 1914; they survived mixtures of lime-sulfur which had been effective in previous years [95]. Resistance to a synthetic insecticide occurred for the first time in Sweden, in 1946, in a house fly strain only two years after DDT had been introduced (Wiesmann 1947 as cited in [96]). A year later, DDT resistance was observed in the mosquito *Cx. molestus* in Italy [96]. Resistance to 338 compounds has been reported in 574 arthropod species from 1910 to 2012, with the highest incidence rate observed in dipteran insects. The highest percentage of resistance cases has been observed in the agricultural sector and the country with the highest percentage of resistance cases is the US [4].

2.2.2 Resistance mechanisms - Physiological

2.2.2.1 Modified pharmacodynamics

Reduced cuticular penetration (*pen*) confers resistance by delaying the penetration of certain compounds (abamectin, carbaryl, chlorthion, DDT, diazinon, dieldrin, malathion, naphthalene, clothianidin and imidacloprid [97, 98] [99] through the insect cuticle [72].

Generally, *pen* offers moderate protection (2-3-fold) from poisoning by itself, and acts as an enhancer of resistance bestowed by other mechanisms especially at lower concentrations of the toxicant [97, 100-102]. In contrast, in AVER house flies however, *pen* conferred >1700-fold resistance to abamectin, characterized by a 2.4-fold decreased rate of penetration [99]. The gene that controls this mechanism is unknown, but the factors responsible have been mapped to autosome 3 in house flies [103]. Topical and penetration assays conducted on the 314 strain (isolated from the F_3 progeny of the backcross, and resistant to tributylin acetate and pyrethroids due to *pen* only) showed a 90% reduced penetration rate of ^{3}H-pyrethrin I [70]. Although several studies have been conducted on *pen* via topical assays and penetration studies to assess its effect on resistance [2, 70, 104-106], it is yet to be determined whether pen can confer protection against an insecticide when administered orally.

2.2.2.2 Altered target site

Nonsynonymous point mutations in structural genes are a common source of target-site resistance. These mutations typically reduce the binding affinity of the toxin for its receptor or alter the normal functional pharmacology of the receptor. The number of possible amino acid substitutions is very limited, thus identical mutations associated with resistance are commonly found in highly diverged taxa [107].

Three mutations in the voltage sensitive sodium channels (*Vssc*) of the house fly cause pyrethroid resistance; L1014F (*kdr*), M918T + L1014F (super-kdr) [108] and L1014H (*kdr-his*) [1]. Knockdown resistance (*kdr*) arises in response to a single point mutation in the voltage sensitive sodium channel gene (*Vssc*) leading to reduced sensitivity to DDT and pyrethroids.

Since this phenomenon was first described in house flies in 1954 in Italy [109], *kdr* has been identified and studied in almost all major agricultural and medically important arthropod pests globally [110]. In house flies, *kdr-his* generally confers the least resistance, whereas super-*kdr* offers the highest levels of resistance to pyrethroids [1, 111]. The *kdr* mutation does not confer resistance to imidacloprid [112].

Reduced sensitivity to organophosphates (OPs) and carbamates occurs because of alterations to the enzyme, AChE. This is a major mechanism of resistance for these insecticides. OPs and carbamates inhibit AChE and prevent its normal function of neurotransmitter degradation at the cholinergic nerve synapse. Reduced sensitivity of AChE is due to mutations that reduce the ability of OPs or carbamates to inhibit AChE [113]. Following the initial description in spider mites [114, 115], this mechanism has been observed in other species; house fly [116, 117], *Cx. Pipiens* [118] and *Anopheles sp*. [113, 118, 119], *Drosophila melanogaster* [120], *Bactrocera oleae* [109], *Leptinotarsa decemlineata* [121], *Aphis gossypii* [122]. In house flies, the mutations identified to confer resistance are: G262A, F327Y, G262V, G365A, and V180L. The G262V mutation confers the highest level of resistance [123].

Mutations in the resistance to dieldrin gene, *Rdl,* confer resistance to fipronil (phenylpyrazole insecticide) and cyclodiene insecticides. Resistance is caused by an A302S mutation in *Rdl* (GABA receptor) [124]. The A302S mutation generates up to a 100-fold resistance to cyclodienes and picrotoxin [124]. Globally, the A302S mutation has been identified in *D. melanogaster* [125, 126], *D. simulans* [125], *Ae. Aegypti* [127], *Anopheles spp.* [128],

Myzus persicae [129], *Bemisia sp.* [130], *B. germanica* [131, 132], *Tribolium castaneum* [133], *Hypothenemus hampei* [134], *Ctenocephalides felis* [135], *Haematobia irritans [136]*, *Diabrotica virgifera* [137], *Plutella xylosta* [138] and *M. domestica* [132]. An A302G mutation in Rdl is found in only *D. simulans* [125] and some *M. persicae* clones [129]. The two Rdl mutations do not only alter the insecticide binding site directly, but also modify allosterically the preferred state of the receptor for insecticide binding, and confer a "dual resistance effect" [139].

The first incidence of an altered target site known to confer imidacloprid resistance is the point mutation Y151S in the nAChRs, *Nlα1* and *Nlα3* of the brown planthopper, which was confirmed by allele-specific PCR [140]. However, studies of this mutation in field strains of the insect failed to detect the mutation [141, 142]. Thereafter, the N133D mutation of N1β1 subunit was shown to confer imidacloprid resistance in the same insect [143]. The mutation, R81T, of the β1 subunit has also been confirmed to confer resistance to imidacloprid in *Myzus persicae* [144], *Aphis gossypii* [145].

2.2.2.3 Metabolic detoxification

Metabolic detoxification of insecticides is one of the most common mechanisms of resistance in insects and can confer high levels of resistance to certain compounds. Cytochrome P450 monooxygenases (P450s) are an important and highly diversified group of hydrophobic heme-containing enzymes [146] concerned with the detoxification/bio-activation of endogenous and exogenous compounds such as pesticides [147]. P450s have a remarkable range of substrates they can metabolize due to the sizeable number of P450s present in each species [147] and also

due to the extensive substrate-specificity of some P450s [148]. In house flies, P450s confer resistance to a number of insecticide classes including neonicotinoids [50, 149, 150]. Carboxylesterases/hydrolases are enzymes involved in the detoxification of OPs, carbamates and pyrethroids [71]. Qualitative and quantitative changes in these enzymes result in resistance in different insects [146, 151]. "The glutathione *S*-transferases (GSTs) are members of a large family of multifunctional intracellular enzymes involved in the detoxification of endogenous and xenobiotic compounds via glutathione conjugation, dehydrochlorination, glutathione peroxidase (GPx) activity or passive/sacrificial binding" [24]. Elevated levels of GST activity in insects confer resistance in insects [152-154], and have been implicated in house flies' resistance to OPs, carbamates and DDT [155-157].

2.2.3 Resistance mechanisms - Behavioral

The ability of insects to alter their patterns of behavior in order to decrease the effect of insecticides has been documented and is thought to be influenced by both dependent [84] and independent stimuli [97]. Behavioral resistance is evident in house flies; house flies fed with imidacloprid formulated bait in a choice assay showed high levels of resistance due to behavioral changes in the flies [158] whereas flies treated topically with fenvalerate avoided knockdown due to their grooming behavior [159].

2.2.4 Molecular basis for resistance

Molecular mechanisms of resistance happen in a number of ways. This could involve increased transcription of the detoxifying enzyme as evidenced in the pyrethroid resistant LPR house fly strain. Metabolism-mediated resistance in this strain is due to CYP6D1 [160] which is controlled by factors on autosome 1 and 2 and is expressed (mRNA and protein) 8-fold higher in the resistant strain compared to the susceptible aabys strain [161].

Gene amplification also enhances resistance; it is the expression of multiple copies of genes. This is a major mechanism of resistance to OPs evident in *Culex* mosquitoes and aphids [162] and involves multiple copies of the gene for carboxylesterase. This mechanism is also active in the brown planthopper, *Nilaparvata lugens* [163] and the sheep blowfly, *Lucilia cuprina* [164].

The occurrence of a premature stop codon can also induce resistance. Spinosad resistance due to a premature stop codon resulting in truncated transcripts, and thus a non-functional nicotinic acetylcholine receptor subunit gene, in *Pxyla6* of the diamondback moth, *Plutella xylostella* [165] and Dα6 of the fruit fly, *D. melanogaster* [166, 167] have been determined.

The molecular basis of behavioral resistance has been difficult to determine until a recent study of the German cockroach. Glucose-averse (GA) cockroaches had become averse to the glucose used as phagostimulant component of baits in toxic traps, severely reducing trap effectiveness. Aversion was shown to be a result of changes in the peripheral gustatory system, whereby in GA cockroaches, glucose stimulated bitter-GRNs (gustatory receptor neurons) which suppressed the responses of sugar-GRNs causing them to avoid baits containing glucose [168].

2.2.5 Interactions between resistance mechanisms

Resistance mechanisms can interact in an additive manner, such as in the model $RR_{additive} = RR_1 + RR_2 + \ldots RR_n - (n - 1)$, where n is the total number of resistance loci and RR denotes the resistance ratio conferred by individual loci [169-171]. Interactions could be synergistic (or antagonistic) when the product of the resistance ratios is greater than (or less than) the expected resistance ratio in relation to an additive type of interaction ($RR_{additive}$) [172-174]. Interactions of multiple resistance loci have been studied in *M. persicae* [175], *D. melanogaster* [176, 177], *Cx. pipiens quinquefasciatus* [178] and house flies, *M. domestica* [63, 103, 179-183]. Knowledge of the number of loci involved in resistance and how they interact greatly facilitates effective resistance management [171, 184].

2.2.6 Genetics and evolution of resistance

The development of resistance in an insect population is generally dependent on the frequency and number of applications of insecticides used against them and the inherent characteristics of the insect species involved. Typically, initial introduction of a control agent with a novel mode of action is very effective in the first few years after which potency reduces over time. Development of resistance is pre-adaptive and not mutational, it simply occurs by selection of rare individuals in a population that can survive the toxicant. [101, 185-187]. Resistance could develop after a short period of use as was in the case of DDT [96] or after many years of use of a compound. How quickly resistance develops depends on the number of genes involved, frequency and intensity of the resistance genes in the population. Factors such as the fitness costs associated with the resistance alleles, the number of loci involved, the intensity of

selection pressure and the pattern of inheritance affect how quickly resistance will develop. How quickly resistance develops depends on the mobility of the pest involved, number of generations per year, characteristics of the chemical being used and treatment thresholds. Ultimately, all these factors would affect the lifespan of effective use of any insecticide.

2.2.7 Management of resistance

Pesticide resistance management attempts to prevent or slow the evolution of resistance development in the field, and enhance reversion of resistant populations to susceptibility with the aim of keeping the resistance level under a threshold [188]. Resistance management is dependent on pest management and pesticide-use strategies to extend the effective life of pesticides. Understanding the physiological properties of the insecticide and the biochemical and genetic basis of resistance mechanisms is essential for choosing an appropriate management strategy. Successful resistance management will lead to appreciable reduction in economic losses, which arise due to pest injury, the cost of control of pests and the formulation of newer insecticides that will effectively control pests.

Georghiou suggested three approaches to resistance management: 1) moderation, 2) saturation and 3) multiple attack [189]. Moderation works well in pest populations; reduces the risk of resistance evolving, by using susceptible alleles to dilute resistant alleles. This approach works because applications are localized during outbreaks, and compounds with short half-lives and low persistence are frequently used. Refugia is key to maintaining susceptible alleles in the population [189].

Saturation is implemented to protect high value crops or control a newly introduced invasive species. This strategy relies on the application of a high dose of insecticide to kill all susceptible individuals and render the resistance allele functionally recessive. Addition of synergists to this high dose will effectively suppress detoxification mechanisms to improve the kill. Implementing this approach early in resistance development when homozygous resistant individuals are rare is optimal as a highly resistant population could arise after intense selection from a small number of homozygous resistant individuals. This plan does not preserve natural enemies as moderation does though [189].

Management by multiple attack depends on the use of insecticides with different modes of action, either in rotation or as a mixture formulation. Here, more than one target site will be saturated, thereby reducing the probability that an individual will be homozygous resistant for all mechanisms present. When mixtures are used, there is a possibility of selection for multiple resistance mechanisms; synergists are used to enhance the potency of at least one compound [189]. Compounds that exhibit negative cross-resistance may enhance this strategy, such that resistance to one compound may enhance susceptibility to the other, thus care should be taken when choosing chemicals to use. A classic example is pyrethroid resistance in house flies caused by increased P450 activity due to CYP6D1. However, this same P450 is able to bioactivate chlorfenapyr and diazinon [190]

2.3 Neonicotinoids

Neonicotinoids are a class of insecticides that exert their toxicity at the nicotinic acetylcholine receptor (nAChR). They mimic acetylcholine and act as selective agonist at the insect nAChRs [191-193]. Neonicotinoids are potent broad spectrum insecticides and members of this class possess contact, stomach and systemic activity [194]. Imidacloprid was introduced to the market in 1991 followed by several others in this class [195]. Resistance to neonicotinoids has been slow to emerge, though currently resistance has been documented due to; (1) metabolic detoxification by cytochrome P450 monooxygenases [98, 196], (2) mutations in the target site [140, 144, 197, 198] and (3) behavioral changes [158, 199].

2.3.1 History

In 1690, nicotine in the form of aqueous extracts from tobacco (*Nicotiana tabacum*) was discovered. The active compound was subsequently isolated in 1828 by Jean Nicot after whom nicotine was named [200, 201]. Nicotine was used as a foliar spray to cover the underside of leaves [202] and was the best available agent to prevent crop damage by piercing-sucking insects for centuries [203]. It acts as a contact and stomach poison, but is not very effective due to its acute toxicity to mammals and other non-target organisms and its ability to be readily absorbed by varied routes of exposure [202-208]. Nicotinoids are compounds similar in structure to nicotine and have an ionizable, basic amine or imine substituent [209]. This group includes anabasine, anabaseine, epibatidine and dihydronicotyrine [210-212]. Nithiazine, the first

synthetic nicotine derivative, is a nitromethylene insecticide with a thiazine ring. It was found to be effective and safer than nicotine, yet it was not photostable which limited use [213].

Introduction of a chloropyridinylmethyl substituent and replacement of the nitromethylene (=$CHNO_2$) group with a nitroimine (=NNO_2) or cyanoimine (=$CHNO_2$) moiety led to enhanced photostability whilst maintaining insecticidal activity [214]. This new class of insecticides was termed neonicotinoids based on their structural similarity to nicotine and nicotine related compounds (nicotinoids) and their action as agonists of the nAChR [215, 216]. Based on maximum inward currents induced by nAChRs activation, neonicotinoids were sub-divided into two groups: (1) compounds with a heterocyclic ring, which were partial agonists, and (2) open-chain compounds which were much more effective agonists [209, 217]. Their sales represents more than one-fourth of the global insecticide market owing to high toxicity to insects and low mammalian toxicity [191, 218-220]. Insecticides in this class include imidacloprid, thiacloprid, thiamethoxam, acetamiprid, clothianidin and dinotefuran. Imidacloprid, the first of this class to be marketed commercially, is the most widely used neonicotinoid and is highly effective against several important pests, including the house fly [194], and was registered for house fly control in the United States in 2004 [40].

2.3.2 Mode of action

Extensive biochemical and electrophysiological studies have confirmed that neonicotinoids act as agonists of the nAChR. 200 nM of imidacloprid effectively displaces α-bungarotoxin (αBGT), a nAChR agonist, in cockroach neurons and from house fly and honey

bee head membrane preparations [192, 215, 221]. Displacement of αBGT by imidacloprid was also observed in other insect species; locusts (Locusta *migratoria*) and the tobacco hornworm (*Manduca sexta*), suggesting a strong interaction of the ligand-binding domain of nAChR and imidacloprid (reviewed in [221]).

Neonicotinoids are selective for insect pests whilst nicotinoids are selective for vertebrates. The inverse selectivity of these compounds hinges on the differential sensitivity of the insect and vertebrate nAChR subtypes and is attributable to their unique chemical features [222-225]. The nitro-, or cyanoimine electronegative pharmacophore of neonicotinoids has a high affinity for the insect nAChR, but exhibit relatively low potency at the vertebrate receptor. Nicotine and other nicotinoids possess an electropositive pharmacophore conferring diminished affinity to insect nAChR, but a high affinity to vertebrate nAChR [222, 226]. Protonated nicotine penetrates the "ion-impermeable" barrier surrounding the insect central nervous system (CNS) poorly. In contrast, the cationic nature of nicotine is a necessary requirement for interaction at the nAChR. The mediocre insecticidal activity of nicotine is consequently attributed to these two contradictory features [203, 215]. Penetration into the insect CNS by neonicotinoids is greater than that of the protonated nicotinoids and is linked to their hydrophobic nature [227] leading to their ability to overcome the protonation obstacle of nicotine in their translocation and target site interaction [221, 228].

2.3.3 Imidacloprid resistance in insects.

The speed and intensity with which imidacloprid was incorporated into control strategies worldwide after it became commercially available led to widespread concerns over the development of resistance [229]. However, resistance to imidacloprid and neonicotinoids has been relatively slow when compared with the rate of resistant development in other insecticide classes [195]. Resistance to imidacloprid is described in different insect species. For example after a third field season of imidacloprid use, Colorado potato beetle, *L. decemlineata* (Say), larvae and adults collected from Long Island NY, were 13.2- and 100.8-fold more resistant than the susceptible strain, respectively. F_1 reciprocal crosses indicated resistance to imidacloprid in adults was inherited as an autosomal incompletely recessive factor [230]. Whiteflies, *Bemesia tabaci* (B-type) exhibited 490-fold resistance to imidacloprid, 94- to >100-fold cross-resistance to nitenpyram, acetamiprid and thiamethoxam, 8-fold cross-resistance to spinosad and no cross-resistance to pyrethroids. Resistance was autosomal and semi-dominant [231]. In the brown planthopper, *N. lugens*, resistance was 964-fold compared with the laboratory strain. F_1 reciprocal crosses indicated that resistance to imidacloprid was inherited as a partially dominant trait. Chi-square analyses of self-bred F_2 and backcross progenies rejected the hypothesis for a single gene control of the resistance. [232].

In house flies, moderate to high levels of resistance to imidacloprid have been observed. Field collected house flies from California exhibited physiological resistance (observed using no choice feeding assays) and behavioral resistance (observed using choice feeding assays) to imidacloprid [158]. In contrast, two surveys of house fly populations across United States failed

to detect physiological resistance [18, 40], though low levels of resistance (2.1- to 12.8-fold) were observed in fly populations from Florida [48]. In Italy, 1.4-fold resistance was detected in a field collected strain [233],whereas 20- to 22-fold resistance was detected in Danish house flies collected from the field [149]. Field collected house flies from China selected with imidacloprid for 21 generations demonstrated 140-fold resistance to imidacloprid and 63.4-fold cross resistance to acetamiprid [234]. Cross-resistance of >4.2-fold to imidacloprid has been observed in the AVER (abamectin-resistant) and LPR (multi-resistant strain with high levels of resistance to pyrethroids) strains and resistance was suppressible by PBO to 0.5-fold in AVER [112]. Laboratory selections of Danish house flies with imidacloprid produced strains 75- to 150-fold resistant, resistance was suppressible by PBO in this instance [149]. Suppression of resistance by PBO in these strains suggests P450 monooxygenases are involved in resistance to imidacloprid. Further laboratory selections of the FDm strain from Florida yielded 331-fold resistance [235]. The genetic basis for resistance in the FDm strain is yet to be determined. Reduced expression of the nAChR subunit *Mdα2* is also associated with imidacloprid resistance in Danish house flies [236].

2.4 Unanswered questions

Studies have determined, that resistance to neonicotinoids especially imidacloprid is evolving [48, 49, 149], and that higher levels of resistance is possible based on observed resistance levels achieved when field collected imidacloprid resistant strains were further selected with imidacloprid in laboratory studies [149, 235]. It has also been determined that neonicotinoid

resistance in Danish house fly populations is mediated by cytochrome P450 monooxygenases [149]. However, the mechanisms underlying neonicotinoid resistance in house fly populations in North America are unknown. The mode of inheritance, fitness costs and cross-resistance patterns of this class are also not known. The factors that confer resistance to neonicotinoid insecticides, and where they map to, are also unknown. The above are all essential questions that need answers, to put in place a management regimen that will be successful and efficient in slowing the evolution of resistance to neonicotinoids.

CHAPTER 3

Genetics and mechanisms of imidacloprid resistance in house flies[1]

3.1 Introduction

The house fly, *M. domestica* is a significant pest affecting animal production facilities and public health. House flies are mechanical vectors of pathogens that cause >100 diseases in livestock, poultry and humans [237-239]. Control of house flies has been addressed in several ways with one of the most widely used being insecticides. However, house flies have shown a remarkable speed in which they are able to evolve resistance to insecticides. Understanding the mechanisms of resistance is important for the development of successful resistance monitoring and management [184].

Neonicotinoids are the most recently registered class of insecticides for house fly control, and they exert their toxicity via interactions with insect nicotinic acetylcholine receptors (nAChR). Neonicotinoids such as imidacloprid are of enormous economic importance globally, especially in the control of pests that have previously developed resistance to other classes of insecticides [218].

Imidacloprid is the most widely used neonicotinoid for house fly control and was originally formulated as a bait. Both physiological resistance (observed using no choice feeding assays) and behavioral resistance (observed using choice feeding assays) to imidacloprid have been detected in house fly populations from California [158]. Two surveys of house fly populations across the USA have failed to detect physiological resistance [18, 40], although

[1]Previously published with minor changes as Kavi, L. A. K., Kaufman, P. E., Scott, J. G. Genetics and mechanisms of imidacloprid resistance in house flies, Pestic. Biochem. Phys., 109 (2014) 64-69. http://dx.doi.org/10.1016/j.pestbp.2014.01.006.

resistance ratios of 2.1- to 12.8-fold were observed in Florida [48]. In Denmark, 20- to 22-fold imidacloprid resistance has been detected in a field collected strain [149].

Laboratory selections of field collected house flies from Denmark [149] and Florida (FDm strain) [235] have produced strains that are 75- to 150-fold and 330-fold resistant to imidacloprid, respectively. Cross-resistance to imidacloprid was suppressible by piperonyl butoxide (PBO) in the AVER strain [112] and imidacloprid resistance in house flies from Denmark was 1.9- to 6.3-fold suppressible with piperonyl butoxide (PBO) [149] suggesting that cytochrome P450s play a role in imidacloprid resistance in these strains. Imidacloprid resistance was 2.0-fold higher in females than males the 791imi (imidacloprid selected) strain [149]. Imidacloprid resistance in Danish house flies also was associated with a reduction in expression of the nAChR subunit *Mdα2* [236]. The genetics and mechanisms involved in the imidacloprid resistant strain from Florida (FDm) have not been investigated.

The aim of this study was to further characterize imidacloprid resistance in the house fly. I found that further selections (of the FDm strain) increased the level of resistance and that resistance levels were higher in females than males. Resistance was unstable and was not overcome with PBO. Resistance mapped to autosomes 3 and 4 and there was ≥100-fold cross-resistance to acetamiprid and dinotefuran.

3.2 Materials and Methods

3.2.1 Chemicals

Imidacloprid (99.5%), spinosad (98.6%), thiamethoxam (99.5%), acetamiprid (99.2%), dinotefuran (98.2%), nitenpyram (99%) and cartap hydrochloride (99.5%) were obtained from

Chem Service (Westchester, PA). Nithiazine (98.94%) was from Wellmark International (Dallas, TX). PBO (90%) was from Sigma-Aldrich (St. Louis, MO).

3.2.2 House fly strains

Two parental strains were used: aabys is a susceptible strain bearing recessive morphological markers ali-curve (*ac*), aristapedia (*ar*), brown body *(bwb*), yellow eyes (*ye*) and snipped wings (*snp*) on autosomes 1, 2, 3, 4 and 5, respectively; KS8 is an imidacloprid resistant strain obtained by consecutive selections of the FDm strain with 1%, imidacloprid-containing QuickBayt® for five generations [235] and then reared with no selection pressure. House flies were reared as previously described [39]. OCR is a cyclodiene resistant strain that is homozygous for the A302S mutation (the strain served as a control for *Rdl* genotyping) and was obtained from samples that were frozen in 2002 [240].

Strains carrying resistance factors from autosome 3 or 4 (from KS8S2) were isolated as follows. Unmated individuals of the appropriate phenotype (aa+ys for chromosome 3 and aab+s for chromosome 4) were isolated from backcrosses of aabys females and F_1 males (details in section 2.4). Two colony cages of ≥ 350 females and ≥ 110 males were set up for each phenotype. Unmated progeny were sorted for appropriate phenotype (aa+ys or aab+s) and caged. Unmated progeny from the sorted flies were selected with 107 µg imidacloprid/g of sugar for 72 hr (to kill heterozygous individuals). For the aa+ys phenotype, 1,647 female (82% mortality) and 1,075 (85% mortality) were treated, whereas for the aab+s phenotype, 1,462 females (83% mortality) and 1,106 males (67% mortality) were treated. Surviving flies were caged and their unmated progeny were sorted for phenotype and caged. Progeny of these flies were sorted for appropriate phenotype and selected with imidacloprid as described above. Survivors of the imidacloprid

selection were used to establish the IR3 (Imidacloprid Resistant chromosome 3) and IR4 (Imidacloprid Resistant chromosome 4) strains.

3.2.3 Bioassays and selections

Sugar cubes (2.33 g, Domino Dots, Domino Food Inc., Yonkers, NY), were treated with 0.25 mL of insecticide (or a solvent only control) and allowed to dry for ≥ 30 min. Imidacloprid, spinosad, thiamethoxam, acetamiprid, dinotefuran, nitenpyram and PBO were dissolved in acetone, whereas cartap hydrochloride and nithiazine were dissolved in water. For the highest concentrations of imidacloprid it was necessary to use multiple 0.25 mL applications (sugar cubes were allowed to dry between applications).

House flies (25, 3-5 d old) were placed in 180 mL waxed paper cups (Solo Cup Co., Lake Forest, IL) covered with nylon tulle (Jo Ann Fabric, Ithaca, NY), and fasted for 6 hr. Then the treated sugar cube was introduced, flies were provided water via a piece of wet cotton on top of the cup (water was provided daily) and they were held at 25°C with 12:12 h light: dark photoperiod. Percent mortality (defined as flies that were ataxic) was determined after 72 hr. Three to six concentrations giving greater than 0% and less than 100% kill were used for each LC_{50} determination. A minimum of four replicates were run for each bioassay. To evaluate the role of P450 monooxygenases in resistance, the P450 inhibitor PBO was applied (1 µg/fly) in 0.5 µl of acetone to the thoracic notum 1 hr before the introduction of the treated sugar cube to the bioassay cup. Bioassay data were pooled and analyzed via standard probit analysis [241], as adapted to personal computer use by Raymond [242] using Abbott's [243] correction for control mortality. The LC_{50} values in the imidacloprid and imidacloprid + PBO bioassays were used to determine the synergistic ratio (SR). In order to compare SRs between different strains it was

necessary to generate and estimate of the variability in the SR. This was done using the 95% CIs of the LC_{50}s to generate a range for the SRs (i.e. SR range = [lower CI from the without PBO assay/upper CI from the with PBO assay − upper CI from the without PBO assay/lower CI from the with PBO assay]). The SR range of each strain was then compared to the SR range of the aabys strain to determine whether they overlapped or not. If they overlapped, then enhancement of toxicity was not significantly different from that of the susceptible strain. If the SR range of the resistant strain was higher and did not overlap with that of aabys, then the effect of PBO was judged to be more significant in the resistant strain. The degree of dominance (D) for imidacloprid resistance was calculated using Stone's equation [244].

Three generations of imidacloprid selection were carried out starting with the KS8 strain. Selections were done using unmated females (collected within 8 hr of emergence) and males. Flies (1 day old) were fasted for 6 hr and then provided with sugar cubes that had been treated with 1.07 mg imidacloprid/g of sugar as described above. Survivors were removed after 72 hr, provided with untreated food and water and were used to generate the next generation.

3.2.4 Linkage analysis

Chromosomes involved in imidacloprid resistance in the KS8S2 strain were assessed by the F_1 male backcross method of Tsukamoto [103], using a total of 7,987 female and male flies. This method involved crossing aabys with KS8S2 (reciprocal crosses were done), backcrossing the F_1 males to unmated aabys females, separating flies by phenotype and then determining percent mortality at a diagnostic concentration of imidacloprid. Because crossing over is very rare in male house flies [58], the method allows the detection and measurement of the

"dominant" effect of each chromosome. Two reciprocal crosses were set up for the linkage analyses: Cross A (aabys female x KS8S2 male) and Cross B (KS8S2 female x aabys male). The F_1 males of these crosses (F_1A and F_1B) were backcrossed with unmated aabys females and the progeny of each cross separated by phenotypes and tested with a diagnostic concentration of 35.4 µg imidacloprid/g of sugar (as described above) to evaluate the effect of each chromosome [103].

3.2.5 Rdl genotyping

Genomic DNA was extracted from heads of individual females of the aabys, OCR, IR3 and IR4 strains or the whole bodies of males of the IR3 and IR4 using the quick fly genomic DNA prep method (www.fruitfly.org) as described previously [245]. The DNA pellet was dissolved in 30-50 µL of EB buffer (Qiagen, Valencia, CA) and stored at -20°C. A 286-bp genomic fragment of *rdl* containing the A302S mutation was obtained by PCR using primers MdRdlF2 (5'-TCTTACAGGAAATTATTCGCGTC-3') and MdRdlR2 (5'-ACTGGCAAAGACCATCACGAAACAC-3') [9] using GoTaq® Green Master Mix from Promega corporation (Madison, WI). The following thermal cycler conditions were used: 95^{o}C for 1 min followed by 35 cycles of 95°C for 30 s, 48°C for 30 s, 72°C for 30 s and a final extension at 72°C for 10 min. An aliquot (5 µL) of the PCR product was treated with 1.1 µL ExoSap (Thermo Fisher Scientific, West Palm Beach, FL), held at 37°C for 15 min and then 85°C for 15 min. Samples were sequenced at Cornell's Biotechnology Resource Center. Individual sequences were manually inspected for the GCT>TCT mutation, which results in the A302S substitution using Chromas Lite (http://technelysium.com.au/).

3.3 Results

3.3.1 Imidacloprid selection and bioassays

Three generations of selection were carried out starting with the KS8 strain. Percent mortality in response to insecticide treatment generally decreased with each selection (Table 1). Resistance ratios increased with each selection, rising from 230 (KS8), to 240 (KS8S1), to 750 (KS8S2), and ultimately reaching 2,300-fold (KS8S3) for females (Table 2). This is the highest imidacloprid resistance level reported in house flies to date. Selection of the KS8S2 strain increased resistance in females, but did not significantly increase resistance in males (Table 2). The LC_{50} values for males were significantly lower than those of females by 4.1- (aabys) to 72-fold (KS8S3) (Table 2). Imidacloprid resistance levels in females were significantly higher than those found in males for the KS8S2, KS8S3, IR3 and IR4 strains, with the largest difference (72-fold) seen between KS8S3 females and males (Table 2).

Table 1 Imidacloprid selections using treated (1.07 mg imidacloprid/g) sugar cubes to produce the KS8S3 house fly strain.

	Number of flies selected (percent mortality)	
strain	female	male
KS8	400 (90)	500 (91)
KS8S1	600 (84)	600 (92)
KS8S2	1,290 (59)	1,140 (75)

The KS8S1 strain was reared for four generations prior to being selected. The KS8S2 strain was reared for nine generations prior to the selection which produced the KS8S3 strain (see Materials and Methods).

Table 2 Toxicity of imidacloprid to several house fly strains and backcrosses via a treated-sugar cube feeding assay.

Strain	Sex	n	LC_{50} (95% CI)[a]	Slope (SE)	RR[b]	♀/♂[c]
aabys	Female	400	0.012 (0.010-0.014)	3.0 (0.3)		4.1
aabys	Male	500	0.0029 (0.0024-0.0035)	2.1 (0.2)		
KS8	Female	1525	2.7 (1.9-3.9)	1.3 (0.2)	230	
KS8S1	Female	1250	2.9 (2.3-3.7)	1.0 (0.1)	240	
KS8S2	Female	600	9.0 (6.4-15)	1.2 (0.1)	750	17
KS8S2	Male	700	0.52 (0.41-0.66)	1.2 (0.1)	180	
KS8S3	Female	1275	28 (17-52)	0.63 (0.1)	2300	72
KS8S3	Male	800	0.39 (0.30-0.50)	1.1 (0.1)	130	
F_1A	Female	700	0.43 (0.34-0.56)	4.9 (0.1)	36	2.2
F_1A	Male	600	0.20 (0.16-0.25)	1.4 (0.1)	70	
F_1B	Female	700	0.23 (0.18-0.29)	1.4 (0.1)	19	0.6
F_1B	Male	600	0.38 (0.30-0.47)	1.4 (0.1)	130	
IR3	Female	600	0.071 (0.059-0.085)	1.8 (0.1)	5.9	9.0
IR3	Male	900	0.0079 (0.0063-0.0099)	1.3 (0.1)	2.7	
IR4	Female	600	0.13 (0.11-0.16)	1.6 (0.1)	11	14
IR4	Male	800	0.0091 (0.0073-0.011)	1.7 (0.1)	3.1	

n = number of flies exposed.

[a]In units of mg imidacloprid/g sugar.

[b] Resistance ratio = LC_{50} of resistant strain/ LC_{50} susceptible strain. All RRs were significantly greater than 1.0 based on non-overlap of LC_{50} confidence intervals.

[c] LC_{50} female/LC_{50} male

The toxicity of seven insecticides that target the nAChR was determined against the imidacloprid susceptible (aabys) and resistant (KS8S3) strains (Table 3). The relative toxicity to the susceptible strain was dinotefuran = nithiazine ≥ thiamethoxam > spinosad > acetamiprid = imidacloprid > nitenpyram = cartap. Cross-resistance was detected for all insecticides, except spinosad. However, the levels of cross-resistance varied considerably from 3- (nithiazine) to 110-fold (acetamiprid).

The inheritance of imidacloprid resistance was evaluated by testing the F_1 progeny of reciprocal crosses between aabys and KS8S2 (Table 2, Figure 1). Given that there was a difference in imidacloprid toxicity between males and females in the parental strains, the degree of dominance (D) was calculated for each sex in both reciprocal crosses. D was 0.1 for F_1A females and -0.1 for F_1B females, indicating imidacloprid resistance is inherited as an intermediate trait in this sex. D values were 0.6 and 0.9 for F_1A and F_1B males, respectively, indicating imidacloprid resistance is inherited as an incompletely dominant to nearly fully dominant trait in this sex.

Table 3 Toxicity of seven insecticides to imidacloprid susceptible (aabys) and resistant (KS8S3) female house flies.

	aabys			KS8S3			
Insecticide	n	LC_{50}[a] (95% CI)	Slope (SE)	n	LC_{50}[a] (95% CI)	Slope (SE)	RR
Acetamiprid	800	0.011 (0.0093-0.013)	1.9 (0.1)	400	1.2 (0.69-1.9)	0.78 (0.19)	110
Dinotefuran	500	0.0012 (0.00094-0.0017)	5.0 (1.5)	500	0.12 (0.076-0.18)	3.3 (1.4)	100
Thiamethoxam	500	0.0017 (0.0015-0.0018)	5.5 (0.5)	700	0.044 (0.040-0.049)	2.8 (0.2)	26
Nitenpyram	500	0.11 (0.067-0.19)	2.1 (0.6)	400	2.5 (1.8-3.5)	2.6 (0.76)	23
Cartap	500	0.14 (0.12-0.16)	2.9 (0.2)	400	2.0 (1.3-2.7)	1.1 (0.2)	14
Nithiazine	400	0.0013 (0.0010-0.0017)	4.4 (1.2)	450	0.0039 (0.0036-0.0045)	4.4 (0.57)	3
Spinosad	600	0.0060 (0.0055-0.0075)	3.9 (0.3)	600	0.0046 (0.0041-0.0051)	3.0 (0.3)	0.77

n=number of flies exposed.

[a] In units of mg imidacloprid/g sugar.

[b] Resistance ratio = LC_{50} of resistant strain/ LC_{50} susceptible strain. All RRs were significantly greater than 1.0 based on non-overlap of LC_{50} confidence intervals, except for spinosad.

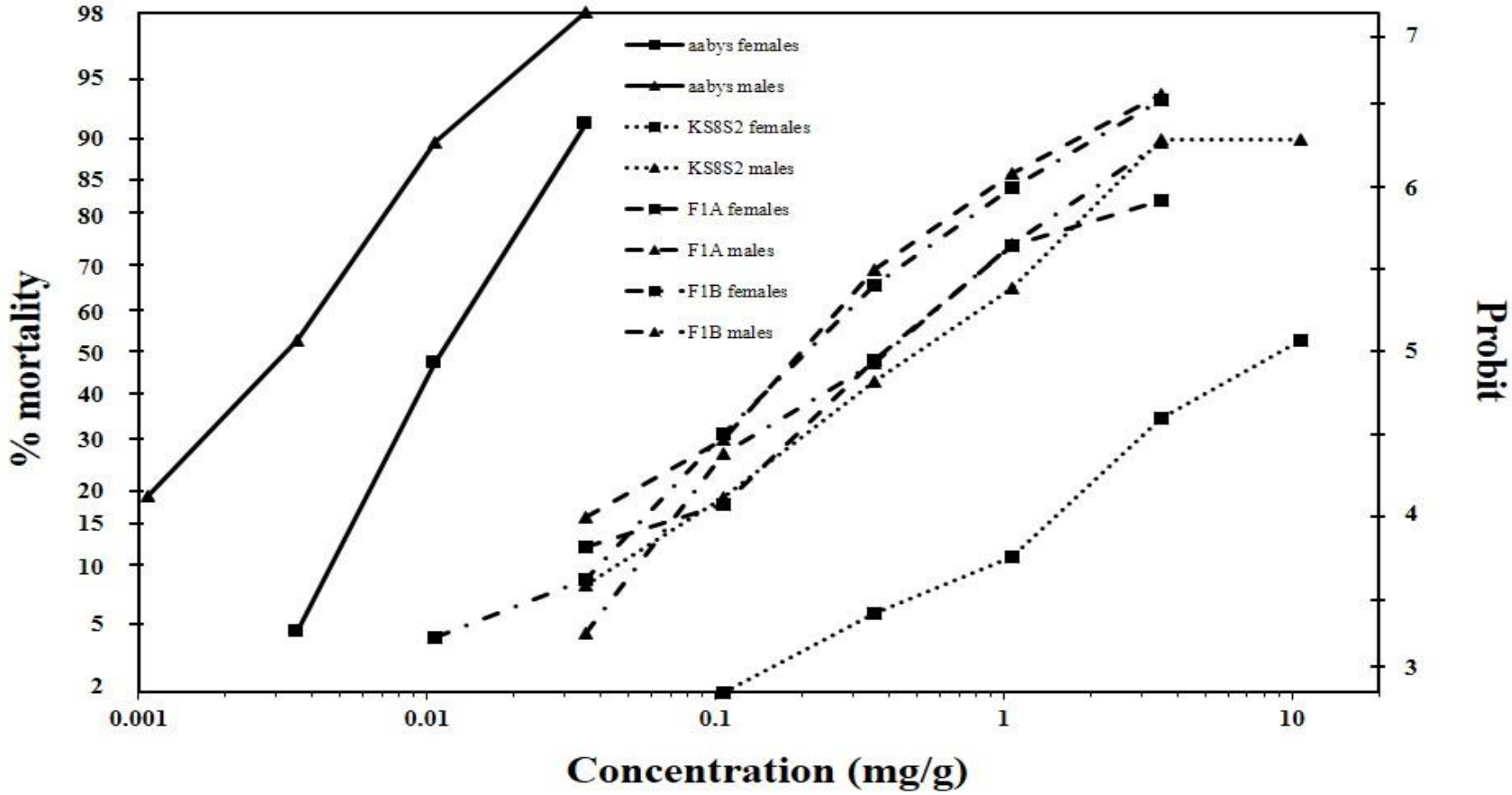

Figure 1 Toxicity of imidacloprid to aabys, KS8S2 and F1 house flies

3.3.2 Linkage analysis of imidacloprid resistance in KS8S2

The linkage analysis revealed that imidacloprid resistance of the KS8S2 strain is due to factors on chromosomes 3 and 4 (Table 4). There was no difference in the results for the linkage analyses between cross A and cross B, so the data were pooled. For cross A, all females were brown bodied and males were black bodied. For cross B, males and females of the backcross exhibited both body colors. Thus, the male determining factor is on chromosome 3 in this strain, which is consistent with previous studies on house flies in Florida [58, 87].

3.3.3 Evaluation of isochromosomal lines

Based on the results of the linkage analysis (above) strains were isolated that had either autosome 3 (IR3) or autosome 4 (IR4) from the KS8S2 strain. Chromosome 3 conferred 5.9-fold resistance in females and 2.7-fold resistance in males. Chromosome 4 conferred 11-fold resistance in females and 3.1-fold resistance in males (Table 2).

3.3.4 Effect of PBO on imidacloprid resistance

PBO treatment resulted in a 3.4-fold synergism of toxicity to the aabys strain (Table 5), suggesting that P450-mediated detoxification is reducing, at least partially, the toxicity of imidacloprid in this strain. PBO treatment resulted in a 6.1-fold synergism of toxicity in KS8S3, but the range of the SRs found for in the KS8S3 (as well as the IR3 and IR4) strains was not significantly different from that of aabys. This suggests that P450-mediated metabolism is not responsible for resistance in these strains.

Table 4 Factorial analysis of the linkage of imidacloprid resistance in the KS8S2 house fly strain.

Autosome(s)	Effect	Mean square	F value
5	171.52	1838.76	6.48
4	586.34	21487.24	75.76*
4+5	-26.11	42.62	0.15
3	227.82	3243.77	11.44*
3+5	-4.74	1.41	0
3+4	37.56	88.15	0.31
3+4+5	-17.94	20.12	0.07
2	113.52	805.44	2.84
2+5	21.09	27.79	0.1
2+4	14.4	12.96	0.05
2+4+5	11.75	8.63	0.03
2+3	-9.01	5.07	0.02
2+3+5	-29.05	52.74	0.19
2+3+4	3.54	0.79	0
2+3+3+5	-5	1.56	0.01
1	51.84	167.95	0.59
1+5	37.76	89.13	0.31
1+4	-8.24	4.24	0.01
1+4+5	6.58	2.7	0.01
1+3	-50.2	157.53	0.56
1+3+5	-27.03	45.65	0.56
1+3+4	-20.25	25.62	0.09
1+3+4+5	-27.95	48.82	0.17
1+2	9.52	5.67	0.02
1+2+5	-22.06	30.41	0.11
1+2+4	-5.23	1.71	0.01
1+2+4+5	24.43	37.31	0.13
1+2+3	-38.42	92.25	0.33
1+2+3+5	-0.73	0.03	0
1+2+3+4	0	0	0
1+2+3+4+5	5	1.56	0.01
Error	283.62		

*Statistically significant at $p < 0.01$ level.

Table 5 Toxicity of imidacloprid + PBO to susceptible and resistant strains of house flies.

Strain	LC_{50}[a] (95% CI)	Slope (SE)	SR[b]	RR[c]
aabys	0.0035 (0.0028-0.0042)	2.0 (0.2)	3.4*	
KS8S3	4.6 (3.3-6.6)	0.82 (0.1)	6.1*	1300
IR3	0.062 (0.042-0.093)	1.7 (0.3)	1.1	18
IR4	0.070 (0.058-0.083)	2.0 (0.2)	1.9*	20

[a] In units of mg imidacloprid/g sugar.

[b] Synergism ratio = LC_{50} without PBO (from Table 1)/ LC_{50} with PBO.

[c] Resistance ratio = LC_{50} of resistant strain/ LC_{50} susceptible strain. All RRs were significantly greater than 1.0 based on non-overlap of LC_{50} confidence intervals.

*Significantly >1.0 based on non-overlap of confidence intervals.

3.3.5 Rdl genotyping

The only known mechanism of resistance on autosome 4 of house flies is the A302S mutation in *Rdl*, which confers resistance to cyclodiene insecticides [125] and fipronil [62, 246]. Therefore, a fragment of *Rdl* containing the A302S mutation was PCR amplified and sequenced from the aabys, OCR (cyclodiene resistant strain), IR3 and IR4 strains. As expected, *Rdl* from the susceptible strain was homozygous for A302 (n=4), while OCR was homozygous for S302 (n=3). The IR3 (n=10) and IR4 (n=14) strains were both homozygous for the A302 susceptible *Rdl* allele.

3.3.6 Stability of imidacloprid resistance

Levels of imidacloprid resistance in the KS8, KS8S2 and KS8S3 strains were evaluated over time (Figure 2). Resistance decreased 9-, 23- and 23-fold in the KS8, KS8S2 and KS8S3 strains over 14, 12 and 6 months, respectively (representing approximately 26, 26 and 13 generations, respectively). Bioassays with the aabys strain found that the LC_{50} varied by $\leq$ 2-fold over the time course of this study.

3.4 Discussion

Imidacloprid selection resulted in a highly resistant strain of house fly, although the resistance was not stable and decreased over the course of several months. Thus, there are resistance alleles present in house flies in Florida that can be selected for and will confer substantial levels of resistance. However, the instability of the resistance suggests that this resistance has a fitness cost (in the absence of insecticide). Therefore, resistance problems could

potentially be mitigated in the field if there were periods of times when imidacloprid (and other neonicotinoids) were not used, to allow the population to revert to susceptibility. The reversion of resistance, especially in the KS8S3 strain suggests that either the selections had not removed all susceptible alleles (which were more fit in the absence of insecticide) or else the mechanism itself is unstable (e.g. esterase-4 resistance to omethoate is due to gene amplification in *Myzus persicae* and is unstable [247]).

The imidacloprid selections resulted in a strain in which females were significantly more resistant than males, suggesting some part of the resistance is sex-linked. However, in the F_1 offspring, LC_{50} values of males from both reciprocal crosses were <2-fold different, as were the LC_{50} values for the F_1 females from both reciprocal crosses. This suggests there are neither maternal nor paternal contributions to the higher levels of resistance (i.e. there is no sex-linked resistance) found in females of the KS8S2 and KS8S3 strains, which is difficult to explain. In the F_1, males were 70- to 130-fold resistant, compared to 19- to 36-fold in females, due to the inheritance of resistance in males being incompletely dominant to nearly full dominant, while resistance in females was inherited as an intermediate trait. Why greater levels of resistance would be observed in females of the KS8S2 and KS8S3 strains (and also in the IR3 and IR4 strains), inheritance of resistance would be different between males and females, and yet there would be neither maternal nor paternal effects on resistance is not clear.

Linkage analysis indicated factors responsible for resistance were on autosomes 3 and 4. Strains containing either autosome 3 or 4 from the KS8S2 strain were isolated and found to be only moderately resistant (5.9- and 11-fold, respectively) compared to the parent strain, from which they were isolated. This suggests that the factors on autosomes 3 and 4 interact in a greater

than additive manner, which is the most common interaction between homozygous resistance factors [171]. No greater than (or less than) additive effect was found in the linkage analysis, and is consistent with the observation that interaction between heterozygous resistance factors is variable [171].

Bioassays using the sugar cube method produced LC_{50} values for the susceptible strain similar to those using dental wicks impregnated with sugar water plus imidacloprid [40]. An advantage of the sugar cube method is that the cubes could be reused for up to four weeks without loss of biological activity (data not shown).

Previous studies with house flies have found two resistance mechanisms on autosome 3 (*pen*, *kdr*) and one on autosome 4 (*Rdl*) [248]. The *kdr* mutation in *Vssc* does not confer cross-resistance to imidacloprid [112]. *Pen* (decreased rate of cuticular penetration) confers resistance to a range of insecticides [249] and may be the factor responsible for resistance in the IR3 (and KS8S2) strain. However, studies of this mechanism have been limited to topical application, and the relative levels of resistance this mechanism can confer when insecticides are ingested are unknown. Our finding that the IR4 strain did not have the A302S *Rdl* mutation is consistent with a previous study that found the *Rdl* mutation did not confer cross-resistance to imidacloprid [112]. Thus, the imidacloprid resistance on autosome 4 (and perhaps autosome 3) appears to be unique.

Failure of PBO to block expression of resistance, suggests that at least part of the imidacloprid resistance must be due to an altered target site. *Drosophila/Musca* homology maps [250] predict that α3 and α7 would be on autosome 3, and that α4 and β1 would be on autosome 4. These genes are candidates for further investigations with the IR3 and IR4 strains. The *Mdα2*

gene that was reported as being reduced in expression in imidacloprid resistant house flies from Denmark [236] is on autosome 2 [251] and therefore is not at the loci for imidacloprid resistance in the KS8S3 strain.

Cross-resistance was detected to all the insecticides acting in nAChRs, except for spinosad. It is difficult to evaluate the levels of cross-resistance observed, relative to the level of resistance to imidacloprid, because the latter was unstable and imidacloprid assays were not run at the same time as the other compounds. Considering that the cross-resistance assays were run in March 2013, the level of imidacloprid resistance in the KS8S3 strain at the time was probably near 2,000-fold (Figure 2). Thus, the cross-resistance levels (even the ≤ 100-fold cross-resistance to acetamiprid and dinotefuran) appear to be substantially lower than the level of resistance to imidacloprid. In terms or resistance management applications, it would be optimal to use insecticides to which there is minimal cross-resistance. In light of our results, it would appear that imidacloprid and spinosad would be suitable materials for a resistance management rotation scheme.

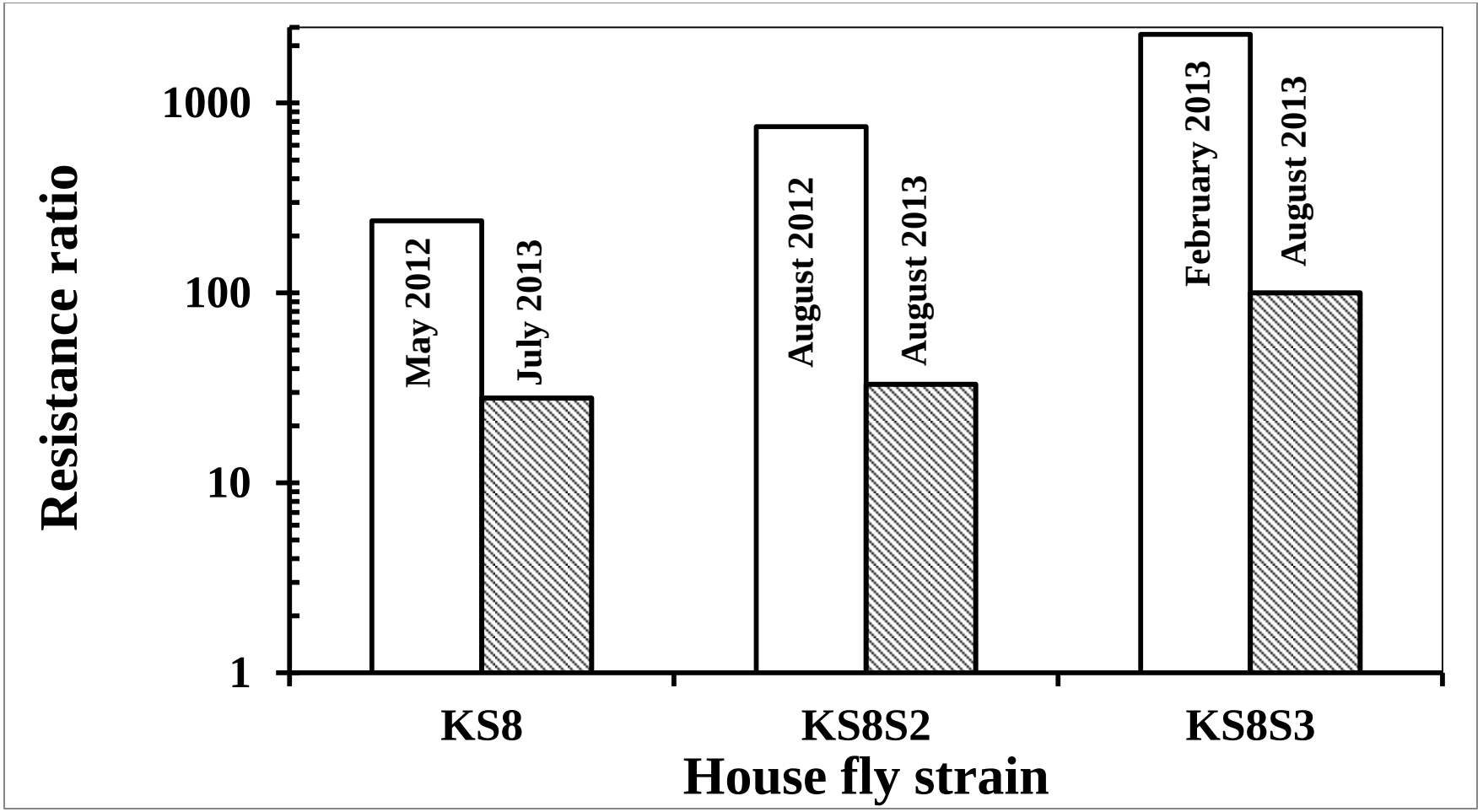

Figure 2 Changes in imidacloprid resistance over time in three strains of house fly.

The dates (month/year) shown for KS8, KS8S2 and KS8S3 represent approximately 26, 26 and 13 generations, respectively.

CHAPTER 4

Future directions

Characterization of imidacloprid resistance in the house fly is far from completion. However, this study has served to answer some of the pertinent questions required for further work to be conducted, for effective resistance management. From this study, resistance mapped to chromosome 3 and 4, but the factors and mechanisms involved are not known.

A first step to investigate the possible mechanisms of resistance will be to examine the nAChRs predicted to be on chromosomes 3 (α3 and α7) and 4 (α4 and β1) based on *Drosophila/Musca* homology maps [250] using information from the house fly genome and transcriptome databases. It would be necessary to identify these nAChR in the house fly, their relative transcript expression levels in aabys, KS8S3, IR3 and IR4 and the possible mutations present that could confer imidacloprid resistance. This same approach revealed, that a 60% lower copy number of Mdα2 in the 766b and 791imi strains compared to the susceptible strain of Danish house flies conferred neonicotinoid resistance, and will be useful in investigating imidacloprid resistance in the house fly. Identifying the mechanisms responsible for resistance in these strains will be important in increasing the knowledge available and drawing inferences from, when compared to other resistance strains in the future.

Besides, it will be necessary to identify what level of protection the factors on autosome 3 and 4 confer to the KS8S3 strain. This can be studied by isolating a strain, with the aa++s phenotype from crosses of the IR3 and IR4 strains. This will be very important in determining

quantitatively the resistance offered and synergistic effect of the two factors on autosomes 3 and 4.

For the factors involved in resistance on chromosome 3, it would be necessary to rule out the effect of *pen* as was done for *kdr*. However, there is no study that has investigated the effect of *pen* via feeding assay. A study to investigate *pen* by feeding assay will be important in ascertaining whether it contributed to resistance, and to what extent.

Due to the unstable nature of imidacloprid resistance in the KS8, KS8S2 and KS8S3 strains, it would be necessary to investigate the fitness costs associated with resistance once the mechanisms for resistance are identified. Knowing that resistance is unstable is not enough, knowledge of the fitness costs associated with resistance in the absence of selection pressure is necessary for effective resistance management.

Cross resistance studies showed that the KS8S3 strain is cross resistant at varying levels to members of the neonicotinoid class. One challenge in this characterization was that imidacloprid bioassays of the strain were not done at the same time as bioassays of the other insecticides. Assessing the current level of resistance and cross resistance to the other neonicotinoids in this strain will help to ascertain whether cross resistance is also reverting as in the case of imidacloprid or not, and what the actual level of cross resistance is.

Another study of interest will be to investigate how quickly resistance will rise after reverting to very low levels. The KS8S3 strain was 2300-fold resistant initially but reverted to 100-fold resistance in only 6-months. Selecting with imidacloprid again will give the chance to study what effect this selection can have and how quickly the previous high levels of resistance

are achieved. This will be necessary for adding to the knowledge required for a rotation based resistance management scheme. Further selections of the KS8S3 strain will also be important to determine whether the unstable nature of resistance is due solely to fitness costs or whether it was due to the heterozygous nature of the KS8S3 population. Selections with imidacloprid will most likely push for a homozygote resistant population unless the heterozygote state is preferred. This will generate more insight into the possible fitness costs involved.

Finally, resistance to imidacloprid is higher in females than in males of the KS8S2 and KS8S3 strains, although there seems to be no maternal or paternal contributions to the higher levels of resistance based on bioassays of the F_1 of reciprocal crosses between aabys and KS8S2. Higher levels of resistance to spinosad in females have also been observed in 791spin strain [93], although the difference was not as big as seen in the KS8S2 and KS8S3 strains. A possible explanation given was that the variation in spinosad resistance between males and females was due to a recessive spinosad resistance factor on autosome 3 [93]. In our case however, the same variation is also exhibited in the IR4 which has resistance factors from only autosome 4. Whether the disparity in resistance between the sexes is due to recessive factors or might just be a function of resistance to insecticides that act at the nAChR is yet to be determined.

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