UNIVERSITY OF CALIFORNIA
RIVERSIDE

Mechanisms Conferring Behavioral Resistance to the Neonicotinoid: Imidacloprid in the House Fly (*Musca domestica* Linnaeus)

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Entomology

by

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March 2021

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ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisor, Dr. Alec Gerry, for his unwavering support and guidance over the years I spent in his laboratory. I know I would not have accomplished all the things I did during my Ph.D. if Alec had not believed in me and fully supported the crazy research topic I pursued. His unwavering positivity made even the bad days in the lab enjoyable.

I would like to thank my dissertation committee members Dr. Anupama Dahanukar and Dr. Jason Stajich, for their support and enthusiasm around my dissertation research. Dr. Anupama Dahanukar was invaluable in providing her time and advice during the early stages of developing my research project. It has always provided me with insightful and constructive feedback on anything I have presented to her. The third chapter of my dissertation would not have been possible without the help of Dr. Jason Stajich. I truly appreciate the countless hours he spent listening to me ramble about this crazy genomics project I wanted to undertake and explain what to some, might seem like simple computational biology concepts. I also appreciate his wiliness to meet often during the Corona Virus (COVID-19) pandemic of 2020 via Zoom to walk me through the analysis of our results.

I would like to thank one of my scientific mentors, friends, and all-around fantastic person Dr. Amy Murillo for her support and encouragement over the past few years. Amy sat across from me in the lab and served as a sounding board when I had any

questions regarding science, life, or college football. She is someone I look up to, inspire to be like, and am genuinely thankful she took me under her wing.

I would like to thank Dr. Bradley Mullens as without him, and I can guarantee I would not be where I currently am. Dr. Mullens not only inspired me to pursue a Ph.D. in the field of medical and veterinary entomology through his passionate teaching but told my now advisor (Dr. Alec Gerry) that I would be a good fit for his research lab. When I look back, I almost do not believe it was by chance that I decided to take Dr. Mullens Medical and Veterinary Entomology course in my final quarter at UCR (Winter 2015). I will forever be thankful for Dr. Mullens as he believed in me before I believed in myself.

I would like to thank my friends and family for their undivided support and encouragement over the past 10+ years, ever since I had the crazy idea of pursuing a Ph.D., I remember back in high school saying that I wanted to get a Ph.D. without any idea about all the hard work, blood, sweat, and tears it would take. A special thanks goes out to my Dad (Kyle), Mom (Rashawn), and Stepmom (Kristi) for never discouraging me from pursuing my dreams, no matter how far-fetched they seemed.

I lastly would like to thank my number one support system through all of this, my wife, Chandler. She embraced the entomologist in me, meaning she accepted (or tolerated) the insects in the freezer, the cow poop on the shoes, Saturdays spent tending fly colonies, and the microscope on the kitchen table for days or weeks at a time sorting flies by phenotype. She served as my copy editor, motivator, cheerleader, and shoulder to cry on through it all. She supported me every step of the way, all while graduating

medical school number one in her class and working 80-hour weeks as a resident physician. For this, I thank you from the bottom of my heart.

Chapter 1 has been published as Hubbard, C. B., and A. C. Gerry (2020) Selection, Reversion, and Characterization of House Fly (Diptera: Muscidae) Behavioral Resistance to the Insecticide Imidacloprid in the Journal of Medical Entomology.

Chapter 2 has been published as Hubbard, C. B., and A. C. Gerry (2021) Genetic evaluation and characterization of behavioral resistance to imidacloprid in the house fly in Pesticide Biochemistry and Physiology.

DEDICATION

To Chandler, Tally, Red, and Lady-

This might not be an easy time

There's rivers to cross and hills to climb

Some days we might fall apart

And some nights might feel cold and dark

And nobody wins who's afraid of losing

And the hard roads are the ones worth choosing

Someday we'll look back and smile

And know it was worth every mile

ABSTRACT OF THE DISSERTATION

Mechanisms Conferring Behavioral Resistance to the Neonicotinoid: Imidacloprid in the House Fly (Musca domestica Linnaeus)

by

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Doctor of Philosophy, Graduate Program in Entomology
University of California, Riverside, March 2021
Dr. Alec C. Gerry, Chairperson

The house fly (*Musca domestica* L.) is a cosmopolitan and synanthropic pest fly species commonly associated with confined animal facilities. It has been implicated in the transmission of over 200 different human and animal pathogens and can be extremely pestiferous in high numbers.

One of the most common methods for house fly control is the use of insecticides, but insecticide resistance is an increasing problem due to over-application of insecticides and lack of rotation among insecticidal chemical classes. House fly resistance to imidacloprid, the most widely used neonicotinoid insecticide available for fly control, has evolved in field populations through both physiological and behavioral mechanisms. In this dissertation I investigated the mechanisms conferring behavioral resistance to imidacloprid.

Behavioral resistance to imidacloprid was documented to present in a field population of flies from a southern California dairy, though the resistance was not uniform among individuals in the population. Flies were selectively bred for behavioral resistance to imidacloprid, without increasing the physiological resistance profile of the selected flies. The rapid selection for behavioral resistance suggests that inheritable alleles conferring behavioral resistance were already present in the wild type fly population collected from the dairy site.

House fly behavioral resistance was further characterized using behavioral observation and feeding preference assays, with resistance determined to be both contact-dependent and specific to the insecticide (imidacloprid) rather than to a non-insecticidal component of a bait matrix as previously documented. The chromosomal location of behavioral resistance factors was then examined through the use of an autosomal linkage analysis. Behavioral resistance was mapped to autosomes 1 and 4 with inheritance of resistance being shown to be neither fully dominant nor recessive. Factors on autosomes 1 and 4 independently conferred contact-dependent avoidance and aversion of imidacloprid.

The molecular mechanisms conferring behavioral resistance to imidacloprid were then investigated using a pooled sequencing approach. In this evolve and resequence experiment we attempted to identify putative selected sites or candidate loci that may be responsible for our selected phenotype by comparing house flies that did not exhibit the behavioral resistance phenotype to house flies that exhibited a high level of behavioral resistance. While 47 genes were identified to have significant differences in SNP

frequencies between the susceptible and resistant populations, these genes either had an unknown function or a reported function that is not expected to alter expression of behavioral resistance to imidacloprid. Additional fundamental and applied research should be conducted to understand further both the complex phenotypic and genotypic nature of behavioral resistance to imidacloprid.

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INTRODUCTION:

The common house fly (*Musca domestica* L.) is a synanthropic fly species that has a cosmopolitan distribution (West 1951, McKie 2017) inhabiting rural and urban environments. House flies are known as a filth fly, a name related to the sites in which they develop. House flies have been shown to develop in many substrates associated with humans and animal agriculture such as feces, food waste, rotting fruits and other garbage (Keiding 1986, Cook et al. 2011).

Biology and Recognition

House flies are holometabolous insects in the family Muscidae and have four life stages. Female flies lay eggs on moist organic debris that can harbor aerobic microbial fermentation, such as feces, food waste, and moist organic material (West 1951). Females will lay eggs in a mass (100-300) and have the ability to lay up to 900 eggs in her lifetime (West 1951). While immature development rate is dependent upon temperature, house fly eggs hatch into L1 larvae generally within 6 -12 hours after deposition. The vermiform larvae ("maggots"') are small, cream-colored, and have a blunt posterior that tapers to a point on their anterior end. The larvae will go through three successive molts before pupating. Late stage L3 larvae will migrate away from their moist development site to a drier location to pupate. The puparium is a hardened outer skeleton of the final larval instar of the pupae.

The pupae are approximately 4-7 mm in length, ranging from light red-brown in color. Within the puparium, the pupae will develop into an adult house fly. Developmental time from egg to adult for the house fly varies dramatically with temperature, and the substrate larvae develop in (Cook et al. 2011, Khan et al. 2012). Studies have shown that house flies can develop from egg to adult in as little as seven days at 33°C (Larsen and Thomsen 1940), but developmental time averages between 10 - 14 days depending on temperature and larval substrate. Adult house flies will emerge from the anterior end of the pupal case by forcing the end of the puparium off with the ptilinum (West 1951). The adult house fly is a medium-sized fly, approximately 3-8 mm in length, generally dull gray in color, with yellow coloring to the sides of the abdomen and four longitudinal black stripes on the thorax, with a strong upward bend in the fourth longitudinal wing vein (vein M1+2) (West 1951). Adult house flies have sponging mouthparts. Flies consume foods by sucking up liquids or will regurgitate with saliva or vomitus onto solid foods to moisten them for subsequent ingestion (West 1951).

Dispersal and Disease

While most house flies will remain on or near animal production facilities from which they developed (Lysyk and Axtell 1986), house flies are also known to disperse from development sites. With a dispersal range of more than 12 km (Parker 1916, Bishopp and Laake 1921, West 1951, Schoof et al. 1952, Quarterman et al. 1954), flies can become serious problems even far from their development site. House fly dispersal away from development sites is driven by a diverse set of factors, including

environmental factors (temperature, humidity, wind) and population pressures such as resource competition (Schoof and Silverly 1954).

The house fly has adapted to living in almost every environment and is known worldwide as a serious nuisance pest species. The production of large numbers of house flies can result in litigation against animal producers or urban waste facilities resulting in economic loss or forfeiture of operation (Thomas and Skoda 1993). House flies are also implicated in transmitting numerous animal and human pathogens, with over 200 different pathogens detected in association with this fly species (Greenberg 1971, 1973, Graczyk et al. 2001, Nayduch and Burrus 2017), including bacteria such as Escherichia coli O157: H7 (Sasaki et al. 2000), Helicobactor pylori (Grübel et al. 1997), and Staphylococcus aureus (Barro et al. 2006); viruses such as rotavirus (Tan et al. 1997); and parasites such as Endolimax nana (Khan and Huq 1978) and Cryptosporidium parvum (Graczyk et al. 1999).

As house flies have been implicated in causing disease outbreaks and cause disturbances to normal animal behavior by feeding around the face and humans working on animal production faculties, it is imperative to control house flies. It has been shown that control of house flies can reduce human illness caused by enteric pathogens (Watt and Lindsay 1948, Chavasse et al. 1999).

Genetics and Genomics

The house fly has five autosomes and two sex chromosomes (X and Y). House flies have a unique polymorphic sex determination system (Sharma et al. 2017). Male

house flies carry a dominant male determining factor (M-factor), which can reside on any of the five autosomes or on either sex chromosome. The M-factor was determined to be Mdmd by Sharma et al. (2017). Mdmd regulates transformer (Md-tra), which is a binary switch that, when active, directs female determination and, when inactive, controls male differentiation. Mdmd has been shown to be present in more than one copy in some cases (Hamm et al. 2015). The house fly genome was successfully sequenced in 2014 to a size of 0.691 Gb (Scott et al. 2014). There are predicted to be 15,345 genes (14,180 protein-coding genes and 1,165 non-coding genes) present in the genome. A large number of immune-related genes were predicted, which is likely due to the close association house flies have with pathogens. A significant expansion of cytochrome P450 detoxication genes and chemoreceptor gene families were also seen relative to D. melanogaster. With the genome of the house fly now available, molecular tools currently utilized to elucidate basic and applied biological questions in other Dipteran species can be used to address knowledge gaps in the house fly and improve house fly management particularly by elucidating the molecular mechanisms that confer insecticide resistance.

House Fly Management

An integrated pest management (IPM) approach should be used to efficiently control house flies in which multiple control measures are implemented. An IPM strategy for controlling house flies will include 1) monitoring, 2) cultural control, 3) biological control, 4) chemical control.

Monitoring

Monitoring for flies can be accomplished using numerous methods targeting immature or adult house flies (Gerry 2020). Each method has positives and negatives, but the key with adult house fly monitoring is to employ monitoring strategies at regular intervals to identify an uptick in adult house fly abundance. If significant numbers of adult flies are being caught, an investigation into immature developmental sites is critical (Gerry et al. 2005, 2011).

Identifying house fly immatures development sites is key to house fly management. Management strategies can be put in place to break the developmental lifecycle before the emergence of adult flies (the pestiferous life stage).

Cultural Control

Cultural control techniques such as manure management are ideal for managing house flies, as manure removal/sanitation eliminates or reduces house fly immatures before they become adults, as the adults are the pestiferous and disease-carrying life stage (Geden et al. 2020). While the complete removal of manure/larval breeding habitats may be practical in a small-scale operation, this can become a logistical challenge in large animal agricultural operations. In many large-scale animal operations, manure is removed from animal pens or animal housing and directly applied to cropland. As this manure is commonly infested with house fly larvae, large outbreaks of flies can still occur (Watson et al. 1998). For these operations, other manure management approaches are needed. For example, controlling the larval development habitats' moisture content is extremely

important as house fly larval development is optimal in substrates with 50-75% moisture (Fatchurochim et al. 1989). Collecting and spreading manure into thin layers in areas that will receive direct sunlight will dry the manure and make it uninhabitable for fly eggs and larvae. If these operations are in an area that receives regular rain, morning fog, or persistent high humidity, this may not be an option Liquefaction of manure within ponds or other large-scale water retention devices may be an option. The overall goal behind proper manure management is to break the insect's life cycle and prevent the emergence of pestiferous adult flies (Axtell 1986, Axtell 1999).

Biological Control

Natural enemies, including predators, parasitoid wasps, parasitic nematodes, and entomopathogenic fungi, exist naturally in the environment have been extensively studied for control of the house fly in all developmental life stages (Geden et al. 2020).

Predators such as the predatory mite *Macrocheles muscaedomesticae* (Scopoli) have been shown to consume upwards of 20 house fly eggs per day per mite. In simulated field conditions, they have demonstrated reductions in total house fly numbers (Axtell 1986).

Pupal parasitoid wasps in the family Pteromalidae, such as *Muscidifurax raptor* or *Spalangia cameroni*, naturally occur throughout the United States. They are also available commercially to augment the natural population as wasp development is significantly slower than their fly hosts. Adult female wasps drill through the puparium and will lay one or more eggs on the developing pupae. The wasp larvae will consume

the pupating fly and only an adult wasp will emerge from the pupal casing (Geden and Hogsette 2006). Parasitoid wasps are somewhat host-specific (Machtinger and Geden 2018), limiting off-target effects. Due to the low dispersal range of these parasitoids and the fact that parasitoids mass-reared in a laboratory setting may not be as competent as wild wasps, their effectiveness as supplemental biological control agents is probably limited to confined areas of house fly development (Machtinger et al. 2015).

Entomopathogenic nematodes in the family Steinernematidae have been extensively reviewed for effectiveness against house flies of all life stages (Kaya and Gaugler 1993, Gaugler 2002, Georgis et al. 2006). Entomopathogenic nematodes can be inexpensively mass-produced, are safe for vertebrates, and have a long storage life. However, studies of their real-world field effectiveness are currently lacking.

Entomopathogenic fungi such as *Metarhizium brunneum*, *Beavueria bassiana*, and Entomophthora muscae have been shown to naturally infect and kill immature and adult house flies (Geden et al. 1995, Watson et al. 1995, Kaufman et al. 2008), While entomopathogenic fungi have been shown in the laboratory to be an effective biological control agent, few studies have shown significant reduction/control of house flies following application of fungi to the environment (Steinkraus et al. 1993, Geden et al. 1993, Six and Mullens 1996), as fungi virulence can be dramatically impacted by environmental conditions (Reis et al. 2008) and the application of fungi has not been optimized for some fungal species, which significantly reduces its ability to be applied in the field. An in-depth review of biological control techniques for the house fly can be found in Geden et al. 2020.

Physical Control

To assist in controlling adult flies, physical controls and barriers can be used to catch and prevent flies from invading indoor spaces. Sticky fly tapes, ribbons, and cards traps can be utilized in areas of high fly activity. These products typically have a sticky surface and take advantage of a fly's natural tendency to land and rest on vertical surfaces (Howard 1911). Adult flies land and become stuck and unable to escape. These traps can be changed periodically when the surface is no longer sticky or is completely covered in flies. The trap's surface can become easily compromised in a dusty environment yielding them ineffective and should be monitored regularly (Anderson and Poorbaugh 1965, Rutz and Axtell 1981. While small sticky ribbons or tapes may become saturated quickly and generally do not have a significant reduction on fly numbers but can provide a way to monitor for the abundance of flies, giant sticky ribbons which are available in rolls have been documented to collect over 9 million flies during a 10-week period, and significantly reduced fly numbers (Kaufman et al. 2001).

Other commonly used physical control methods include the use of attractant-based traps. This trapping system evokes the house fly's highly evolved olfactory system (Scott et al. 2014). Highly volatile and attractive substances such as fish heads, watermelon rinds, corncobs, molasses, milk, yeast, grain, and blood have long been used as lures to draw in and capture flies in traps (inverted cone, jar, jug) (Pickens et al. 1973, Mulla et al. 1977, Pickens and Miller 1987), but have drawn complaints due to their offensive smell. More recently specific volatile blends of trimethylamine, ammonia, indole, linoleic acid, (z)-3-hexenyl acetate, and benzaldehyde, have been identified as

attractive to flies while also less objectionable to users when used in trapping systems (Hung et al. 2019). (Z)-9-tricosene, a cuticular hydrocarbon found in some populations of female house flies (Carlson 1971), has also been shown to attract flies when added to traps, and currently is the most common commercial feeding-attractant (Geden et al. 2020). Attractants utilized in physical traps have also shown efficacy when combined with a toxicant in granular sugar baits.

Chemical Control

In conjunction with the previously described control methods, insecticides are commonly utilized to control immature and adult life stages of the house fly. Insecticides differ in mode of action, knockdown speed, toxicity, off-target effects, and persistence in the environment. They can be formulated and applied in many forms, including sprays, dusts, ear tags, and baits. Currently, the most common active ingredients used for fly control include the synthetic pyrethroids for space sprays/treatments and the neonicotinoids formulated into consumable baits (Geden et al. 2020). Often house fly management is attempted with only the use of insecticides due to their low cost, ease of application, rapid action, and perceived effectiveness. However, with the constant use of insecticides, resistance is widespread in field populations of house flies (Keiding 1975, Keiding 1999).

Insecticide Resistance

The World Health Organization defines insecticide resistance as "the development of an ability in a strain of an organism to tolerate doses of toxicant which would prove lethal to the majority of individuals in a normal (susceptible) population of the species" (World Health Organization Expert Committee on Insecticides 1957). The development of insecticide resistance occurs rapidly under high insecticidal pressure conditions, lack of chemical class rotation, and no refugia from insecticide exposure (Georghiou 1972, Zhu et al. 2016, Hubbard and Gerry 2020). Insecticide resistance to all insecticidal classes available for control of the house fly has been documented (Keiding 1999, Darbro and Mullens 2004, Kaufman et al. 2006, Gerry and Zhang 2009, Kaufman et al. 2010, Seraydar and Kaufman 2015, Murillo et al. 2015, Scott 2017, Freeman et al. 2019, Hubbard and Gerry 2020). Insecticide resistance has been documented to evolve rapidly under high selection pressure (e.g. Hubbard and Gerry 2020). It has been determined to be caused by well-characterized physiological changes (e.g., target site insensitivity or increasing production of toxin-metabolizing enzymes) as well as through inherited behavioral traits which cause the insect to reduce contact with or consumption of insecticides (Gerry and Zhang 2009, Wasik and Gerry 2010, Seraydar and Kaufman 2015, Hubbard and Gerry 2020).

Physiological Resistance

In the 1940s and into the 1950's Dichlorodiphenyltrichloroethane (DDT), an organochlorine, was used heavily for house fly control (Keiding 1999). DDT's acts by binding and opening sodium ion channels, causing the neurons to spontaneously fire,

resulting in insect spasm and eventual death. This heavy use of DDT resulted in the rapid development of resistance DDT and other organochlorines by 1946 (March and Metcalf 1950). In the 1960's and 1970's after the discontinuation of DDT, high levels of resistance to the chemical class continued in many countries. The mechanisms behind this resistance were two-fold: 1) knockdown resistance (KDR), which is caused by point mutations in the voltage-gated sodium channel (VGSC) which result in reduced target-site sensitivity, and 2) detoxification of the DDT by dehydrochlorination through the enzyme DDT-ase (Clark et al. 1984).

Following the development of house fly resistance to DDT, other insecticides were quickly adopted, but resistance to these newer insecticides was also quickly observed (reviewed by Keiding 1999). Physiological resistance to all major classes of insecticides has been documented in house flies, including Organochlorines, Organophosphates, Carbamates, Pyrethrins, Pyrethroids, Neonicotinoids, Spinosyns, and Indoxacarb for adult control (Geden et al. 2020).

Behavioral Resistance

While physiological resistance to insecticides has been the primary resistance mechanism responsible for the failure of many insecticides applied for control of insects, behavioral resistance has also been documented for more than 70 years. In some instances, it may prove to be as important or more important as a resistance mechanism. Behavioral resistance was first documented by Gahan et al. (1945) when *Anopheles quadrimaculatus* and *Aedes aegypti* were shown to avoid cage surfaces treated with

DDT, whereas mosquitoes placed into untreated control cages rested on cage walls. A similar mosquito behavior was also observed by Trapido (1954) when examining DDT resistance in *Anopheles albimanus*.

Behavioral resistance can be generally defined as "those actions, evolved in response to the selective pressures exerted by a toxicant, that enhance the ability of a population to avoid the lethal effects of that toxicant (Lockwood et al. 1984). Behavioral resistance can be categorized as either stimulus-independent or stimulus-dependent (Georghiou 1972). Stimulus-independent behavioral resistance comes from a behavior that leads to the natural avoidance of an environment or situation where an insect might be exposed to an insecticide. For example, mosquitoes selected for exophilic habits avoid contact with insecticides applied indoors (Fouet et al. 2018). Among anopheline populations in areas in which indoor residual sprays (IRS) and long-lasting insecticidal nets (LLIN) are in constant use (Takken 2002, Gatton et al. 2013), mosquitoes have been observed feeding outdoors during the early evening. This outdoor feeding phenotype has significantly reduced the effectiveness of the IRS and LLIN because the mosquitoes are no longer making contact with the toxicant treated surfaces indoors.

Whereas stimulus-dependent behavioral resistance involves the heightened ability of an insect to detect and limit contact with a toxic substance, perhaps due to a repellent or irritant property of the toxic substance, its formulation, or presentation leading to an aversive response (Georghiou 1972).

Excito-Repellency

An excito-repellency response is a behavioral change elicited by an organism (insect) after coming near or making casual contact with a surface treated with an insecticide. This response results in the organism's 'avoidance' of an area treated with an insecticide, caused by noncontact (spatial) repellency or contact excitation (irritancy) (Roberts et al. 1997, Boonyuan et al. 2016). Excito-repellency responses have been documented in numerous arthropod species, including mosquitoes (Kongmee et al. 2004, Chareonviriyaphap et al. 2013, Gatton et al. 2013, Boonyuan et al. 2016), horn flies (Lockwood et al. 1985, Byford et al. 1987, Sparks et al. 1989, Zyzak et al. 1996), kissing bugs (Diotaiuti et al. 2000), spider mites (Penman et al. 1988) and bed bugs (Romero et al. 2009, Agnew and Romero 2017).

Aversion to insecticides or components formulated into toxic food baits

The most well-studied behavioral resistance phenotype involves insects, or other animals heightened ability to detect and limit contact with a toxic food material. This limited contact reduces or eliminates consumption of the toxicant, dramatically increasing survival of the organism. This form of behavioral resistance to insecticides or components of toxic food baits has been documented in numerous vertebrate species such as the red fox (*Vulpes vulpes*) (Kinnear et al. 2017, Allsop et al. 2017), European rabbit (*Oryctolagus cuniculus*) (Oliver et al. 1982), brushtail possum (*Trichsurus vulpecula*) (Ogilvie et al. 2000), gerbils (*Tatera indica indica* and *Meriones hurrianae*) (Prakash and Jain 1971), pocket gophers (*Thomomys bottae navus*) (Howard et al. 1967), prairie voles

(*Microtus ochrogaster*) (Horak et al. 2018), brown rat (Rattus norvegicus) (Gaines and Hayes 1952, Brunton et al. 1993), Sprague Dawley laboratory rats (inbred *Rattus norvegicus*) (Howard et al. 1968, Prescott et al. 1992), and roof rats (*Rattus rattus*) (Howard et al. 1968), as well as a number of insects such as fungus growing termites, (*Macrotermes gilvus*) (Iqbal and Evans 2018), German cockroach (*Blattella germanica*) (Silverman and Bieman 1993, Silverman and Selbach 1998, Wada-Katsumata et al. 2013, Wada-Katsumata et al. 2014, Wada-Katsumata et al. 2018), and the house fly (*Musca domestica*) (Freeman and Pinniger 1992, Learmount et al. 1996, Darbro and Mullens 2004, Gerry and Zhang 2009, Mullens et al. 2010, Hubbard and Gerry 2020).

Vertebrate Behavioral Resistance

Within the vertebrate pest control community, poison baiting is a common method to control pest animals. Poison baits are easy to deploy, cost-effective, and result in rapid mortality of the target pest (Allsop et al. 2017). Baiting vertebrates comes with the same challenges experienced with baiting arthropods. The target species must find the bait placed in the environment, consume it, and ingest a sufficient quantity of the toxin to cause death (Allsop et al. 2017). It has been documented that while animals will find baits, they often avoid consuming them due to behavioral aversion to the bait. This aversion has been reported to be caused by learned avoidance based on previous exposure to baits or social learning and innate (inherited) behavioral avoidance (Galef and Laland 2005, Allsop et al. 2017).

Learned avoidance of poison baits is also commonly seen when attempting to control vertebrate pest populations. Learned aversion or bait shyness can be seen after an animal survives exposure to a poison bait and associates the poison's negative effects with a characteristic of the poison such as appearance, taste, or smell (Gustavson 1977). Bait shyness has been shown to develop in as little as a single exposure to a toxic bait like zinc phosphide (Horak et al. 2018).

Innate aversion to poison baits is defined as a "non-learned instinctive aversion that is a result of selection pressures on a species and refers to heritable characteristics of individuals within the target population (Allsop et al. 2017)." Often innate aversion is observed as an inherited personality trait of some individuals within a population such as neophobia or wariness towards unfamiliar objects in a familiar environment. This behavior can result in reduced contact with poison baits placed into an animal's native habitat. Continuous placement of poison baits in the environment was shown to select for highly neophobic brown rat populations. This neophobic behavior provided a survival advantage, as those that were leery of the bait avoided consuming the toxic material and survived to procreate.

German Cockroach Behavioral Resistance (Glucose Aversion)

German cockroach (*Blattella germanica*) control has long been difficult to accomplish due to the close association German cockroaches have with humans and pets (Wada-Katsumata 2018), making the use of spray/fog-based insecticides dangerous and not practical. Beginning in the 1980's insecticidal baits began to be utilized for German

cockroach control (Wada-Katsumata 2018). Insecticidal baits formulated with an insecticide and phagostimulant were ideal for cockroach control. They posed fewer health risks than spray-based insecticides and took advantage of the cockroaches need to feed for nymphal development and adult reproduction (Wada-Katsumata et al. 2018). Not long following the introduction of insecticidal baits, their efficacy began to wane due to physiological and behavioral resistance in the cockroach populations. The German cockroach developed an aversion to D-glucose, the nutrient matrix in which the insecticide hydramethylnon had been mixed (Silverman and Bieman 1993). Silverman and Bieman documented German cockroaches avoided ingesting toxic baits or diet mixtures containing D-glucose (glucose). This aversion to glucose was shown not to be a learned behavior as commonly seen in the vertebrate community but instead was found to naturally occur and be inherited as an autosomal incompletely dominant trait controlled by a single major gene on autosome 9 (Silverman and Bieman 1993, Ross and Silverman 1995). This was the first documented case of an organism naturally developing an aversion to a known nutrient/phagostimulant as a resistance mechanism to avoid lethal exposure to a pesticide. Behavioral aversion to glucose has now been demonstrated in numerous cockroach populations worldwide (from Florida to South Korea) and is thought to have independently arisen (Silverman and Ross 1994, Wada-Katsumata et al. 2013).

Glucose averse German cockroaches were shown to reject glucose solutions despite extreme food deprivation resulting in high cockroach mortality (Silverman and Selbach 1998).

This aversion resulted in the failure of insecticidal baits containing glucose, necessitating the reformulation of many baits (Silverman and Liang 1999, Wang et al. 2004).

Aversion to glucose was further characterized and shown to be processed through chemosensory appendages including the antennae and mouthparts (paraglossae > labial palps > maxillary palps), as glucose acted as a deterrent (similar to caffeine and DI water) when placed onto the mouthparts and antennae of glucose averse cockroaches (Wada-Katsumata 2013). Glucose was also shown to stimulate both sweet and bitter gustatory receptor neurons (GRN) in the peripheral gustatory system indicating that resistant cockroaches interpreted glucose as both a phagostimulant and a deterrent (Wada-Katsumata 2013). It is hypothesized that the bitter GRN acquired sensitivity to glucose due to a structural modification of gustatory receptor in the bitter GRN that allows for the detection of glucose, or a glucose gustatory receptor is misexpressed in the bitter GRN (Wada-Katsumata et al. 2018). This gain-of-function mutation which confers protection of German cockroaches from insecticides containing glucose, is currently being further evaluated through the functional analysis of the gustatory receptors in the German cockroach (Wada-Katsumata et al. 2018).

House Fly Behavioral Resistance

House fly behavioral resistance to insecticides or insecticide bait matrixes has been well documented for more than 70 years (Sparks et al. 1989). House fly behavioral resistance to an insecticide (DDT) was first documented in 1949 by King and Gahan. The

authors observed that in dairy barns where DDT residues were not giving satisfactory fly knockdown, large numbers of flies were seen resting on untreated floors, equipment, and feed troughs, instead of on treated walls and ceilings. Similar observations were documented by Missiroli (1950) and Bruce and Decker (1950).

Similarly, behavioral resistance to the organophosphate Malathion was observed after a few years of use of this insecticide. Kilpatrick and Schoof (1958) determined that house flies from a Savannah, Georgia farm were behaviorally averse to a 2% malathion solution dispensed on plywood placed in the environment. Flies would readily approach the plywood but failed to land and make contact with the material, but readily landed on plywood treated with another organophosphate Dichlorvos (DDVP). A similar behavioral aversion to malathion was also observed on a separate farm in Georgia (Schoof and Kilptrick 1958). Malathion averse house flies were shown to visit bait treated with malathion significantly less than susceptible flies in laboratory assays (Fay et al. 1958). In one study, three field-collected behaviorally resistant fly colonies completely avoided a 1% malathion-poisoned sugar milk-egg bait compared with unpoisoned baits (Smith and Yearian 1964). Behaviorally resistant flies were never observed directly landing on the bait, and those that landed near the bait approached and contacted the bait flew off in a matter of one or two seconds (Fay et al. 1958). Behavioral resistance to malathion was also shown to be caused by a reduction in feeding on malathion treated sugar. Flies were allowed to feed on P^{32}-labelled sucrose that contained malathion. Consumption of the radiolabeled sucrose treated with malathion was measured. Surviving flies (malathion-resistant) were shown to have consumed only small amounts of radiolabeled malathion

treated sucrose to a susceptible house fly colony (Schmidt and Labrecque 1959). Behavioral resistance to DDT is spatial aversion or an excito-repellency response elicited by the fly, as is seen in other insects exhibiting behavioral resistance to these compounds (Gahan et al. 1945, Kongmee et al. 2004). Behavioral resistance to malathion seems to be expressed as a combination of excito- repellency and an aversion to the insecticide or components in the food bait.

House Fly Aversion to Insecticides or Components Formulated into Toxic Food Baits

With the advent of new insecticides and insecticidal classes, behavioral resistance commonly began to be documented due to house flies developing an aversion to consume the insecticide or components of insecticidal food bait, as was previously reported in the German cockroach (Silverman and Bieman 1993). Freeman and Pinniger (1992) examined behavioral resistance to the organophosphate bait (Alfacron®, A.I. azamethiphos) in house fly populations collected in the United Kingdom. Through the examination of single fly feeding responses to blank bait (all inert ingredients of the Alfacron® bait), sugar, and technical grade azamethiphos, and the Alfacron® bait, the authors concluded that aversion was likely to formulation components or contaminants in the insecticidal bait matrix instead of to the active ingredient azamethiphos, as the fly feeding response to the blank bait included the inhibition of the proboscis extension response (PER) and resulted in 0 total seconds feeding, whereas flies readily fed on the sugar and azamethiphos bait formulation.

Behavioral resistance to Alfacron® and Golden Malrin® (A.I. methomyl) (another commonly utilized fly bait) was confirmed to be prevalent in house fly populations across the United Kingdom (Chapman et al. 1993). When behavioral resistance to an insecticide or bait is suspected, a non-insecticidal food source (e.g., sucrose alone) is simultaneously provided with the bait, allowing flies to feed on either food source (choice feeding assay) (Learmount et al. 1996, Gerry and Zhang 2009). Learmount et al. (1996) examined 36 field-collected house fly colonies collected throughout the United Kingdom, testing them for physiological resistance (no-choice test) and behavioral resistance (choice-tests) to Alfacron® and Golden Malrin®. Behavioral resistance to Alfacron® was present in the number of house fly populations tested. Seventeen strains exhibited fly knockdown of <50% in choice-tests. Behavioral resistance to Golden Malrin® was documented in nine strains of house flies tested, with eight strains exhibiting reduced knockdown when exposed to a choice-test, and one strain that was Golden Malrin® was completely ineffective against. This study confirmed the importance of laboratory testing that allows for the discrimination between physiological and behavioral resistance.

Interestingly, Darbro and Mullens (2004) documented a similar aversive response to methomyl-treated bait (Golden Malrin®) when flies from several California locations were tested in choice behavioral feeding assays. Eight strains of flies were tested for aversion to methomyl. Results show females from seven of the eight strains and males from six strains showed a significant preference for sugar over the methomyl bait (Darbro and Mullens 2004). Though it is unknown if behavioral resistance to Golden Malrin® was to the insecticide (methomyl) or other components of the bait formulation, as documented

in the Freeman and Pinniger (1992), these results corroborate the findings of Learmount (1996). Methomyl can control flies in no-choice tests, but in a more realistic field scenario (choice-test) the flies are significantly averse to the chemical (Learmount et al. 1996), which is likely why Golden Malrin® was observed to fail to control house flies in the United Kingdom and California.

House Fly Behavioral Resistance to Imidacloprid

Imidacloprid is an insecticide in the neonicotinoid class of insecticides, IRAC code 4A. This insecticide class binds competitively and irreversibly to the nicotinic acetylcholine receptor, leading to a paralysis of the insect (Jeschke and Nauen 2005). The neonicotinoids are currently the most widely used insecticidal class across all of agriculture, used in seed coating, topical application, and baits for insects (Yamamoto 1999, Sparks and Nauen 2015), due to their novel mode of action and relatively new synthesis. Imidacloprid has been formulated into granular fly baits for fly control since late 2002 (U.S. Environmental Protection Agency 2002, Jeschke and Nauen 2005).

House fly resistance to imidacloprid was reported soon after the commercial availability of imidacloprid fly baits, with evidence of physiological resistance (Kaufman et al. 2006) and behavioral resistance (Gerry and Zhang 2009). Gerry and Zhang assessed physiological and behavioral resistance to technical grade imidacloprid from a field-collected cohort of house flies (BS) collected from a dairy in San Jacinto, California. Physiological and behavioral resistance was compared to an imidacloprid-susceptible house fly colony (UCR fly strain) collected in 1982 from a dairy in Mira Loma, California. While results indicated that BS flies' physiological resistance profile was

moderate (Resistance ratio (RR) = 10), flies exhibited high levels of behavioral resistance to imidacloprid. In choice assays, low mortality rates never exceeding 35% were observed even when BS flies were exposed to a sugar treated with a concentration of imidacloprid 50 X LC_{99} of the UCR fly strain.

Behavioral resistance to imidacloprid was later documented by Mullens et al. 2010 when accessing the efficacy of a novel Metaflumizone bait compared to commonly utilized baits for fly control, Elector® (A.I. Spinosad), and QuickBayt® (A.I. imidacloprid). Mullens documented that during field trials, flies seldom visited or fed on imidacloprid containing baits. During laboratory trials, field flies spent significantly less time feeding on imidacloprid baits than susceptible laboratory flies.

Seraydar and Kaufman (2015) investigated the role and type of behavioral mechanisms that play a role in resistance to imidacloprid-containing baits. Following the selection of an imidacloprid resistant house fly colony with QuickBayt®, a significant decrease in mortality was seen when the flies were provided a choice-assay with sucrose and QuickBayt®, indicating a behavioral aversion to the bait. Flies were observed equally contacting QuickBayt® and sucrose, likely eliminating repellency as the form of stimulus-dependent behavioral resistance observed. The authors hypothesize that behavioral resistance may be to the bait matrix components, as was observed with an aversion to Alfacron® (Freeman and Pinniger 1992), such as the bittering agent included in the bait, Bitrex®, which may deter mammals from consuming it.

Due to the complexities of selecting for the behavioral resistance phenotype, the complex and proprietary blend of materials utilized in the bait matrix, and the conflicting

publication results which state that flies may or may not contact imidacloprid containing baits the same as other baits or phagostimulants, the actual mechanism conferring behavioral resistance to imidacloprid in the house fly is yet to be understood.

The purpose of my dissertation is to further our understanding of the mechanisms that confer behavioral resistance to the neonicotinoid insecticide imidacloprid in the house fly. The elucidation of the mechanisms may allow for the development or selection of insecticide chemistries that limit or delay behavioral resistance selection by house flies or other pests. As an area of study, understanding behavioral resistance to insecticides and poisons is in its infancy. Still, I hope to inspire, guide and shed light on this complex yet fascinating research area by directed studies focused on house fly behavioral resistance to the insecticide imidacloprid. In chapter 1, a protocol is described to rapidly select house fly populations for a high degree of inherited behavioral resistance or behavioral susceptibility to imidacloprid when formulated into a sucrose food source while leaving physiological resistance to imidacloprid relatively unchanged. Behavioral resistance to imidacloprid is further characterized using video observation of the feeding behavior of these behaviorally resistant fly populations. In chapter 2, the inheritance and genetics of behavioral resistance to imidacloprid is described. Chromosome(s) carrying factors conferring behavioral resistance to imidacloprid are identified utilizing autosomal linkage analysis. In chapter 3, a pooled sequencing approach was used to attempt to identify molecular/ genetic changes in behaviorally resistant house fly colonies that may contribute to behavioral resistance to imidacloprid.

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CHAPTER 1

Selection, reversion, and characterization of house fly (Diptera: Muscidae) behavioral resistance to the insecticide imidacloprid

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ABSTRACT

Insecticide resistance in pest populations is an increasing problem in both urban and rural settings caused by over-application of insecticides and lack of rotation among chemical classes. The house fly (*Musca domestica* L.) is a cosmopolitan fly species implicated in the transmission of numerous pathogens, and which can be extremely pestiferous when present in high numbers. The evolution of insecticide resistance has long been documented in house flies, with resistance reported to all major insecticide classes. House fly resistance to imidacloprid, the most widely used neonicotinoid insecticide available for fly control, has been selected for in field populations through both physiological and behavioral resistance mechanisms. In the current study, house flies collected from a southern California dairy were selectively bred for behavioral resistance to imidacloprid, without increasing the physiological resistance profile of the selected flies. Flies were also successfully selected for behavioral susceptibility to imidacloprid. The rapid selection for either behavioral resistance or behavioral susceptibility suggests that inheritable alleles conferring behavioral resistance were already present in the wild type fly population collected from the dairy site. The methods used for the specific selection of behavioral resistance (or susceptibility) in the fly population will be useful for further studies on the specific mechanisms conferring this resistance. House fly behavioral resistance was further investigated using behavioral observation and feeding preference assays, with resistance determined to be both contact-dependent and specific to the insecticide (imidacloprid) rather than to a non-insecticidal component of a bait matrix as previously documented.

INTRODUCTION

The common house fly (*Musca domestica* L.) (Diptera: Muscidae) is a synanthropic fly species that has a cosmopolitan distribution (West 1951). House flies are associated with urban environments and animal production where feces, food waste, and rotting fruit are abundant (Keiding 1986). These flies are a known nuisance species and have also been implicated in the mechanical transmission of over 200 different pathogens (Thomas and Skoda 1993, Geden and Hogsette 2001, Malik et al. 2007, Nayduch and Burrus 2017). With a dispersal range of more than 5 km (Parker 1916, Bishopp and Laake 1921, West 1951, Schoof and Siverly 1954) flies can be a serious problem even at a substantial distance from their development sites, where fly nuisance can result in litigation against animal producers resulting in economic loss or forfeiture of operation (Thomas and Skoda 1993).

Toxic fly baits (granular/scatter baits) are one of the more commonly applied insecticide formulations for control of adult house flies. Fly baits contain a toxicant formulated into a phagostimulant matrix (usually sucrose-based) to induce feeding (Darbro and Mullens 2004). Toxicants used in fly baits are generally fast-acting insecticides, though a few slower acting insecticides (e.g., spinosad) have been used as well (Zahn et al. 2019). Fly baits are either placed into a bait station or are scattered on the ground in areas of high fly activity. In a natural environment where many alternative food sources are available to flies, the selection of fly populations that exhibit reduced contact with the bait or that limit bait consumption following contact with the bait can significantly impact bait effectiveness (Morrill 1914, Ferguson et al. 2014, Parker et al.

2015). The development of insecticide resistance occurs rapidly under conditions of high insecticidal pressure, lack of chemical class rotation, and no refugia from insecticide exposure (Georghiou 1972, Zhu et al. 2016).

Insecticide resistance is defined by the World Health Organization as "the development of an ability in a strain of an organism to tolerate doses of toxicant which would prove lethal to the majority of individuals in a normal (susceptible) population of the species" (World Health Organization Expert Committee on Insecticides 1957). In house flies, the inheritance of physiological adaptations that alter insecticide target sites or increase the production of toxin-metabolizing enzymes can lead to insecticide resistance (Liu and Scott 1997, Rinkevich et al. 2006, Zhang et al. 2018, Ma et al. 2019). These physiological resistance mechanisms in house flies have been well studied (Scott 2017), and resistance to all major classes of insecticides has been documented (Keiding 1999, Darbro and Mullens 2004, Kaufman et al. 2006, 2010, Murillo et al. 2015, Freeman et al. 2019). However, there is evidence that insects may also inherit behavioral traits to reduce contact with or consumption of insecticides (Gerry and Zhang 2009, Wasik and Gerry 2010, Seraydar and Kaufman 2015).

Neonicotinoids are a class of insecticides that bind competitively and irreversibly to the nicotinic acetylcholine receptor, leading to paralysis of the insect (Jeschke and Nauen 2005). Currently, neonicotinoids are the most widely used insecticides in the world (Sparks and Nauen 2015) and include the insecticide imidacloprid, which has been formulated into granular baits for fly control since late 2002 (U.S. Environmental Protection Agency, 2002). House fly resistance to imidacloprid was reported within a few

years of the commercial availability of imidacloprid fly baits, with evidence for both physiological resistance (Kaufman et al. 2006) and behavioral resistance (Gerry and Zhang 2009). Similarly, physiological and behavioral resistance has also been reported to imidacloprid in several other insect species (Wen and Scott 1997, Wang et al. 2002, Tan et al. 2008, Shi et al. 2011, Iqbal and Evans 2018).

Behavioral resistance can be categorized as either stimulus-independent or stimulus-dependent (Georghiou 1972). Stimulus-independent behavioral resistance comes from a behavior that leads to the natural avoidance of an environment or situation where an insect might be exposed to an insecticide. For example, mosquitoes selected for exophilic habits avoid contact with insecticides applied indoors (Fouet et al. 2018). Whereas stimulus-dependent behavioral resistance involves the heightened ability of an insect to detect and limit contact with a toxic substance, perhaps as the result of a repellent or irritant property of the toxic substance, its formulation, or presentation leading to an aversive response (Georghiou 1972).

House fly susceptibility to fly baits is typically evaluated using a feeding assay, where adult flies are offered only a fly bait or sucrose combined with technical grade insecticide (no-choice feeding assay). During the assay, flies are given sufficient time to discover and feed on the insecticide-treated food. Surviving flies are suspected to be physiologically resistant to the toxicant at the dose provided (Kaufman et al. 2006). However, flies exhibiting an aversive or repellent response to the insecticide-treated food will also survive in a no-choice assay, at least until they starve. When behavioral resistance to an insecticide or bait is suspected, a non-insecticidal food source (e.g.,

sucrose alone) is simultaneously provided, allowing flies to feed on either food source (choice feeding assay) (Learmount et al. 1996, Gerry and Zhang 2009). Flies expressing aversion or repellent behaviors toward the bait or insecticide may "choose" to feed on only the non-toxic food source, resulting in survival even in the absence of physiological resistance traits. Surviving flies are thus deemed to be behaviorally resistant to the insecticide relative to a susceptible population of flies which readily feed on the insecticide-treated food.

While behavioral resistance to various insecticidal products has been documented in field fly populations for more than 50 years (e.g., Schoof and Kilpatrick 1958, Schmidt and Labreoque 1959, Smith and Yearian 1964, Learmount et al. 1996, Darbro and Mullens 2004, Gerry and Zhang 2009), a clear and deliberate approach to laboratory selection for behavioral resistance has not been previously reported. Furthermore, methods to describe and study the mechanisms conferring this novel form of resistance are not well developed due to the difficulty of developing rigorous protocols to study the complex nature of insect behaviors as they relate to resistance (Sparks et al. 1989, Zalucki and Furlong 2017). To study the mechanisms of behavioral insecticide resistance, it is desirable to select for flies expressing a high degree of a behavioral resistance phenotype when exposed to an insecticide. But laboratory selection for insecticide resistance can result in both increased physiological as well as behavioral resistance of the selected flies, complicating interpretation of results when using traditional no-choice as well as choice feeding assays (Seraydar and Kaufman 2015).

The goal of the present study was to develop and implement a protocol to rapidly select house fly populations for a high degree of inherited behavioral resistance or behavioral susceptibility to imidacloprid when formulated into a sucrose food source while leaving physiological resistance to imidacloprid relatively unchanged. The selected behavioral resistance phenotype was subsequently characterized using video observation of the feeding behavior of these fly populations. The selection of house fly colonies with a homozygous behavioral resistance genotype to imidacloprid will make possible future studies to determine the genetic/molecular basis of behavioral resistance to imidacloprid.

MATERIALS AND METHODS

Reference Fly Colonies

A wild-type (WT) fly colony was established in 2015 following the collection of approx. 500 mixed-sex adult house flies by sweep net from multiple locations on a dairy near the southern California town of San Jacinto. Flies were transferred to a mesh cage, provided food (50:50 sucrose and dehydrated milk) and water ad libitum, and transported to the laboratory where they were held for 5 d to allow female flies time to complete egg development. Eggs were subsequently collected from many of the female flies by placing a small plastic food dish containing tissue paper soaked in evaporated milk into the mesh cage for a 24 h period. Eggs were rinsed from the tissue paper and placed into immature rearing pans with the colony thereafter maintained in insectary rooms at 27°C, 14:10 L:D, 35% RH, and following standard rearing practices (Zahn and Gerry 2018).

An imidacloprid-susceptible house fly colony (UCR fly strain) collected in 1982 from a dairy in Mira Loma, California, and maintained in colony at UCR without

insecticide exposure since this time was used to determine relative insecticide susceptibility of WT and selected fly strains in this study. The UCR colony was housed in a separate insectary room from other fly colonies but otherwise maintained with the same environmental conditions and rearing practices as other colonies in this study.

Imidacloprid susceptibility bioassays

Adult house flies (3-5 d-old) were aspirated from a colony cage and chilled briefly in a -20°C freezer. Flies were then sorted by sex on a chill table, and 25 female flies were placed into each of five 230-mL glass jars (VWR International, catalog # 16195-008) (n=125 total flies per trial). Each jar contained a 4-cm dental wick (Richmond Dental Co., Charlotte, NC) soaked in water and either a single 15 mL paper soufflé cup (Amerifoods Trading Co., Los Angeles, CA) containing 1 g of granular sucrose formulated with technical grade imidacloprid (CAS: 138261-41-3, Chem Service Inc., West Chester, PA) ("no-choice" bioassay) or both a soufflé cup containing sucrose with imidacloprid and a second soufflé cup containing only sucrose ("choice" bioassay). Sucrose formulated with imidacloprid was made by dissolving into acetone the desired test concentration of imidacloprid per g sucrose to be used in each trial and then applying the acetone-imidacloprid solution to granular sucrose, mixing thoroughly to ensure even dispersal of the insecticide through the sucrose and then placing the mixture in a fume hood for 24 h to allow the acetone to evaporate. The mixture was then thoroughly homogenized before removing 1g of the sucrose-imidacloprid mixture to place into each soufflé cup. The sucrose only food option was similarly prepared with acetone but

without the addition of imidacloprid. An additional five glass jars each with 25 flies (n=125 total flies) were set up as a negative control, with flies provided a 4-cm dental wick soaked in water and either 1 or 2 (for no-choice or choice bioassay, respectively) soufflé cups containing only granular sucrose prepared without imidacloprid as above. Glass jars were covered with mesh netting and flies were allowed to freely feed within the jars. Bioassays were performed under standard colony rearing conditions (described above) with dental wicks rehydrated at 24 and 48 h. Mortality was recorded at 72 h, with individual flies scored as dead if they were unable to right themselves. Mortality was pooled for all five treatment or control jars, and Abbott's formula was used to correct for control mortality using R version 3.3.0 (R Core Team 2017).

Both no-choice and choice bioassays were performed using varying concentrations of imidacloprid until a minimum of five different imidacloprid concentrations produced a corrected mortality from 1-99% in each assay. Probit analysis was used to estimate the dose of imidacloprid needed to kill 50% (LC_{50}) and 95% (LC_{95}) of flies.

Selection for behavioral resistance to imidacloprid

Approximately 5,000 house fly pupae from the 3rd filial generation (F_3) of the WT colony were collected from several immature rearing pans, thoroughly mixed, and equally distributed into five adult fly rearing cages to establish five separate colonies for independent selection of behavioral resistance to imidacloprid. Resistance selection was performed separately for each of the five fly colonies (“Behavioral Resistance Strains”

BRS1-BRS5 fly strains) to evaluate whether more than one behavioral resistance mechanism might be selected using our protocol. Adult flies within 8 h of eclosion, and therefore unmated (Murvosh et al. 1964), were aspirated from their cage, chilled for 8 min at -20°C, sorted by sex on a chill table, and ~300 male and 300 female flies were placed into sex-specific cages provisioned with food and water for 3-5 d to mature.

After reaching maturity, flies were starved for 14 h and then exposed to a behavioral resistance selection assay. In this assay, flies were provided a soufflé cup containing 3 g of sucrose alone and a second soufflé cup containing 3 g of sucrose formulated with imidacloprid at a "selection dose" concentration of 4,000 µg/g (3x LC_{95} for the WT colony in a no-choice bioassay). Flies were exposed to the selection assay for 72 h under standard colony conditions. The very high concentration of imidacloprid used in this assay ensured that surviving flies did not feed on the sucrose-imidacloprid food offered. After 72 h surviving male and female flies were combined into a single adult cage, provided food and water ad libitum, and allowed to mate for 7 d before eggs were collected. Each of the fly strains (BRS1-BRS5) was selected in this way every 3 filial generations to complete 10 selections. Following the 5^{th} and 10^{th} (last) selections, each BRS strain was tested for altered susceptibility to imidacloprid using both the no-choice bioassay (to test for physiological resistance) and choice bioassay (to test for behavioral resistance) as described in the susceptibility bioassay section above.

A significant difference in susceptibility to imidacloprid among behaviorally resistant and reference fly strains was determined by non-overlapping 95% confidence intervals in calculated LC values for all fly strains for which LC values could be

determined. Resistance of selected fly strains relative to the WT and UCR reference fly strains was determined by dividing the LC value of a selected fly strain by the LC value of a reference fly strain to give a resistance ratio (RR), with a RR >1 indicating an increase in resistance to imidacloprid.

Selection for behavioral susceptibility to imidacloprid

Recently emerged (unmated) adult WT colony flies were aspirated from their cage and sorted by sex on a chill table. Adult flies were placed as individual mating pairs (one male, one female) into one of 50 mating chambers (947 mL polypropylene deli containers, Pro-Kal, Kalamazoo, MI, USA) with a removable plastic lid and a bottom modified by adding a fiberglass screen. Mating chambers were inverted (screen side up), and provisioned with food (1:1, sucrose: dehydrated milk) and water placed into 37 mL soufflé cups (Solo Cup Company, Urbana, IL) for 7 d, after which larval media was provided for egg deposition. Larval media was moistened every 24 h until removal at 72 h. Eggs in larval media were mixed with 500 mL of fresh media. Offspring from each mating pair were reared separately following standard rearing procedures (Zahn and Gerry 2018). After eggs were removed from each mating chamber, food was also removed, and the mating pair of flies were starved for 14 h. Mating pairs were then exposed to the "behavioral resistance selection assay", but for only 24 h to identify flies that quickly consumed the sucrose-imidacloprid food and thus lacked a behavioral resistance phenotype. When both adults in a mating pair died during the selection assay, the offspring of this mating pair was anticipated to similarly lack a behavioral resistance

phenotype. All offspring of mating pairs that died were combined into a single colony of "Behaviorally Susceptible Strain" flies (BSS fly strain). The BSS fly strain was selected in this way seven times before evaluating overall behavioral susceptibility as described below. Due to low numbers of BSS strain flies in post-selection generations, imidacloprid susceptibility assays to determine an LC value were not performed on this strain.

Overall imidacloprid susceptibility of selected strains

Differences in overall susceptibility to imidacloprid among all fly strains (UCR, WT, BSS, BRS1-5) were determined by fly survival in a choice bioassay with flies provided a soufflé cup containing 1 g sucrose alone and a second soufflé cup containing 1 g sucrose formulated with imidacloprid at the selection dose of 4,000 µg/g, with mortality evaluated after 72 h. The assay was replicated 5 times for each fly strain, with 25 female flies utilized in each replicate. Mortality was analyzed via Fisher's exact test with a Bonferroni correction applied for multiple comparisons ($P<0.00185$) to determine whether fly strains differed in their susceptibility to the selection dose of imidacloprid.

Observation of behavioral resistance phenotype

Adult house flies (3-5 d-old) were starved in their colony cage for 14 h, then sorted on a chill table into groups of 25 same-sex flies placed into a 120 x 25 mm Petri dish that was then placed into the center of a Plexiglass observation chamber (50 x 18.25 x 18.5 cm) held in an insectary room at 27°C and 35% RH. A weigh dish (Fisherbrand Polystyrene Weighing Dishes, Number 02-202-101) containing 1 g of sucrose and a

second weigh dish containing sucrose formulated with imidacloprid at the selection dose of 4,000 µg /g sucrose were randomly assigned for placement at 13 cm from each sidewall of the observation chamber. A second observation chamber with the treatment positions reversed was simultaneously set up to mitigate possible bias in treatment position.

Flies were allowed 15 min to acclimate in the covered Petri dish before initiating the observation assay, after which the Petri dish cover was removed, and flies were allowed to move freely throughout the chamber for 2 h while their movement was recorded using a video camera (Hero 5 Black, GoPro, San Mateo, CA). The observation assay was replicated 10 times for each fly sex over three fly generations for each imidacloprid-resistant fly strain (WT, BRS1-BRS5). The video was analyzed using Behavioral Observation Research Interactive Software (BORIS) (https://www.boris.unito.it/) (Friard and Gamba 2016), recording the number of times a fly landed on each food dish (landing events) and the amount of time each fly spent on the food dish (contact time). Landing events evaluate attraction or repellency of the offered materials, while contact time is a surrogate for time spent exploring, tasting, and feeding on the material. A single fly could have more than one landing event, should it disengage from a food dish, and then subsequently land on a food dish again during the observation period. Differences in landing events and contact time between treated and untreated food dishes were analyzed separately for each fly sex using a Wilcoxon matched-pairs test.

With no differences in treatment position among paired observation chambers during initial analyses, each observation chamber utilized was subsequently analyzed as a separate replicate.

Specificity of behavioral resistance to imidacloprid

To determine specificity of the selected behavioral resistance mechanism to imidacloprid, a feeding preference study was performed for each imidacloprid-resistant fly colony (WT, BRS1-BRS5) comparing house fly consumption of sucrose-containing imidacloprid to consumption of sucrose-containing another compound in the neonicotinoid insecticide class (dinotefuran). Like imidacloprid, dinotefuran is currently available as a toxicant in fly bait for control of house flies (QuikStrike® fly bait; Wellmark International, Shaumburg, IL, USA). Adult house flies (3-5 d-old) were starved in their colony cage for 14 h, then sorted on a chill table into 5 groups of 25 female flies each (total of 125 flies) that were subsequently placed into inverted 947 mL polypropylene deli containers with a removable plastic lid and a bottom modified by adding a fiberglass screen. Flies were provided water, 1 g of sucrose mixed with 4,000 µg dinotefuran (Cas:165252-70-0, Chem Service Inc., West Chester, PA, USA) in a 37 mL soufflé cup and a second soufflé cup with 1 g sucrose mixed with the selection dose of 4,000 µg imidacloprid. The dinotefuran-sucrose was colored red while the imidacloprid-sucrose was colored blue using food grade coloring solution (McCormick & Co., Inc. Hunt Valley, MD) resulting in the color being present in the abdomen of flies feeding on

a food dish. Flies feeding on both food dishes would have a purple abdomen, while unfed flies would lack color (recorded as "clear").

Flies were allowed to feed on either insecticide-treated sucrose dish for 24 h, by which time 100% fly mortality was observed in all replicates. Dead flies were subsequently sorted via abdomen color as an indication of feeding activity: red, blue, purple, or transparent (Bantel and Tessier 2016). A feeding preference was calculated for all fly strains using the formula ($P_{D/I} = N_{Red} + 0.5N_{Purple}$)/ ($N_{Red} + N_{Blue} + N_{Purple}$), where $P_{D/I}$ is the preference of flies to feed on the dinotefuran-sucrose food over the imidacloprid-sucrose food, and N represents the number of individuals with the indicted abdomen color. A $P_{D/I}$ value > 0.5 indicates a fly feeding preference for the dinotefuran-sucrose, while a $P_{D/I}$ value < 0.5 indicates a fly feeding preference for the imidacloprid-sucrose. For each fly strain, a difference from no feeding preference ($P_{D/I} = 0.5$) was calculated by one sample t-test. In preliminary studies, five replicates of 125 house flies showed no feeding preference for sucrose alone when colored with either the red or blue food coloring (P=0.7496), so any feeding preference between the two treatments was due to the presence of the insecticide. A pictorial overview of the methods described below can be found in figure 1.4.

RESULTS

Prior to selection for behavioral resistance, the field-collected WT fly strain already exhibited both physiological and behavioral resistance to imidacloprid relative to the UCR susceptible fly strain. WT flies had an LC_{50} = 619 (no-choice bioassay) and LC_{50} = 11700 (choice bioassay), while UCR Susceptible flies had an LC_{50} = 19 (no-

choice) and LC_{50} = 48 (choice), resulting in a no-choice bioassay RR of 33 and a choice bioassay RR of 244 (Table 1.1). Though not shown in Table 1.1, WT flies had a LC_{95} = 1263 for a no-choice assay while the LC_{95} for a choice bioassay could not be calculated due to low mortality at even the highest imidacloprid dose utilized (15,000 ug/g sucrose).

Behavioral resistance was very rapidly selected in each of the behaviorally resistant fly strains (BRS1-BRS5), with mean fly survival for selected fly strains during the behavioral resistance selection assay increasing from 2.1 to 72.7% for males and 28.7 to 90% for females in just 5 selection cycles, and ultimately reaching 91.4% and 99.83% survival of male and female flies, respectively by the 10^{th} and final selection cycle (Figure 1.1). Due to very low mortality (<20%) of the final selected BRS fly strains in a choice bioassay, even at the highest imidacloprid dose tested (15,000 ug/g sucrose), neither the LC50 nor LC95 could be determined for BRS selected fly strains and therefore the RR also could not be calculated for BRS stains relative to either the UCR or WT flies. Importantly, physiological resistance to imidacloprid of selected fly strains was not increased by the behavioral resistance selection process, with selected fly strains even exhibiting a slightly decreased resistance to imidacloprid in no-choice assays (RR<1) following the final selection (Table 1.1).

Rapid selection of the BSS fly strain for behavioral susceptibility to imidacloprid was also achieved in this study. After just seven selection cycles, survival of the BSS strain when challenged in a choice feeding assay at the imidacloprid selection dose of 4,000 ug/g sucrose was significantly reduced relative to the WT strain and all selected BRS strains, with survival being similar to the UCR susceptible fly strain (Figure 1.2).

In behavioral observation assays, there were no differences in the number of flies landing on sucrose-imidacloprid food dishes relative to sucrose only food dishes for all fly strains (n=10; P>0.05) (Table 1.2). WT flies also did not differ in their contact time between the two food dishes. In contrast, all behaviorally resistant fly strains (BRS1-BRS5) had significantly reduced contact time with the sucrose-imidacloprid dish relative to the sucrose only food dish (n=10; P<0.05). Male BRS3 flies showed non-significantly reduced contact time with the sucrose-imidacloprid relative to sucrose only food dish, (n=10; P=0.1602). Both landing events and contact time could not be analyzed for UCR and BSS strain flies due to rapid death of flies that landed in the sucrose-imidacloprid dish, with flies often dying within the dish impacting landing by other flies and resulting in a contact time that was not related to feeding behavior.

In feeding preference assays, the WT and behaviorally susceptible fly strains (BSS and UCR) exhibited no preference for imidacloprid or dinotefuran (n=5; P>0.05), with preference indices ($P_{D/I}$) = 0.5, 0.49, and 0.5 respectively. Whereas behaviorally resistant fly strains exhibited a significant preference (n=5; P< 0.001) for dinotefuran over imidacloprid with $P_{D/I}$ = 0.79, 0.89, 0.74, 0.90, and 0.82 for BRS1-BRS5, respectively (Figure 1.3).

DISCUSSION

While behavioral resistance to insecticides or components of toxic food baits has been previously reported in numerous insect species including house flies (Freeman and Pinniger 1992, Learmount et al. 1996, Darbro and Mullens 2004, Gerry and Zhang 2009, Mullens et al. 2010), cockroaches (Silverman and Selbach 1998, Wada-Katsumata et al.

2013, Wada-Katsumata et al. 2014, Wada-Katsumata et al. 2018), fungus-growing termites (Iqbal and Evans 2018), as well as in mammal species including the invasive red fox (Allsop et al. 2017) and the brown rat (Brunton et al. 1993). Behavioral resistance has also been documented to be expressed as an excito-repellency response in mosquitoes (Chareonviriyaphap et al. 2013, Gatton et al. 2013), horn flies (Byford et al. 1987, Sparks et al. 1989, Zyzak et al. 1996), and bed bugs (Romero et al. 2009, Agnew and Romero 2017). However, separation of behavioral resistance from physiological resistance mechanisms in resistant pest populations is challenging and reported resistance phenotypes may include both behavioral and physiological resistance mechanisms.

This is the first study to successfully select specifically for behavioral resistance to an insecticide without increasing the physiological resistance of the selected insect population. We show that behavioral resistance is specific to an insecticide (imidacloprid) rather than to a non-insecticidal component of a bait matrix as previously documented for house flies (Freeman and Pinniger 1992) and German cockroaches (Silverman and Bieman 1993). Behavioral resistance to insecticides should be considered as important or perhaps even more important than physiological resistance in some cases, since behavioral resistance cannot be overcome simply by increasing the concentration of insecticide applied.

Selectively breeding flies for increased physiological resistance is commonplace when looking to elucidate resistance mechanisms (e.g. Kaufman et al. 2010, Kavi et al. 2014, Zhu et al. 2016, Khan 2019, Reid et al. 2019), but selection for increased behavioral resistance alone has not been previously demonstrated. This was

accomplished using a selection process where flies were offered a food choice of sucrose alone or sucrose with a very high dose of insecticide, so that only flies consuming sucrose alone survived to populate the next generation. A very high level of behavioral resistance was achieved in the selected fly strains (BRS1-BRS5) following just 5-10 selection cycles. Similarly, a behaviorally susceptible fly strain (BSS) was obtained through a selection process where only the offspring of flies that died following a short exposure to the two food choices populated the next generation, with a high level of behavioral susceptibility achieved within just seven selection cycles. The level of behavioral susceptibility achieved was similar to that of the insecticide susceptible fly colony (UCR strain) that we have maintained in the laboratory since 1982. This study therefore differs from previous studies which selected house flies for resistance to imidacloprid using a selection process where flies were offered only sucrose with imidacloprid (no-choice) resulting in selection for physiological resistance with little or no opportunity for selection of behavioral resistance (Kaufman et al. 2010, Seraydar and Kaufman 2015). This study also differs from previous studies in that reversion of insecticide resistance was rapidly achieved using an active selection process, rather than through a passive process where susceptible genotypes are anticipated to have higher fitness in the absence of insecticidal pressure (e.g. Seraydar and Kaufman 2015).

The development of fly strains exhibiting a strong behavioral resistance phenotype has allowed us to better understand the complex nature of behavioral resistance to imidacloprid. For example, the very rapid selection for behavioral resistance or behavioral susceptibility in the current study suggests that the WT fly population

already contained natural genetic variation which was capable of conferring the behavioral resistance phenotype to selected fly strains. Further, the similar landing rate for resistant and susceptible fly strains on food dishes containing imidacloprid-treated sucrose and on food dishes with sucrose alone suggests that behaviorally resistant flies cannot detect imidacloprid prior to physical contact with the treated food source. However, behaviorally resistant flies showed greatly reduced time spent in contact with (and presumably feeding on) the imidacloprid-treated sucrose relative to the wild type flies suggesting detection of imidacloprid results in rapid disengagement with the toxic food source. This behavioral avoidance of imidacloprid-treated sucrose explains the very low mortality recorded in the imidacloprid susceptibility choice feeding bioassays performed in the current study, even when a very high dose of imidacloprid was used. In contrast, both UCR Susceptible and BSS selected susceptible flies readily fed on the imidacloprid-treated sucrose and rapidly died during observation assays.

It is important to emphasize that the behaviorally resistant selected flies are still physiologically susceptible to imidacloprid, i.e., if they were to consume sucrose formulated with imidacloprid at the offered dose, they would die. Interestingly, Darbro and Mullens (2004) documented a similar aversive response to methomyl-treated bait when flies from several California locations were tested in a choice feeding assay, but it is unclear if the aversion was to the insecticide or other components of the bait used. Freeman and Pinniger (1992) also described an aversive behavior in house flies but concluded that aversion was likely to formulation components or contaminants in the insecticidal bait matrix instead of to the active ingredient azamethiphos. Aversion to a

component (glucose) of an insecticidal bait matrix was also the mechanism of behavioral aversion in German cockroaches (Silverman and Bieman 1993).

All fly strains selected in this study for behavioral resistance to imidacloprid (BRS 1-5) demonstrated a resistance phenotype specific to this insecticide rather than to the more general neonicotinoid chemical class. Selected flies were not behaviorally resistant to the neonicotinoid dinotefuran in behavioral observation assays where these flies showed a strong preference to feed on dinotefuran over imidacloprid, while behaviorally susceptible and wild type fly strains (UCR, BSS, and WT) had no preference for sucrose formulated with either insecticide. Feeding preference assays have traditionally been used to determine the contributions of gustatory receptors to perceiving different tastants in Drosophila (Bantel and Tessier 2016, Chen et al. 2019), but can be used to determine feeding preference between any two food materials as was performed in this study.

Given that the resistance phenotype is expressed soon after contact with imidacloprid but not with dinotefuran, it seems likely that behavioral resistance is due to specific detection of imidacloprid by a chemoreceptor that initiates an aversion response by the fly. These receptors are likely either on the fly tarsus or proboscis allowing the fly to detect the imidacloprid insecticide without ingestion (Deither 1976), particularly as the high imidacloprid dose used in these studies might be expected to kill flies even following very limited consumption of the treated sucrose. However, other mechanisms for imidacloprid detection and the subsequent aversive response cannot be ruled out. For example, it is possible that behavioral resistance occurs in response to imidacloprid

binding at the nicotinic acetylcholine receptor site, though this seems unlikely as it would require ingestion of at least some of the insecticide. If this were the case, consumption of dinotefuran by behaviorally resistant flies could be due to the drastically different chemical structures of imidacloprid and dinotefuran resulting in different response when these compounds are bound to the receptor site. Dinotefuran uniquely possesses a nonaromatic ring, one oxygen capable of forming hydrogen bonds and an asymmetric carbon (Kiriyama et al. 2003, Matsuda et al. 2020). However, significance of the structural differences between the two chemicals with respect to the target-site actions has yet to be determined.

While the focus of this study was the selection for and characterization of behavioral resistance to imidacloprid, we can also assess the change in physiological resistance to imidacloprid of the wild type parent population since flies from this same southern California dairy were also collected and tested for resistance to imidacloprid in 2008 (Gerry and Zhang 2009). Although records of past insecticide use on this dairy are not available, granular baits containing imidacloprid or dinotefuran continue to be applied for fly control at this dairy as well as throughout the region (Gerry A, personal observations). Since 2008, physiological resistance to imidacloprid in wild flies at this dairy site more than tripled relative to the UCR Susceptible fly strain from a RR = 10.3 in 2008 to a RR = 33 in 2015 (this study). While this increase in resistance to imidacloprid might seem substantial, the imidacloprid concentration (5,000 µg/g bait) in the commercial fly bait QuickBayt® (Bayer Healthcare LLC, Shawnee Mission, KS, U.S.A.) is more than 2x the LC_{95} value for WT flies in the current study using a no-choice

bioassay, suggesting that QuickBayt would still be effective to kill flies if physiological resistance were the only mechanism contributing to imidacloprid resistance. While in comparison to the modest increase in physiological resistance to imidacloprid from 2008 to 2015, the large increase in behavioral resistance over this same time period indicates that behavioral resistance mechanisms are conferring greater protection to the flies.

Imidacloprid was first registered as a commercial fly bait (QuickBayt® with 0.5% imidacloprid and 0.1% (Z)-9-tricosene) in November 2002 (EPA, 2002). Efficacy studies in subsequent years demonstrated initial effectiveness of this bait (Butler et al. 2007), followed by rapid loss of effectiveness as a result of increasing fly resistance (Gerry and Zhang 2009, Mullens et al. 2010). Murillo et al. (2015) made visual counts of flies landing on commercial fly baits offered to flies at a southern California dairy and recorded a four-fold greater number of flies on a fly bait containing dinotefuran (QuikStrike; Wellmark International, Shaumburg, IL, USA) relative to the imidacloprid fly bait QuickBayt. Interpreting this outcome based on the current study, flies may have visited the two bait materials in similar numbers, but behaviorally resistant flies encountering the imidacloprid bait would quickly depart from the imidacloprid bait while they would remain and feed on the dinotefuran bait, resulting in lower numbers of flies on the imidacloprid bait at each observation time. Behavioral resistance to an insecticide can therefore skew interpretation of bait attractiveness studies which score house fly attraction by instantaneous fly counts on the offered bait materials. Behaviorally resistant flies might also be incorrectly assumed to be physiological resistant to the offered

insecticide in these field studies due to low fly mortality in the treatment arena if time of contact with the bait or bait consumption is not also determined.

Future studies should focus on the genetic mechanisms for inherited behavioral resistance to insecticides and on the specific mechanisms for detection and response to imidacloprid. Elucidation of these mechanisms may allow for development or selection of insecticide chemistries that limit or delay the selection for behavioral resistance by house flies or other pests.

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TABLES AND FIGURES

Table 1.1: Physiological and behavioral susceptibility to imidacloprid of reference fly strains (UCR, WT) and fly strains selected from WT strain for behavioral resistance (BRS1-BRS5). [1] Flies were provided food dishes with sucrose-imidacloprid only (no-choice) or with separate food dishes containing either sucrose-imidacloprid or sucrose only (choice). Significant differences in the lethal concentration (LC) value among fly strains were determined by non-overlapping 95% confidence intervals (CI) of the LC values and are indicated within columns by capital letters for no-choice bioassays and lower case for choice bioassays. [2]Resistance ratio (RR) = LC_{50} of fly strain (row) / LC_{50} of WT or UCR reference fly strain (column). Values that could not be calculated due to lack of sufficient fly mortality even at the highest imidacloprid dose tested (> 15,000 µg/g sucrose) are indicated as not determined (ND).

Assay Type[1]	Fly Strain	n	Slope (SE)	LC50 (95% CI) (µg/g)	RR (LC50) WT[2]	RR (LC50) UCR[2]
No-choice	UCR	875	1.4 (0.2)	19 (10 - 38) A	-	-
No-choice	WT	1375	2.6 (0.1)	619 (586 - 651) C	-	33
No-choice	BRS 1	875	2.4 (0.1)	539 (495 - 583) B	0.87	28
No-choice	BRS 2	750	2.3 (0.1)	473 (430 - 516) B	0.76	24
No-choice	BRS 3	750	2.1 (0.1)	487 (436 - 538) B	0.79	25
No-choice	BRS 4	750	1.9 (0.1)	536 (479 - 594) BC	0.87	28
No-choice	BRS 5	750	2.2 (0.1)	438 (395 - 480) B	0.71	23
Choice	UCR	875	1.1 (0.2)	48 (40 - 55) a	-	-
Choice	WT	750	1.6 (0.1)	11700 (10400 - 12900) b	-	244
Choice	BRS 1	750	ND	>15000	ND	ND
Choice	BRS 2	750	ND	>15000	ND	ND
Choice	BRS 3	750	ND	>15000	ND	ND
Choice	BRS 4	750	ND	>15000	ND	ND
Choice	BRS 5	750	ND	>15000	ND	ND

Table 1.2: Mean ± SE landing events and contact time (in seconds) on dishes containing sucrose alone or sucrose with imidacloprid (4,000 µg/g) over a 2 h observation period. [1]N indicates the number of replicates tested (25 flies/replicate). Differences between treatments in the number of landing events or contact time by fly strain and sex were determined by Wilcoxon matched-pairs test with a significant difference indicated by P-value in bold font.

		Landing Events (Lands ± SE)			Contact Time (Time ± SE)		
Strain	N[1]	Sucrose	Imidacloprid	P-value[†]	Sucrose	Imidacloprid	P-value
WT ♂	10	5.8 ± 1.7	5.3 ± 1.9	0.78	30.8 ± 9.2	15.9 ± 5.0	0.19
BRS 1 ♂	10	27.8 ± 6.9	22.6 ± 3.4	0.29	121.3 ± 70.1	3.4 ± 2.7	**0.002**
BRS 2 ♂	10	7.5 ± 2.1	11.1 ± 3.5	0.48	146.6 ± 58.6	1.9 ± 0.5	**0.004**
BRS 3 ♂	10	7.4 ± 2.2	6.0 ± 1.8	0.71	94.9 ± 36.3	32.9 ± 18.8	0.16
BRS 4 ♂	10	4.3 ± 0.9	6.4 ± 2.1	0.3	83.8 ± 24.9	8.1 ± 3.2	**0.004**
BRS 5 ♂	10	7.6 ± 1.9	7.3 ± 2.2	0.58	107.6 ± 40.9	3.2 ± 0.7	**0.002**
WT ♀	10	10.0 ± 1.9	10.3 ± 1.7	>0.99	128.7 ± 93.9	34.7 ± 8.2	0.56
BRS 1 ♀	10	14.5 ± 3.1	16.1 ± 3.2	0.75	45.5 ± 12.1	5.6 ± 2.3	**0.002**
BRS 2 ♀	10	9.5 ± 3.3	12.8 ± 2.4	0.29	265.6 ± 81.8	3.2 ± 1.1	**0.002**
BRS 3 ♀	10	5.9 ± 1.6	7.2 ± 1.5	0.34	121.4 ± 37.5	4.6 ± 1.1	**0.002**
BRS 4 ♀	10	7.3 ± 1.4	5.3 ± 0.6	0.26	40.9 ± 7.5	9.1 ± 4.2	**0.002**
BRS 5 ♀	10	8.0± 1.4	4.8 ± 0.8	0.09	67.8 ± 17.9	7.5 ± 2.5	**0.002**

Figure 1.1: Survival of male (a) and female (b) flies from each BRS fly strain during imidacloprid behavioral resistance selection assay over 10 selection cycles. Selection 1 indicates survival of the field-collected WT house flies during the first selection assay. Surviving offspring from each selection assay comprised a selection and populated the next generation.

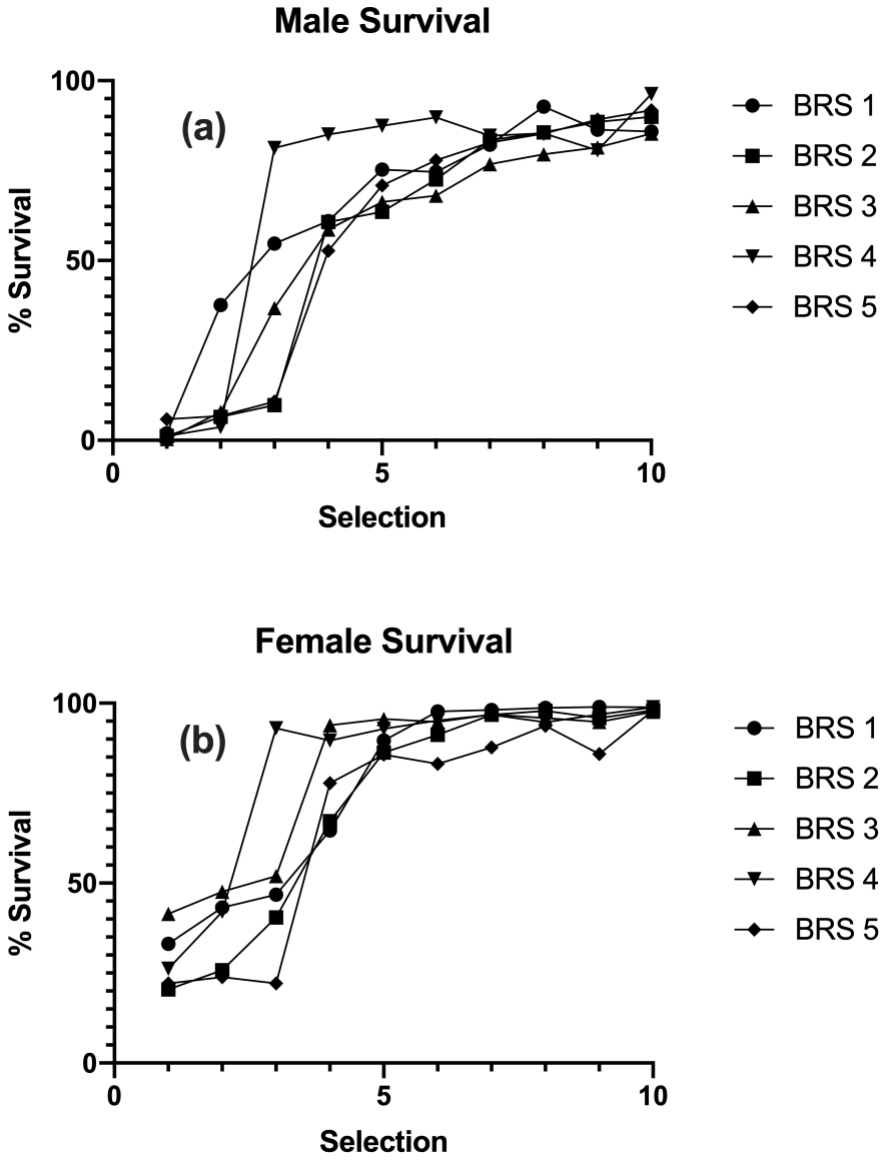


Figure 1.2: Relative behavioral resistance to imidacloprid by fly strain as indicated by fly survival following 72 h exposure to a choice feeding assay with paired food dishes containing either sucrose alone or sucrose treated with imidacloprid at a dose of 4,000 µg per g sucrose. Fly strains were selected from a field-collected population of flies (WT) for behavioral resistance (BRS1-BRS5; 10 selections) or behavioral susceptibility (BSS; 7 selections) to imidacloprid. The UCR fly strain is a susceptible reference house fly strain maintained in colony at UC Riverside since 1982. *Different letters indicate significance ($P<0.00185$) after Bonferroni correction for multiple comparisons.

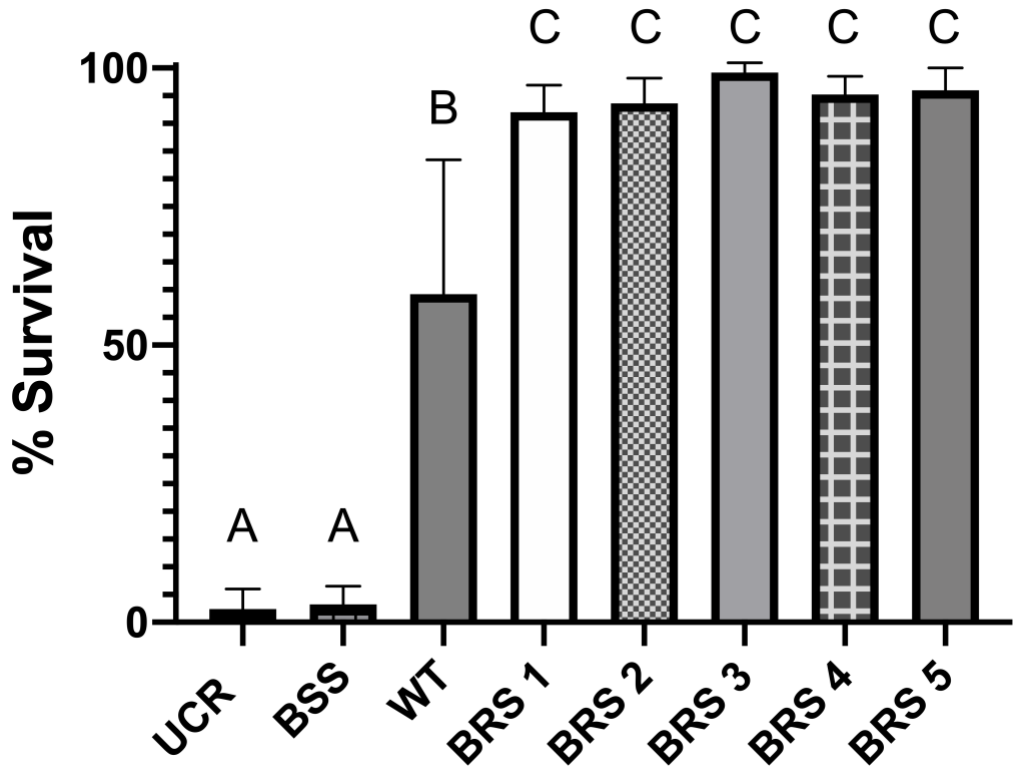


Figure 1.3: Feeding preference index ($P_{D/I}$) for flies provided a choice to feed on either sucrose with 4,000 µg/g imidacloprid or sucrose with 4,000 µg/g dinotefuran. A $P_{D/I}$ value >0.5 indicates a greater proportion of flies feeding on the sucrose-dinotefuran, while a $P_{D/I}$ = 0.5 indicates that flies fed equally on the two insecticide-treated sucrose foods. A significant preference among the two food choices for each fly strain was determined by one-sample t-test (***=P<0.001).

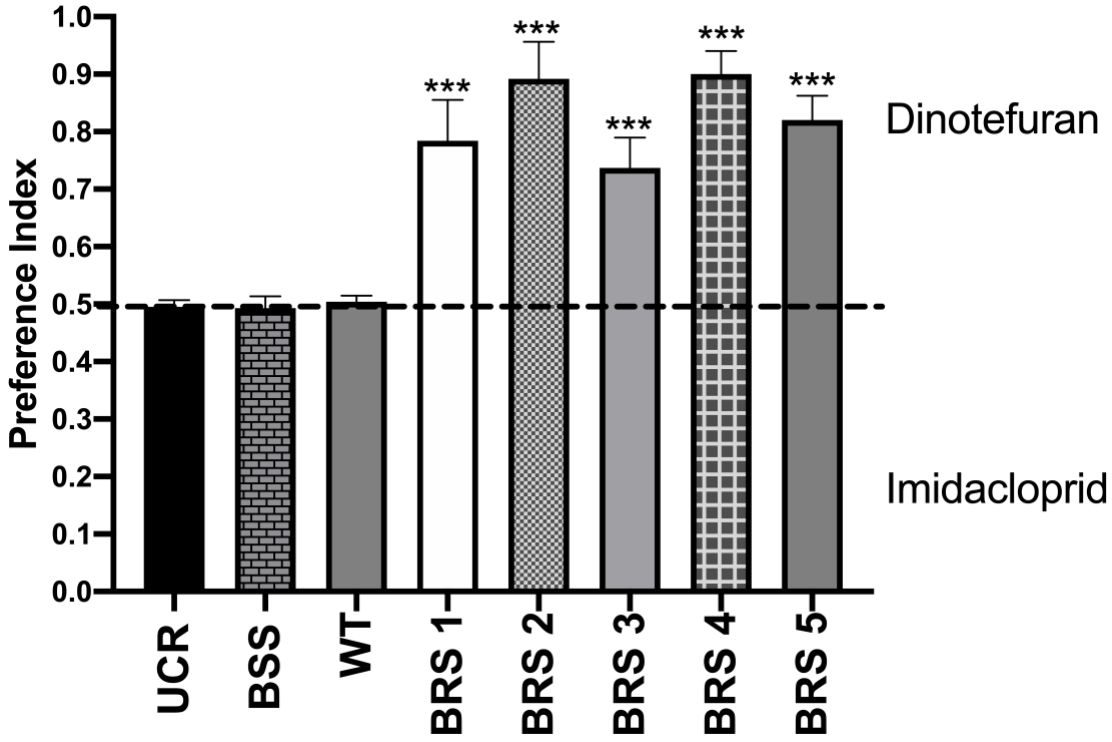
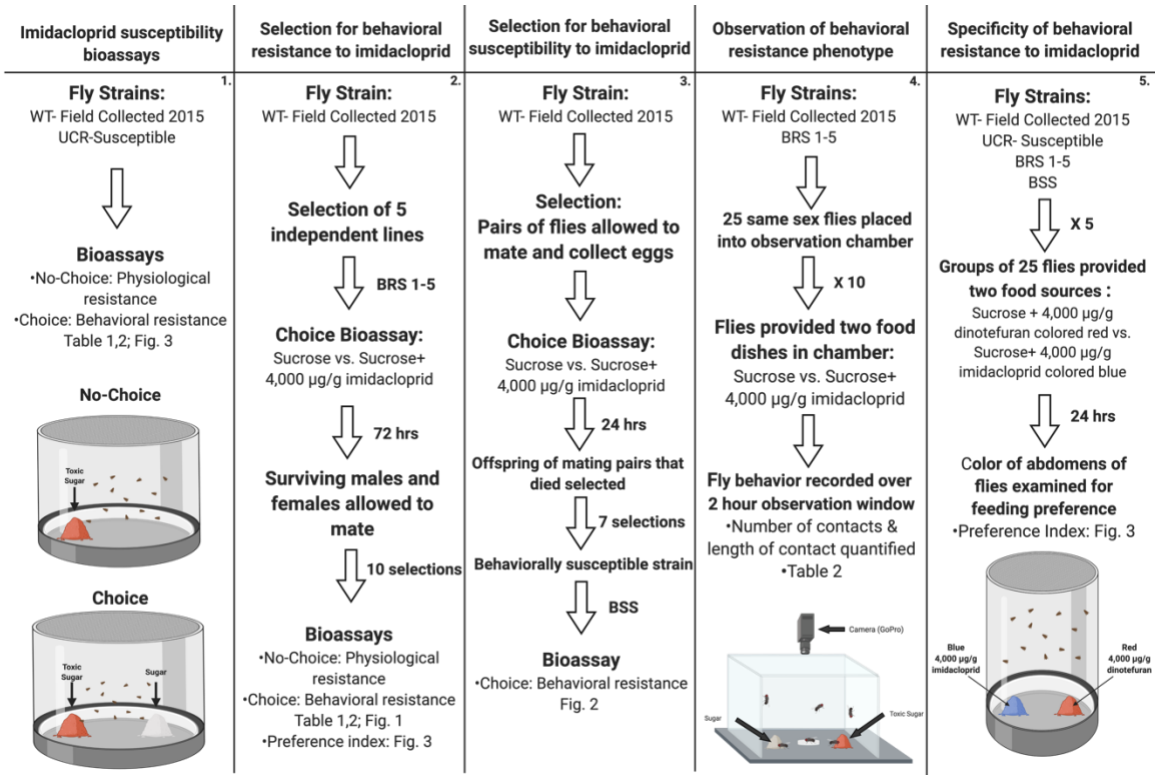


Figure 1.4: Flow chart describing experiment workflow with graphic representation created with BioRender.com



CHAPTER 2

Genetic evaluation and characterization of behavioral resistance to imidacloprid in the house fly.

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ABSTRACT

Insecticide resistance in pest populations is an increasing problem in both urban and rural settings due to over-application of insecticides and lack of rotation among insecticidal chemical classes. The house fly (*Musca domestica* L.) is a cosmopolitan pest fly species implicated in the transmission of numerous pathogens. The evolution of insecticide resistance long has been documented in house flies, with resistance reported to all major insecticide classes. House fly resistance to imidacloprid, the most widely used neonicotinoid insecticide available for fly control, has evolved in field populations through both physiological and behavioral mechanisms. Previous studies have characterized and mapped the genetic changes that confer physiological resistance to imidacloprid, but no study have examined the genetics involved in behavioral resistance to imidacloprid to date. In the current study, several approaches were utilized to characterize the genetics and inheritance of behavioral resistance to imidacloprid in the house fly. These include behavioral observation analyses, preference assays, and the use of genetic techniques for the identification of house fly chromosome(s) carrying factors. Behavioral resistance was mapped to autosomes 1 and 4. Inheritance of resistance was shown to be neither fully dominant nor recessive. Factors on autosomes 1 and 4 independently conferred contact-dependent avoidance of imidacloprid and a feeding preference for sugar alone or for sugar with dinotefuran, another neonicotinoid insecticide, over imidacloprid. This study serves as the first linkage analysis of a behavioral trait in the house fly and provides new avenues for research regarding inherited behavior in the house fly and other animals.

INTRODUCTION

The house fly (*Musca domestica* L.) is a cosmopolitan and synanthropic fly species that is a significant pest of animal agricultural operations and in urban waste storage facilities (West 1951, Thomas and Skoda 1993, Geden and Hogsette 2001). House flies may cause considerable nuisance to communities near their developmental sites (Thomas and Skoda 1993) and are implicated in transmitting numerous animal and human pathogens (reviewed by Nayduch 2017). Failure to control adult flies can result in litigation against animal producers or urban waste facilities as flies disperse from development sites to surrounding communities, due to the potential for nuisance and pathogen transmission (Thomas and Skoda 1993).

Adult house flies are often controlled using insecticides when adult fly populations exceed acceptable abundance or activity levels (Geden and Hogsette 2001, Gerry 2020). However, over-use of insecticides for house fly control has resulted in house fly resistance development to nearly all major insecticide classes (Keiding 1999, Darbro and Mullens 2004, Kaufman et al. 2006, Scott et al. 2013, Murillo et al. 2015, Freeman et al. 2019). In the house fly, insecticide resistance can occur through selection for well-characterized physiological resistance mechanisms including upregulation of detoxifying enzymes (e.g., P450's or GST's) or structural alteration at insecticide binding sites that reduces accessibility of the binding site or impairs insecticide binding to the target site (target site insensitivity) (Liu and Scott 1997, Rinkevich et al. 2006, Zhang et al. 2018, Ma et al. 2019). More recently, there is increasing evidence that insecticide resistance in the house fly also can be acquired through inherited changes in behavior that

reduce house fly consumption of insecticidal food baits (Darbro and Mullens 2004, Gerry and Zhang 2009, Seraydar and Kaufman 2015, Hubbard and Gerry 2020).

Currently, neonicotinoids are the most widely utilized insecticide class in the world (Sparks and Nauen 2015). These insecticides bind irreversibly to the nicotinic acetylcholine receptor, inhibiting normal binding of acetylcholine, disrupting nerve function, and resulting in paralysis and insect death (Jeschke and Nauen 2005). In the house fly, physiological resistance to the neonicotinoid imidacloprid has been linked to the overexpression of a microsomal glutathione S-transferase gene on chromosome 3, and to an unknown trans-regulatory gene on chromosome 4, which results in overexpression of a galactosyltransferase-like gene (Reid et al. 2018). In contrast, behavioral resistance mechanisms have been largely overlooked and specific molecular mechanisms conferring house fly behavioral resistance to imidacloprid have yet to be identified. However, the phenotypic behaviors responsible for behavioral resistance to imidacloprid were recently determined to be both contact-dependent and specific to imidacloprid (Hubbard and Gerry 2020).

Wild house fly populations demonstrated behavioral resistance to imidacloprid within a few years of the commercial availability of imidacloprid-containing fly bait (Gerry and Zhang 2009), with resistance due to reduced fly feeding on the bait (Mullens et al. 2010). While physiological and behavioral resistance mechanisms may both contribute to the overall insecticide resistance profile of wild house flies, resistance to imidacloprid formulated into food bait was shown to be primarily due to a change in fly behavior, at least for one wild house fly population in southern California (Hubbard and

Gerry 2020). From 2008 to 2015, wild house flies from a southern California dairy developed a modest 3-fold increase in physiological resistance to imidacloprid, a level that is insufficient for these flies to survive exposure to a commonly-utilized commercial fly bait (QuickBayt; Bayer Healthcare LLC, Shawnee Mission, KS) with an imidacloprid concentration that is 3X the dose needed to kill > 95% of these flies in no-choice feeding assays. However, when provided a choice of food bait with or without imidacloprid, these wild flies exhibited a high level of contact-dependent avoidance of the food containing imidacloprid (Hubbard and Gerry 2020). This behavioral resistance provided a high degree of protection from the insecticide in the food bait and supports earlier reports of reduced fly feeding on imidacloprid baits (Mullens et al. 2010). Behavioral resistance is therefore suspected to be a primary mechanism behind imidacloprid resistance in house flies in southern California.

The objective of the current study was to characterize the genetics of behavioral resistance to imidacloprid in a house fly strain that was highly selected for behavioral resistance to imidacloprid presented in food bait and specifically, to identify the house fly chromosome(s) carrying factors conferring behavioral resistance to imidacloprid.

MATERIALS AND METHODS

Chemicals

Imidacloprid (99.50%; CAS: 138261-41-3) and dinotefuran (99.50%; CAS: 165252-80-0) were obtained from Chem Service Inc., West Chester, PA.

Parental house fly strains

Six house fly strains were used as parental strains in this study: five strains exhibiting strong behavioral resistance to imidacloprid (BRS 1-5) (Hubbard and Gerry 2020) and an insecticide susceptible strain (aabys) carrying the 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 (Scott et al. 2014). The BRS 1-5 strains were selected for behavioral resistance to imidacloprid from a wild house fly population collected from a southern California dairy. The selection process is detailed in Hubbard and Gerry (2020). Briefly, selection was achieved using a choice feeding assay with flies starved for 14 h and then subsequently provided a food dish containing sucrose and a second food dish containing sucrose mixed with a very high concentration of imidacloprid (4,000 µg/g sucrose; 3X LC95 for the wild fly population in a no-choice feeding assay). Sucrose mixed with imidacloprid was made by dissolving into acetone the desired concentration of imidacloprid per g sucrose and then applying the acetone-imidacloprid solution to granular sucrose, mixing thoroughly to ensure even dispersal of the insecticide through the sucrose. This mixture then was placed in a fume hood for 24 h to allow the acetone to evaporate. The sucrose only food option was similarly prepared with acetone but without the addition of imidacloprid. Only flies that did not consume the offered sucrose mixed with imidacloprid during the 72-h choice feeding assay period survived to reproduce. Flies were selected in this way every three filial generations for 10 selections resulting in a high degree of behavioral resistance to imidacloprid with no increase in physiological resistance of selected fly lines. Behavioral resistance to

imidacloprid was subsequently maintained in BRS 1-5 strains by exposing flies every four filial generations using the same choice-feeding assay described above. Flies were otherwise reared and maintained under standard rearing conditions (Zhan and Gerry 2018).

Linkage analysis of behavioral resistance to imidacloprid

The F_1 male backcross method of Tsukamoto (1964) was used to determine house fly chromosome(s) that were carrying factors contributing to the selected behavioral resistance in each BRS fly strain (Figure 2.1). Each fly strain selected for behavioral resistance (BRS 1-5) was subjected to the same methodology described below. Reciprocal crosses of a BRS fly strain to the aabys fly strain were performed to give heterozygous F_1 offspring. The F_1 offspring express dominant phenotypes, including normal house fly morphology. Males from F_1 offspring were then backcrossed with aabys females to give backcross (BC) offspring displaying 2^5=32 different phenotypes (chromosome combinations). These BC flies (3-5 d old) were exposed en masse to the choice feeding assay described above and mortality of flies by phenotype was assessed after 72 h. This method allows for determination of the dominant effect of each house fly chromosome containing a recessive morphological marker as crossing over is rare in male house flies (Hamm et al. 2005, Kavi et al. 2014). As no significant chromosomal effect differences were seen between reciprocal crosses in each fly strain, data was combined for each reciprocal cross. For all selected fly strains (BRS 1-5), linkage

analysis indicated that factors conferring behavioral resistance to imidacloprid are located on autosomes 1 and 4 (Table 2.1 and 2.2).

To determine the level of behavioral resistance to imidacloprid inherited by the heterozygous F_1 flies, five replicates of 25 female F_1 offspring from each reciprocal cross were exposed to the choice feeding assay described above. As no differences in survival were noted between reciprocal crosses ($p<0.55$), reciprocal crosses were pooled for further analysis.

Selecting BC fly lines with phenotypes linked to behavioral resistance

Given the same phenotypes were associated with behavioral resistance in all BRS fly strains, a single fly strain (BRS 1) was chosen for further study. The F_1 backcross method was again performed to generate BC flies of each phenotype. The BC flies were separated by phenotype and by sex within 8 h of emergence to prevent mating (Murvoch 1964), with flies expressing a phenotype indicating inheritance of only BRS autosome 1, 4, or 1 and 4 (+abys, aab+s, +ab+s) placed into separate cages supplied with food and water *ad libitum*. At 3-5 d old, flies were starved for 14 h then exposed to the choice feeding assay described previously for a first, purifying selection. Surviving male and female flies of the same phenotype were combined into a single cage to mate, with offspring of these flies again separated by phenotype and sex and exposed at 3-5 d old to the choice feeding assay. Male and female flies of the same phenotype that survived this second purifying selection were combined into a single cage and allowed to mate, establishing three separate BC fly lines each carrying only the BRS fly strain autosomes 1

and/or 4 that are linked to behavioral resistance to imidacloprid; hereafter referred to as fly lines A1, A4 and A1/4, respectively.

Evaluating behavioral resistance to imidacloprid of selected BC fly lines

Evaluation of behavioral resistance to imidacloprid in fly lines A1, A4, and A1/4 follows methodology described previously (Hubbard and Gerry 2020) to quantify the level of resistance, assess the resistance phenotype, and to determine specificity of behavioral resistance to imidacloprid relative to another neonicotinoid insecticide (dinotefuran) that is also commercially available as a component of insecticidal house fly bait (QuikStrike®; Wellmark International, Shaumburg, IL, USA). Dinotefuran has a drastically different chemical structure than imidacloprid, including having a nonaromatic ring, one oxygen capable of forming hydrogen bonds and an asymmetric carbon (Matsuda et al. 2020). This chemical was evaluated in the current study because it is in the same chemical class as imidacloprid and it was commonly used on the dairy farm where the behaviorally resistant flies used in the current study were collected (Hubbard and Gerry 2020).

Degree of behavioral resistance to imidacloprid

To determine the degree of behavioral resistance, 125 flies (3-5 d old) from each fly line and sex were placed into separate cages and exposed to the choice feeding assay described above. An additional 125 flies from each fly line and sex were placed into separate cages and provided the sucrose only food option to control for acetone toxicity

and fly mortality unrelated to the imidacloprid treatment. With <3% fly mortality in control treatments, no mortality corrections were needed. The assay was replicated for each fly line during 5 consecutive filial generations. Mortality differences by sex and strain were evaluated using two-way analysis of variance with a Tukey's post hoc test for separation of means.

Observation of behavioral resistance phenotype

Adult house flies were starved for 14 h prior to being sorted into groups of 25 same sex flies, placed into a Petri dish positioned into the center of a plexiglass observation chamber (50 x 18.25 x 18.5 cm). Flies were provided two weigh dishes placed equidistant from either sidewall of the observation chamber, one containing only sucrose with the other containing sucrose formulated with imidacloprid at the choice feeding assay dose (4,000 µg/g sucrose). A second observation chamber ran concurrently with the treatment positions reversed to mitigate positional effects. Flies were recorded via video camera as they moved throughout the chamber during a two-hour observation window. The assay was replicated 8 times (4 replicates per sex) over two filial generations for each fly line. Analysis of video recordings was completed using open source video analysis software (Friard and Gamba 2016), where the number of times a fly landed on each dish (landing events) and the amount of time each fly spent on the food dish (contact time) were documented. Differences in landing events and contact time between the sucrose only food dish and the sucrose-imidacloprid food dish were analyzed for each fly line using a Wilcoxon matched-pairs test. With no difference between males

and females for number of landing events ($p<0.1682$) or length of contact time ($p<0.0728$) on a particular food dish, data were combined for the sexes within each fly line for remaining analyses.

Specificity of behavioral resistance to imidacloprid

Feeding preference assays were performed for each isolated fly line and for the aabys susceptible fly strain. Flies were exposed to a choice feeding assay to compare fly consumption of sucrose mixed with either imidacloprid or dinotefuran (a related neonicotinoid insecticide). House flies (3-5 d old) were starved overnight (14 h), sorted into groups of 25 same sex individuals and placed into assay chambers. Each assay chamber was provisioned with water, and two soufflé cups, one containing sucrose treated with imidacloprid (4,000 µg/g sucrose), and the second containing sucrose treated with dinotefuran at the same concentration (4,000 µg/g sucrose). Both insecticides were mixed with sucrose following the same methods as described previously except that a small amount of either red or blue food grade coloring solution (McCormick & Co., Inc. Hunt Valley, MD) also was added to separate the treatments visually. Two assay chambers were utilized concurrently with the treatment positions and color assigned to each treatment reversed in order to mitigate both positional and treatment color effects. Flies were allowed 24 h to feed after which dead flies were sorted via abdomen color (blue, red, or purple [fed on both treatments]) and a feeding preference index (PI) was calculated for the fly line/strain (Bantel and Tessier 2016) using the formula ($P_{D/I} = N_D + 0.5N_P)/ (N_D + N_I + N_P)$, where $P_{D/I}$ is the preference of flies to feed on sucrose with

dinotefuran over sucrose with imidacloprid and N = the number of individuals feeding on either sucrose with dinotefuran (N_D), sucrose with imidacloprid (N_I), or on both treatments as indicated by a purple abdomen color (N_P). $P_{D/I} = 0.5$ indicates no fly preference for sucrose with either insecticide, while $P_{D/I} > 0.5$ indicates preference for sucrose with dinotefuran, and $P_{D/I} < 0.5$ indicates a preference for sucrose with imidacloprid. For each fly line/strain a total of 10 replicates were performed for each sex over three filial generations. For each fly line/strain, differences in the PI between sex or coloring solution were evaluated using a Kruskal-Wallis test. With no significant difference for any fly line/strain between sex ($p>0.2090$) or coloring solution ($p>0.2383$), all replicates for each fly line/strain were combined for analysis using one sample t-test to determine a feeding preference for either insecticide ($P_{D/I} \neq 0.5$). Differences in feeding preference between fly line/strain were determined via Kruskal-Wallis test with Dunn's multiple comparisons post-hoc test.

RESULTS

Linkage analysis of behavioral resistance to imidacloprid

Autosomal linkage analysis indicated that behavioral resistance to imidacloprid is linked to factors on autosomes 1 and 4 in each BRS 1-5 fly strain (Table 2.1-2.5). With no differences between reciprocal crosses for any fly strain, reciprocal cross data was combined for linkage analysis. Survival of each BC phenotype in the choice feeding assay demonstrates agreement with the linkage analysis with percent survival of BC flies generally as follows: flies with BRS autosomes 1 and 4 > BRS autosome 4 > BRS autosome 1 > neither BRS autosome 1 or 4 (Figure 2.2).

Evaluating behavioral resistance to imidacloprid of selected fly lines

Female F_1 offspring exposed to imidacloprid averaged 22.7 ± 3.7% survival across all F_1 reciprocal crosses in comparison to an average of 1.6 ± 0.9% for the susceptible (aabys) parent strain and 96.0 ± 0.7% for the behaviorally resistant (BRS 1-5) parent strain (Figure 2.3). Survival data reported for BRS strain flies is from Hubbard and Gerry (2020) and is reproduced here for comparison.

Survival of flies carrying resistance factors on autosome 1 (A1) differed significantly by sex ($p< 0.05$) with female survival (64.2 ± 4.2%) nearly three times that of male survival (23.8 ± 4.9%). Survival was not different by sex for flies carrying resistance factors on autosome 4 (A4) or on both autosomes 1 and 4 (A1/4) with percent survival for A4 males and females 43.4 ± 4.1% and 56.0 ± 6.6%, respectively and for A 1/4 males and females of 66.4 ± 11.4% and 84.2 ± 8.6%, respectively (Figure 2.4).

Observational analysis of behavioral resistance phenotype

For all three selected BC fly lines, the number of landing events on food dishes with sucrose or sucrose-imidacloprid was not significantly different ($n=8$; $z <1.26$; $p>0.23$) (Figure 2.5a). However, fly contact time with the sucrose-imidacloprid food dish was significantly lower than for the sucrose only food dish for all three fly lines ($n=8$; $z<2.24$; $p<0.02$) (Figure 2.5b).

Specificity of behavioral resistance to imidacloprid

The aabys parent strain flies exhibited no statistical preference for feeding on sucrose with either dinotefuran or imidacloprid ($P_{D/I}$ = 0.51, p=0.3286), whereas all selected BC fly lines had a significant preference (p<0.0001) for feeding on sucrose with dinotefuran over sucrose with imidacloprid with $P_{D/I}$ = 0.73, 0.67, and 0.71 for A1, A4, and A1/4, respectively (Figure 2.6). The feeding preference for all BC fly lines was not different from the BRS1 (resistant) parent strain (p>0.99) (data for BRS1 from Hubbard and Gerry 2020), while the feeding preference for all BC fly lines and the BRS1 parent strain were significantly different (p<0.006) from the aabys (susceptible) parent strain.

DISCUSSION

Behavioral resistance by insects to food baits containing insecticides has been documented in the German cockroach (*Blattella germanica* (L.) (Silverman and Bieman 1993, Wang et al. 2004, Wada-Katsumata et al. 2013) and in the house fly (Freeman and Pinniger 1992, Learmount et al. 1996, Darbro and Mullens 2004, Gerry and Zhang 2009, Mullens et al. 2010, Hubbard and Gerry 2020), but the underlying mechanisms that lead to expression of behavioral resistance can be difficult to determine due to challenges associated with studying these behavioral traits (Sparks et al. 1989, Zalucki and Furlong 2017).

Behavioral resistance in house flies is genetically inherited and is expressed as a contact-dependent avoidance behavior that reduces the length of time that flies are in contact with and feeding on the insecticide imidacloprid added to a sucrose food bait (Hubbard and Gerry 2020). Resistant house flies will readily feed on sucrose food bait

when imidacloprid insecticide is not present. The German cockroach can similarly inherit contact-dependent aversion to food bait containing insecticide. However, the aversion response by the German cockroach is elicited by the phagostimulant (glucose) rather than the insecticide in the food bait (Silverman and Bieman 1993). In resistant German cockroaches, a gain-of-function mutation resulted in glucose stimulating both sugar and bitter gustatory receptor neurons in the peripheral gustatory system, with resistant cockroaches interpreting glucose as both a phagostimulant and a deterrent (Wada-Katsumata et al. 2013).

The current study is the first to identify the chromosomal location associated with any behavioral trait in house flies. Previously, linkage analysis has been used to determine genetic locations associated only with physiological insecticide resistance in house flies (Zhang 1997, Shono et al. 2004, Tian 2011, Kavi et al. 2014, Feng et al. 2018), though chromosomal or genomic locations have been determined for factors conferring behavioral traits in other animal systems including *Drosophila melanogaster* Meigen (Hirsch 1959, Hirsch and Erlenmeyer Kimling 1962, Greenspan 2004, Sisodia and Singh 2005), *B. germanica* L. (Ross and Silverman 1995), *Culex pipiens* L. and *Cx. quinquefasciatus* Say (Kilpatrick et al. 2007), *Anopheles arabiensis* Giles (Main et al. 2016), *Lasioglossum albipes* (Fabricius) (Kocher et al. 2018) and *Homo sapiens* L. (Carhuatanta et al. 2014).

Behavioral resistance in the selected house fly strains was neither fully dominant nor recessive (Tsukamoto 1983) as indicated by an intermediate level of behavioral resistance in the F1 flies relative to the susceptible (aabys) and resistant (BRS) parent fly

strains. However, the specific degree of dominance (Stone 1968) for behavioral resistance could not be calculated since a single high dose of insecticide was used in these studies, but also because LC_{50} values could not be calculated for the BRS fly strains using a choice feeding assay due to the high degree of behavioral resistance in these fly strains (Hubbard and Gerry 2020). The similarity of phenotypic expression (all 32 phenotypes were expressed) between male and female BC flies from reciprocal crosses supports that the male determining factor in each BRS fly strain is present on the Y chromosome, as previously documented for flies from southern California (Hamm et al. 2005, 2015; Meisel et al. 2016).

In the current study, house fly behavioral resistance to imidacloprid was linked to factors on autosomes 1 and 4. Physiological resistance mechanisms in the house fly also have been linked to autosomes 1 and 4, including factors on autosome 1 that confer physiological resistance to the organochlorine lindane (Georghiou 1965), the organophosphate fenitrothion (Rupes and Pintervova 1975), and pyrethroids (Liu and Scott 1995) and factors on autosome 4 that confer physiological resistance to the phenylpyrazole fipronil (Wen and Scott 1999), to cyclodienes (Ffrench-Constant et al. 1993) and to imidacloprid (Kavi et al. 2014). While imidacloprid resistance in the house fly has been linked to autosome 4 for factors conferring both behavioral resistance (current study) and physiological resistance (Kavi et al. 2014), these resistance factors are likely unrelated since the BRS fly strains used in the current study were specifically selected for increased behavioral resistance to imidacloprid and these fly strains did not

have an increase in physiological resistance to imidacloprid as a result of the selection process.

An additive interaction between resistance factors (Hardstone and Scott 2010) located on chromosome 1 & 4 was observed with flies of both sexes from fly line A1/4 (containing resistance factors on both genes) having a higher survival rate than flies from lines A1 or A4 when flies were exposed to a choice feeding assay. The A1 male flies exhibited the lowest survival (23.8%) in the choice feeding assay, with survival being significantly higher for A1/4 males (66.4%) and females from all fly lines (56-84.2%). All fly line and sex combinations had lower survival relative to their BRS 1 parental fly strain (Hubbard and Gerry 2020), suggesting there may be trans regulation of resistance factors or the presence of minor resistance factors on other autosomes not inherited by the selected fly lines.

Behavioral observation assays demonstrated that the behavioral resistance phenotype expressed by all selected fly lines (A1, A4, A1/4) was similar to that of the BRS 1 fly strain they were selected from as reported by Hubbard and Gerry (2020). The frequency of flies landing on sucrose alone was not different from the frequency of flies landing on sucrose mixed with imidacloprid, indicating flies express no aversion or avoidance response prior to fly contact with imidacloprid. All flies in the selected fly lines spent significantly less time in contact with the dish containing sucrose mixed with imidacloprid relative to the dish with sucrose alone. In addition all fly lines preferred to feed on sucrose mixed with the neonicotinoid dinotefuran over sucrose with imidacloprid, likely due to the specific detection of and aversion to imidacloprid, while dinotefuran is

either not detected or does not elicit an aversion response by these flies (Hubbard and Gerry 2020). Dinotefuran has a very different chemical structure relative to imidacloprid (Matsuda et al. 2020), perhaps resulting in different binding sites on the nicotinic acetylcholine receptor for these two chemicals (Kiriyama et al. 2003).

Although selected resistance factors on both autosome 1 and 4 resulted in a similar behavioral phenotype (contact-dependent avoidance of imidacloprid), it is likely that there are at least two factors contributing to the imidacloprid detection and avoidance. While it is currently unknown what genes/genetic elements associated with either autosome 1 or 4 may be responsible for the detection of imidacloprid and the resulting behavioral resistance response, it has been hypothesized that changes to the chemosensory system of the house fly may be responsible. With the expansive chemoreceptor repertoire of the house fly including 87+ odorant binding proteins (OBPs), 85 genes encoding 86 odorant receptors, 79 genes encoding 103 gustatory receptors, and 110 ionotropic receptors (Scott et al. 2014), mutations in genes controlling chemosensory response may have emerged that elicit or enhance an aversive (non-feeding) response to imidacloprid in behaviorally resistant fly lines. Prior work with the fruit fly (*D. melanogaster*) and with the German cockroach has shown that genetic mutations can lead to changes to chemoreceptors resulting in altered insect behavior including food aversion and suppression of food consumption (Wada-Katsumata et al. 2014, French et al. 2015, Chen et al. 2019). The current study extends this body of information to show that imidacloprid aversion by house flies is also under genetic control and identifies the

autosomes which carry resistance factors in the house fly associated with the aversion response.

This study provides a foundation to study the genetic control of behavioral resistance to insecticides in the house fly. Future studies should identify the genetic loci associated with behavioral resistance to imidacloprid on autosomes 1 and 4, and determine the specific molecular mechanisms conferring house fly behavioral resistance. A pooled sequencing approach could be utilized to examine genetic differences among susceptible and behaviorally resistant fly lines as described by Kofler and Schlötterer (2014). If a small number of genetic loci are identified to be causative, molecular methods to rapidly screen house flies (and perhaps other insects) for behavioral resistance to imidacloprid could be developed.

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TABLES AND FIGURES

Table 2.1: Autosomal linkage analysis for behavioral resistance to imidacloprid in the BRS 1 house fly strain.

Autosome (s)	Effect	Mean square	F Value
5	28.54	50.91	0.31
4	-320.16	6406.41	**39.12***
4+5	-28.19	49.66	0.30
3	41.29	106.57	0.65
3+5	-63.67	253.37	1.55
3+4	5.80	2.10	0.01
3+4+5	-4.08	1.04	0.01
2	53.18	176.73	1.08
2+5	58.45	213.54	1.30
2+4	23.30	33.94	0.21
2+4+5	-34.19	73.05	0.45
2+3	109.35	747.37	4.56
2+3+5	64.29	258.32	1.58
2+3+4	48.01	144.03	0.88
2+3+4+5	-0.73	0.03	0.00
1	-209.18	2734.89	**16.70***
1+5	53.02	175.68	1.07
1+4	16.00	15.99	0.10
1+4+5	-69.04	297.89	1.82
1+3	44.98	126.45	0.77
1+3+5	26.02	42.31	0.77
1+3+4	-23.38	34.17	0.21
1+3+4+5	-5.53	1.91	0.01
1+2	-2.01	0.25	0.00
1+2+5	-61.98	240.07	1.47
1+2+4	-11.84	8.76	0.05
1+2+4+5	-77.94	379.66	2.32
1+2+3	-78.77	387.82	2.37
1+2+3+5	3.27	0.67	0.00
1+2+3+4	0.00	0.00	0.00
1+2+3+4+5	0.73	0.03	0.00
Error	5240.78		

*****Bold numbers and asterisk indicate statistical significance ($p<0.01$).

Table 2.2: Autosomal linkage analysis for behavioral resistance to imidacloprid in the BRS 2 house fly strain.

Phenotypes	Effect	Mean square	F-Value
5	49.65	154.08	0.67
4	-274.16	4697.71	**20.37***
4+5	-89.79	503.89	2.18
3	-50.42	158.87	0.69
3+5	-128.29	1028.68	4.46
3+4	48.54	147.28	0.64
3+4+5	46.67	136.10	0.59
2	-65.62	269.10	1.17
2+5	59.28	219.61	0.95
2+4	-18.86	22.23	0.10
2+4+5	-17.71	19.61	0.09
2+3	-14.79	13.67	0.06
2+3+5	-8.00	4.00	0.02
2+3+4	15.24	14.51	0.06
2+3+3+5	-16.82	17.68	0.08
1	-167.92	1762.29	**7.64***
1+5	77.18	372.34	1.61
1+4	67.21	282.29	1.22
1+4+5	-73.92	341.47	1.48
1+3	-16.90	17.85	0.08
1+3+5	31.73	62.91	0.08
1+3+4	55.64	193.51	0.84
1+3+4+5	13.03	10.61	0.05
1+2	72.32	326.91	1.42
1+2+5	64.42	259.34	1.12
1+2+4	-24.72	38.18	0.17
1+2+4+5	-69.11	298.50	1.29
1+2+3	-27.37	46.81	0.20
1+2+3+5	-4.91	1.51	0.01
1+2+3+4	0.00	0.00	0.00
1+2+3+4+5	16.82	17.68	0.08
Error	7380.54		

*****Bold numbers and asterisk indicate statistical significance ($p<0.01$).

Table 2.3: Autosomal linkage analysis for behavioral resistance to imidacloprid in the BRS 3 house fly strain.

Phenotypes	Effect	Mean square	F-Value
5	-15.47	14.95	0.23
4	-127.77	1020.26	**15.70***
4+5	-28.68	51.42	0.79
3	9.65	5.82	0.09
3+5	28.73	51.59	0.79
3+4	22.87	32.70	0.50
3+4+5	-17.77	19.73	0.30
2	-34.90	76.12	1.17
2+5	-39.40	97.03	1.49
2+4	25.42	40.40	0.62
2+4+5	28.34	50.20	0.77
2+3	36.19	81.85	1.26
2+3+5	17.36	18.84	0.29
2+3+4	1.50	0.14	0.00
2+3+3+5	-6.42	2.57	0.04
1	-126.05	993.03	**15.28***
1+5	25.02	39.14	0.60
1+4	74.86	350.25	5.39
1+4+5	5.63	1.98	0.03
1+3	-4.63	1.34	0.02
1+3+5	79.39	393.94	0.02
1+3+4	32.35	65.42	1.01
1+3+4+5	-30.60	58.52	0.90
1+2	60.76	230.74	3.55
1+2+5	-2.56	0.41	0.01
1+2+4	45.35	128.54	1.98
1+2+4+5	36.99	85.51	1.32
1+2+3	0.12	0.00	0.00
1+2+3+5	13.04	10.63	0.16
1+2+3+4	0.00	0.00	0.00
1+2+3+4+5	6.42	2.57	0.04
Error	2079.39		

*****Bold numbers and asterisk indicate statistical significance ($p<0.01$).

Table 2.4: Autosomal linkage analysis for behavioral resistance to imidacloprid in the BRS 4 house fly strain.

Phenotypes	Effect	Mean Square	F-Value
5	141.79	1256.48	5.52
4	-392.11	9609.55	**42.21***
4+5	-83.89	439.85	1.93
3	-58.77	215.90	0.95
3+5	-56.46	199.25	0.88
3+4	46.70	136.28	0.60
3+4+5	4.92	1.52	0.01
2	-100.98	637.33	2.80
2+5	-37.34	87.16	0.38
2+4	73.87	341.01	1.50
2+4+5	93.59	547.45	2.40
2+3	-101.57	644.76	2.83
2+3+5	3.30	0.68	0.00
2+3+4	51.86	168.10	0.74
2+3+3+5	-40.28	101.42	0.45
1	-234.47	3435.95	**15.09***
1+5	-1.65	0.17	0.00
1+4	-58.76	215.79	0.95
1+4+5	-8.42	4.43	0.02
1+3	-49.21	151.32	0.66
1+3+5	39.24	96.24	0.66
1+3+4	90.05	506.86	2.23
1+3+4+5	-75.64	357.60	1.57
1+2	-40.01	100.04	0.44
1+2+5	-30.66	58.74	0.26
1+2+4	85.90	461.17	2.03
1+2+4+5	7.93	3.93	0.02
1+2+3	-29.06	52.78	0.23
1+2+3+5	-73.13	334.29	1.47
1+2+3+4	0.00	0.00	0.00
1+2+3+4+5	40.28	101.42	0.45
Error	7285.13		

*****Bold numbers and asterisk indicate statistical significance ($p<0.01$).

Table 2.5: Autosomal linkage analysis for behavioral resistance to imidacloprid in the BRS 5 house fly strain.

Phenotypes	Effect	Mean Square	F-Value
5	19.40	23.52	0.10
4	-404.33	10217.65	**45.49***
4+5	-5.49	1.88	0.01
3	-34.82	75.76	0.34
3+5	30.16	56.87	0.25
3+4	15.37	14.76	0.07
3+4+5	-43.19	116.59	0.52
2	-79.92	399.15	1.78
2+5	68.48	293.10	1.30
2+4	-16.35	16.71	0.07
2+4+5	-57.45	206.29	0.92
2+3	32.02	64.08	0.29
2+3+5	64.34	258.70	1.15
2+3+4	17.02	18.11	0.08
2+3+3+5	12.37	9.56	0.04
1	-212.01	2809.37	**12.51***
1+5	47.97	143.84	0.64
1+4	61.87	239.26	1.07
1+4+5	-33.81	71.45	0.32
1+3	21.18	28.03	0.12
1+3+5	3.41	0.73	0.12
1+3+4	-29.80	55.50	0.25
1+3+4+5	-18.45	21.29	0.09
1+2	86.86	471.51	2.10
1+2+5	-2.80	0.49	0.00
1+2+4	-18.66	21.77	0.10
1+2+4+5	-36.30	82.36	0.37
1+2+3	56.52	199.67	0.89
1+2+3+5	36.67	84.03	0.37
1+2+3+4	0.00	0.00	0.00
1+2+3+4+5	-12.37	9.56	0.04
Error	7187.60		

*****Bold numbers and asterisk indicate statistical significance ($p<0.01$).

Figure 2.1: Pictorial representation of the modified F_1 male backcross method of Tsukamoto (1964) for each behaviorally resistant (BRS) fly strain crossed with the insecticide susceptible (aabys) fly strain to determine which house fly chromosomes carry factors in the BRS fly strain conferring behavioral resistance to the insecticide imidacloprid. (Created with BioRender.com)

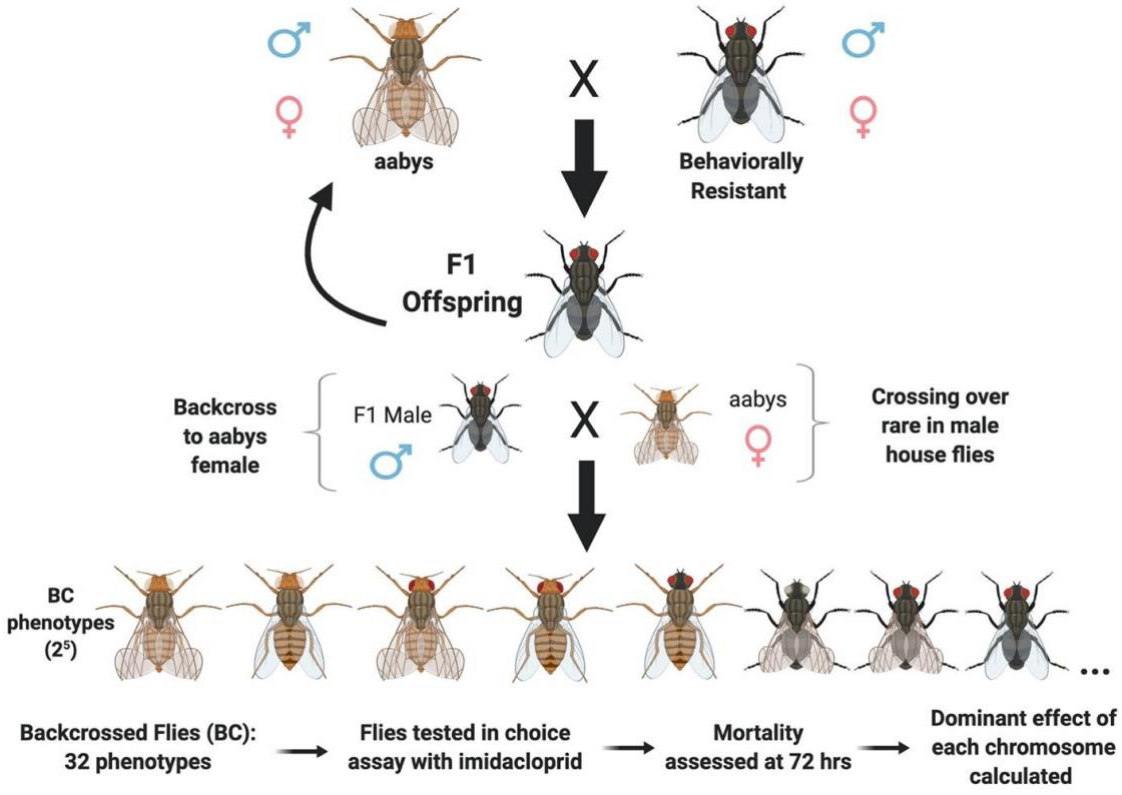


Figure 2.2: Mean percent survival ± SE of backcross flies (5 BRS fly strains x 2 reciprocal crosses) by phenotype (chromosomal combination) following a 72 h choice feeding assay with flies provided both a food dish containing sucrose alone and a second food dish containing sucrose with a high concentration of imidacloprid (4,000 µg/g sucrose). Choice feeding assay was performed to determine the "dominant effect" of each house fly autosome (linkage analysis).

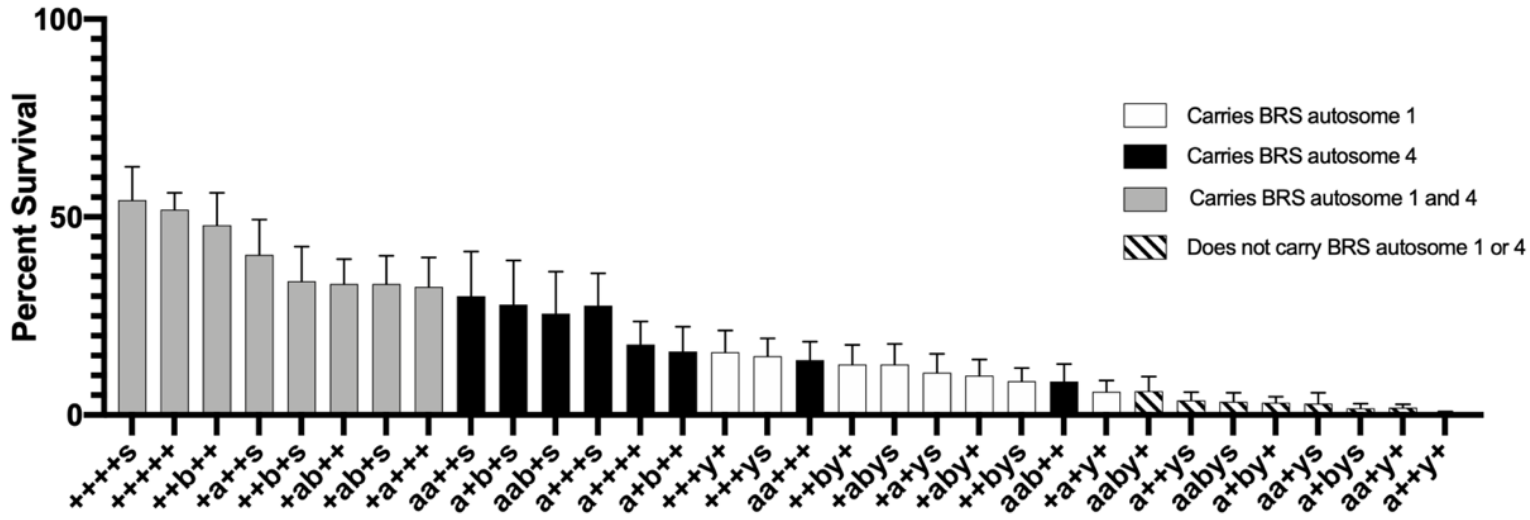


Figure 2.3: Mean percent survival ± SE of female aabys (susceptible), BRS 1-5 (behaviorally resistant), and each F_1 cross of aabys x BRS strain flies following a 72 h choice feeding assay with flies provided both a food dish containing sucrose alone and a second food dish containing sucrose mixed with a high concentration of imidacloprid (4,000 µg/g sucrose). Data for BRS 1-5 survival from Hubbard and Gerry (2020) and shown here for comparison.

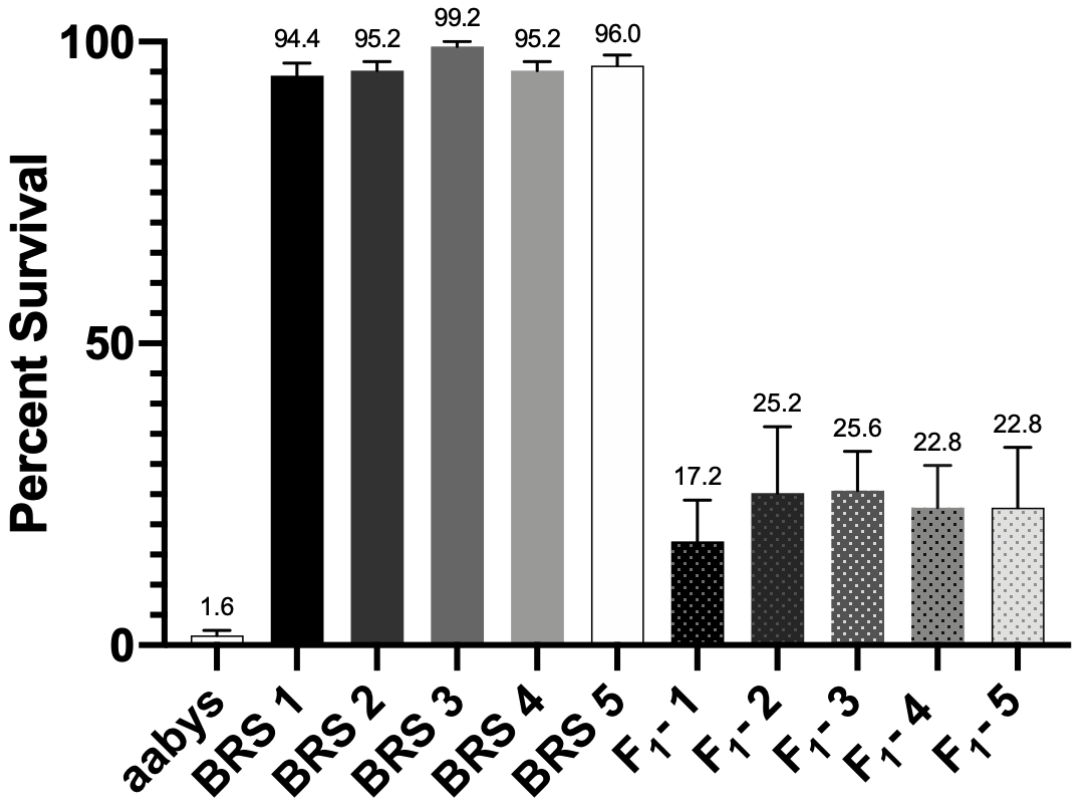


Figure 2.4: Mean percent survival ± SE of house flies carrying autosomes shown by linkage analysis to be associated with behavioral resistance when flies are subjected to a choice feeding assay with paired food dishes containing either sucrose or sucrose mixed with imidacloprid at 4,000 µg/g sucrose. Different letters indicate significance ($p<0.05$).

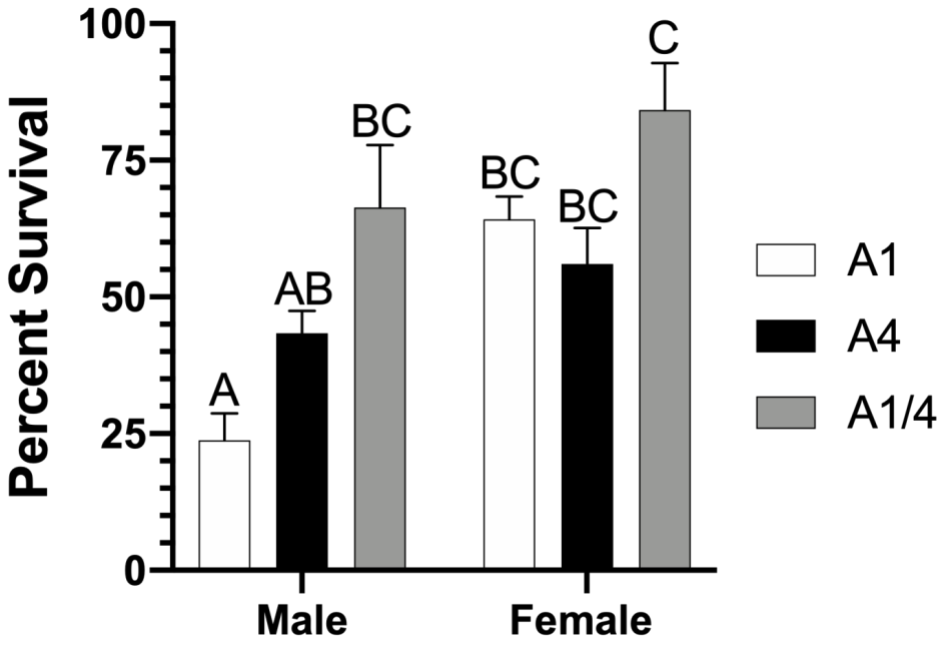


Figure 2.5: Mean ± SE landing events (a) and contact time (b) on paired food dishes containing either sucrose alone or sucrose with imidacloprid (4,000 µg/g sucrose) over a 2-h observation window. Differences between food dish treatments within fly lines were determined by Wilcoxon matched-pairs test (ns = not significant, * = $p<0.05$, *** = $p<0.001$).

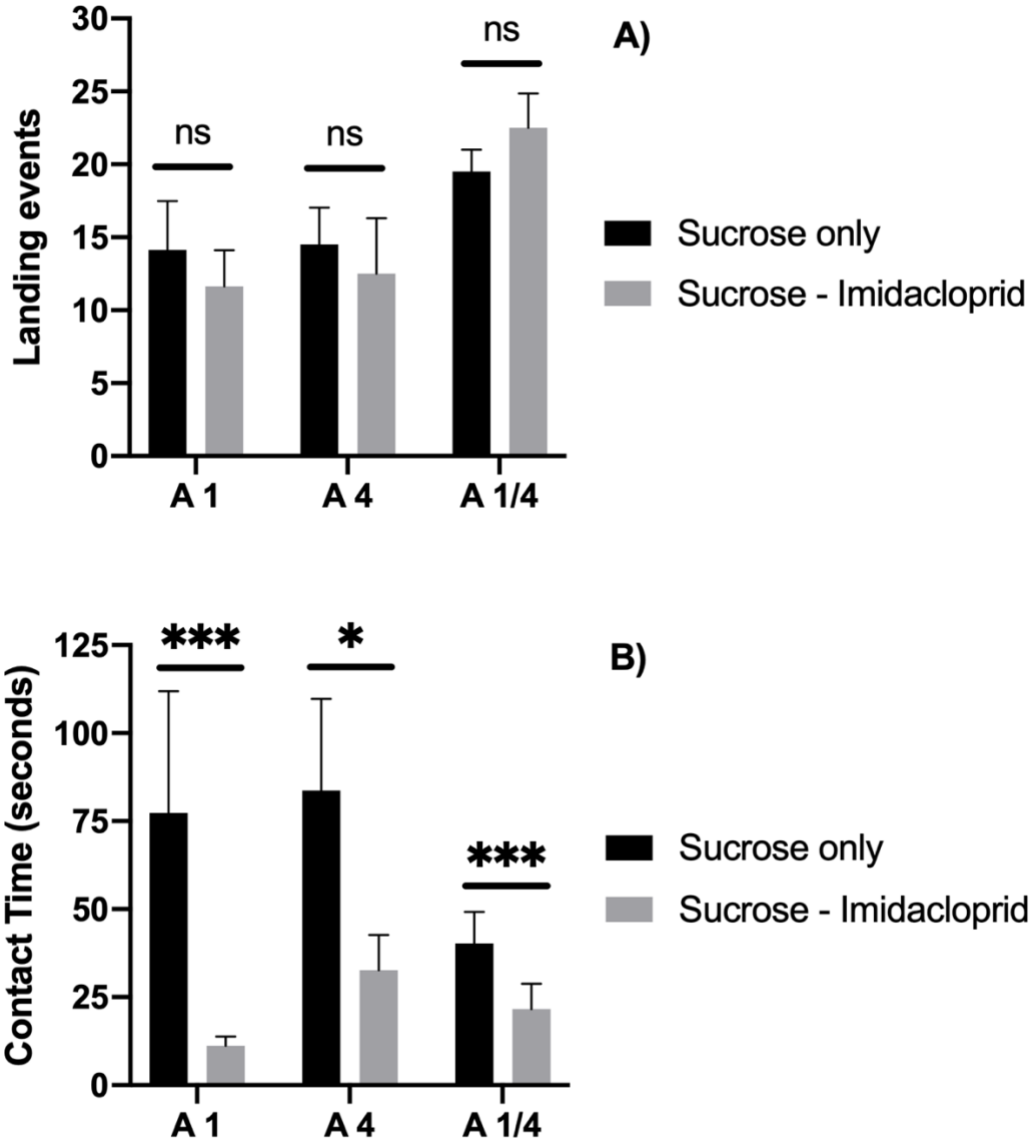
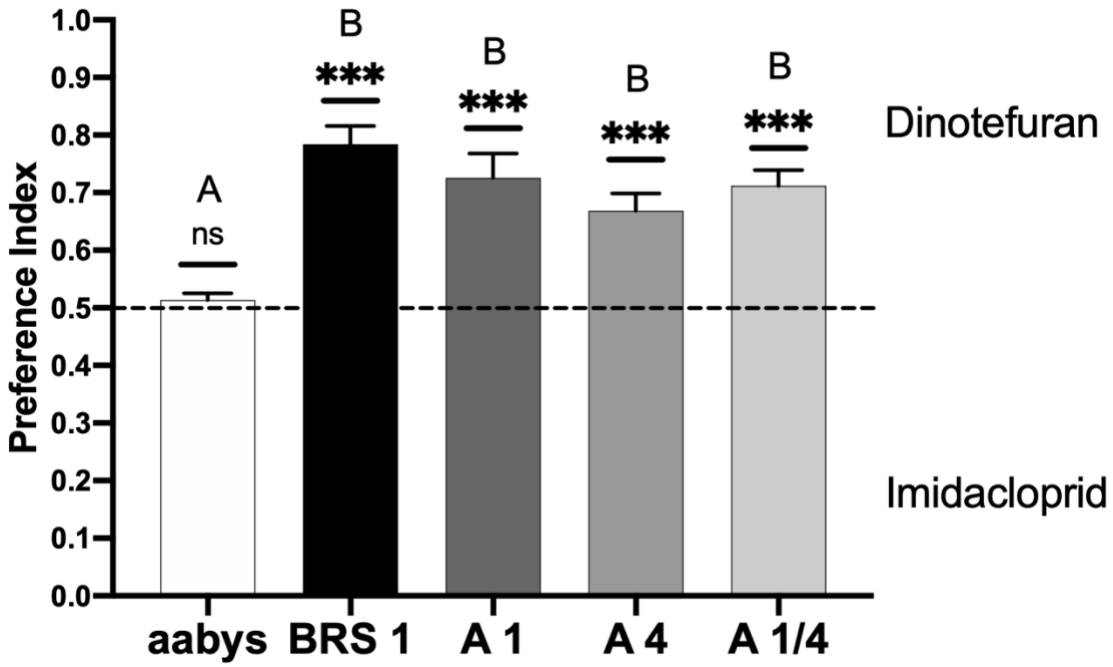


Figure 2.6: Fly feeding preference index (PI) with fly lines/strains provided a choice to feed on either sucrose with dinotefuran or sucrose with imidacloprid at the same concentration of 4,000 µg/g sucrose. For comparison, data for parental fly strain BRS 1 is also shown (from Hubbard and Gerry 2020) in this figure. A significant feeding preference for any single fly line/strain is indicated by *** ($p<0.001$) following one-sample t-test for PI ≠ 0.5. Different letters above each column indicates significant difference in feeding preference among fly lines/strains.



CHAPTER 3

Attempting to unravel the molecular complexities of behavioral resistance to imidacloprid in the house fly (*Musca domestica* L.)

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ABSTRACT

Insecticide resistance in pest populations is an increasing problem in urban and rural settings caused by the over-application of insecticides, a lack of rotation among chemical classes, and aspects of pest biology including rapid generation time that allow for rapid selection of resistant populations. The degree of insecticide resistance in selected populations may be determined by both physiological and behavioral resistance. Genetic mechanisms that determine the expression of behavioral resistance are relatively understudied. The common house fly (*Musca domestica* L.) is a synanthropic fly species with a cosmopolitan distribution inhabiting rural and urban environments that is implicated in the transmission of over 200 different human and animal pathogens. Behavioral resistance by house flies to the neonicotinoid insecticide imidacloprid, formulated into a fly bait widely used against house flies, is reported to be associated with factors on house fly chromosomes 1 and 4.

In the current study, the molecular mechanisms conferring behavioral resistance to imidacloprid were investigated using a pooled sequencing approach to compare genetic variation between an imidacloprid-susceptible house fly strain and five house fly strains that were highly selected in the laboratory for expression of behavioral resistance to imidacloprid. While 47 genes were identified to have significant differences in SNP frequencies differences between the susceptible and resistant populations, these genes either had an unknown function or a reported function that is not expected to alter expression of behavioral resistance to imidacloprid.

Additional fundamental and applied research should be conducted to understand further both the complex phenotypic and genotypic nature of behavioral resistance to imidacloprid.

INTRODUCTION

The house fly (*Musca domestica* L.) is a synanthropic fly species with a cosmopolitan distribution (West 1951, McKie 2017) inhabiting rural and urban environments. It has adapted to living in almost every environment and is known worldwide as a serious nuisance pest species and a potential mechanical vector of nearly 200 human and animal pathogens (Nayduch and Burrus, 2017). The production of large numbers of house flies can result in litigation against animal producers or urban waste facilities resulting in economic loss or forfeiture of operation (Thomas and Skoda 1993).

When house fly populations exceed acceptable levels, management is often conducted almost exclusively using insecticides due to the low cost of insecticides, ease of application, rapid action, and perceived effectiveness (Geden and Hogsette 2001, Gerry 2020). Due to overuse of insecticides for fly control, house flies have developed resistance to all major insecticide classes (Keiding 1999, Darbro and Mullens 2004, Kaufman et al. 2006, Scott et al. 2013, Murillo et al. 2015, Freeman et al. 2019) and resistance is widespread in field populations worldwide (Keiding 1975, Keiding 1999).

Insecticide resistance in the house fly has long been documented to occur through well-characterized physiological resistance mechanisms such as structural alteration at insecticide binding sites, which impairs the insecticide from binding to the target site and through the increased expression of detoxifying enzymes (Lie and Scott, 1997, Rinkevich et al. 2006, Ma et al. 2019). In recent years though, there is mounting evidence that insecticide resistance in the house fly can result from inherited changes to house fly behaviors, which reduce fly contact with or consumption of insecticidal food baits (Gerry

and Zhang 2009, Wasik and Gerry 2010, Seraydar and Kaufman 2015, Hubbard and Gerry 2020, Hubbard and Gerry 2021).

Neonicotinoids are currently the most widely used insecticidal class across all of agriculture (Sparks and Nauen 2015). This insecticide class binds competitively and irreversibly to the nicotinic acetylcholine receptor, leading to a paralysis of the insect (Jeschke and Nauen 2005). The neonicotinoid imidacloprid has been widely utilized in granular fly baits following its licensure in late 2002 (U.S. Environmental Protection Agency 2002). House fly resistance to imidacloprid was documented within the first few years following widespread use of the commercially available fly bait. Resistance to imidacloprid was demonstrated to be caused by both physiological (Kaufman et al. 2006) and behavioral resistance mechanisms (Gerry and Zhang 2009).

To support an insecticide resistance management plan, the mutations responsible for the resistance need to be identified. Reid et al. (2019) determined that physiological resistance to imidacloprid was linked to the overexpression of a microsomal glutathione S-transferase gene and an unknown trans-regulatory gene, which results in overexpression of a galactosyltransferase-like gene on chromosome 3 and 4, respectively. Recently, Hubbard and Gerry (2020) experimentally selected a population of field-collected house flies to exhibit a high level of behavioral resistance to imidacloprid, and subsequently determined behavioral resistance to imidacloprid was contact-dependent and specific to imidacloprid. Behavioral resistance was then linked to factors on autosomes 1 and 4 in the house fly utilizing an autosomal linkage analysis (Hubbard and

Gerry 2021). Still, to date, the molecular mechanisms conferring behavioral resistance to imidacloprid have yet to be identified.

With advancement in sequencing technology (Miller et al. 2007) and the successful sequencing and annotation of the house fly genome (Scott et al. 2014), the examination of genetic drivers of complex traits can be examined through genome-wide analyses (Kofler et al. 2011). In experiments that explore the selection of a phenotypic trait from a polymorphic starting population (i.e., selecting for behavioral resistance from a field-collected fly colony), the main challenge is to distinguish between selected and neutral variants (Schlötterer et al. 2015). This can be achieved through whole-genome sequencing of a large number of individuals from selected and non-selected populations, however such an analysis is still cost-prohibitive. Recently, researchers have begun utilizing a modified sequencing method pooling the DNA of multiple individuals from a population of interest followed by sequencing the pooled DNA (pool-seq). This method, when combined with lab based experimental evolution was coined as "evolve and resequence" by Turner et al. (2011). This technique was reviewed by Schlötterer et al. 2014 and has been proven to be cost-effective while yielding accurate genome-wide allele frequency estimates (Rellstab et al. 2013).

The present study's goal was to identify the genetic basis of house fly behavioral resistance to imidacloprid using a genome-wide pooled-sequencing approach to identify altered loci that may be responsible for our selected phenotype (Figure 3.1).

MATERIALS AND METHODS

House fly strains

Six house fly strains were used in the current study. WT is a fly strain established in 2015 following the collection of approximately 500 mixed-sex adult house flies by sweep net from multiple locations on a diary near San Jacinto, California. The WT house fly strain exhibits a moderate level of physiological resistance to the neonicotinoid imidacloprid. While behavioral resistance to imidacloprid was documented in this fly strain, fly survival in choice-bioassays was variable, indicating that not all individuals in the population of flies collected exhibited the behavioral resistance phenotype. Five fly strains (BRS 1-5) were independently selected for behavioral resistance to imidacloprid from the WT strain, with all selected fly strains ultimately exhibiting a high level of behavioral resistance as demonstrated by >90% survival of flies exposed to a choice feeding assay containing sucrose alone and with a high concentration of technical grade imidacloprid (4,000 µg/g sucrose: 3X LC95 for the wild fly population in a no-choice feeding assay) (Hubbard and Gerry 2020).

Factors conferring behavioral resistance to imidacloprid were subsequently identified by autosomal linkage analysis to be on house fly autosomes 1 and 4 in all five behaviorally resistant fly strains (Hubbard and Gerry 2021). All fly strains carry the male determining factor on the Y chromosome, which facilitated resistance analysis as autosomes did not carry sex-determining factors.

Selection of flies for sequencing

Adult flies within 8 h of eclosion, and therefore unmated (Murvosh et al. 1964), were aspirated from their cage, chilled briefly at -20°C, sorted by sex on a chill table, and ~400 female flies were placed into two strain-specific cages (~200 each) provisioned with food and water for 3-5 d to mature.

After reaching maturity, flies were starved for 14 hours, after which one fly cage from each fly strain was exposed to a choice feeding assay in mass. Flies were provided 3 g of sucrose treated with and without imidacloprid at a concentration of 4,000 µg/g as previously described in Hubbard and Gerry 2020. In the second fly cage, flies were provisioned with sucrose alone to serve as a negative control. Sucrose formulated with imidacloprid was made by dissolving into acetone 4,000 µg/g of imidacloprid (CAS: 138261-41-3, Chem Service Inc., West Chester, PA), then applying the acetone-imidacloprid solution to granular sucrose. The mixture was then thoroughly mixed to ensure even dispersal of the insecticide through the sucrose and then placing the mixture in a fume hood for 24 h to allow the acetone to evaporate. The mixture was then thoroughly homogenized before removing 3g of the sucrose-imidacloprid mixture to place into each soufflé cup. The sucrose alone was similarly prepared with acetone but without imidacloprid. The sucrose alone option was prepared this way to confirm acetone was not causing fly mortality. No mortality was observed in fly cages provided sucrose alone and were subsequently disposed of.

Flies were exposed to the choice assay for 72 hours before removing flies from cages. Dead flies from the WT choice assay (phenotypically susceptible) were removed

from the cage floor and placed directly into a sterile 50 mL falcon tube. Surviving flies from each behaviorally resistant fly strain (phenotypically resistant) were aspirated from their cages, chilled briefly at -20 °C, then placed into sterile 50 mL falcon tubes. All flies were transported back to the lab, where they were placed and held at -80 °C before DNA extraction.

DNA-Sequencing

Heads (50) were removed from female flies using a sterile #10 scalpel (Medline, SKU: STMDS15210) and pooled for each fly strain (WT, BRS 1-5). Pooled heads were homogenized using a motorized grinder, and DNA was extracted using Zymo-Spin IIC-XLR columns (Zymo Research, Irvine, CA) following the Quick-DNA Microprep Kit instruction manual. DNA purity and concentration were confirmed using a Nanodrop 2000 (ThermoFisher Scientific) before being transported on ice to the UCR Genomics Sequencing Core for library preparation. One library per fly strain (six in total) were prepared using the NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina following the manufacturer's instructions. The final libraries were assessed by Agilent Bioanalyzer 2100 to determine library quantity and fragment size distribution before sequencing.

Following library preparation, samples were shipped on dry ice to Novogene Corporation Inc, Sacramento, CA for genome sequencing. Libraries were pooled at equal molarity and sequenced on four Illumina Hiseq lanes generating 150bp paired end reads, with <76 Gb raw data produced per library ~100x coverage (Table 3.1).

Pool-Sequencing analysis

To analyze our pooled sequencing data, we utilized the PoPoolation2 program developed by Kofler et al. (2011) to compare allele frequency differences between two or more populations. Briefly, genomic reads were mapped to the reference house fly genome using the burrows-wheeler alignment tool (BWA-MEM) (Li 2013). Using SAMtools, ambiguously mapped reads were removed, and a synchronized file was created as the main input file for PoPoolation2 (Li et al. 2009). This file contains the allele frequencies for each population at each base in the reference genome. Allele frequency differences were then calculated for each single nucleotide polymorphism (SNP) across the genome to identify SNPs that were consistent across all behaviorally resistant fly strains (BRS 1-5), as likely contributors to the observed behavioral resistance to imidacloprid in these fly strains. Loci that might confer behavioral resistance were identified by Cochran-Mantel- Haenszel (CMH) test in PoPoolation2 (Kofler et al. 2011) to test for consistent and significant difference in allele frequency among independent populations BRS fly strains 1-5 (Kofler and Schlötterer 2014). The CMH test was selected for this analysis as it was shown to perform the best when compared to other tests commonly utilized in evolve and resequence studies (Kofler and Schlötterer 2014). Mean CMH p-values for SNPs within gene locus boundaries were calculated for every gene and genes were assigned to house fly chromosomes based on scaffold assignments to Muller elements provided by Dr. Richard Meisel, University of Houston (unpublished data).

RESULTS

DNA Sequencing

Sequencing of the six DNA libraries (WT, BRS 1-5) produced 100.9, 76.0, 87.8, 99.7, 113.9, and 91.3 Gb of raw data, respectively (Table 3.1). High-quality sequence reads were produced across all samples with a Q30% >90.04%.

Pool-Sequencing analysis

On a genome-wide scale, a total of 3,677,007 SNPs were identified, with a total of 692,019 SNPs being identified as significantly different between the susceptible WT strain and selected BRS 1-5 strains across all house fly chromosomes (Figures 3.2-3.6). A significance threshold was set by utilizing the Bonferroni correction method (Benjamini and Hochberg 1995) the most conservative method for selecting a threshold p-value (Kaler and Purcell 2019), to reduce the likelihood of false positives that can arise from population structure and family relatedness among tested populations as in this study. As a previous study (Hubbard and Gerry 2021) determined that factors conferring behavioral resistance were located on chromosomes 1 and 4, we were especially interested to see if any regions on chromosomes 1 and 4 exhibited significant differences between susceptible and resistant strains. The very large number of significantly different SNPs across all chromosomes prevented identification of specific loci that might be associated with house fly behavioral resistance to imidacloprid.

By examination of mean CMH p-values for SNPs within gene locus boundaries of every gene, there were 47 genes identified to be significantly different between our

susceptible and all resistant populations (Table 3.2, Figure 3.7), with 10 genes on chromosome 1 and 8 genes on chromosome 4 (Table 3.2). Inspection of the predicted function of these genes (Table 3.2), suggests that none of the identified genes is likely to be associated with increasing contact-dependent aversion to imidacloprid that is expressed in behaviorally resistant flies, though the function of some of these genes is unknown.

DISCUSSION

Behavioral resistance to a toxic food material in which the insect or animal limits contact with or consumption of the toxicant is the most well-studied behavioral resistance phenotype. This novel form of resistance has been documented to occur in numerous vertebrate and invertebrate pest species, including the red fox (*Vulpes vulpes* L.) (Kinnear et al. 2017, Allsop et al. 2017), brushtail possum (*Trichsurus vulpecula* Kerr) (Ogilvie et al. 2000), brown rat (*Rattus norvegicus* Berkenhout) (Gaines and Hayes 1952, Brunton et al. 1993), fungus growing termites, (*Macrotermes gilvus* Hagen) (Iqbal and Evans 2018), German cockroach (*Blattella germanica* L.) (Silverman and Bieman, 1993, Wada-Katsumata et al., 2013) and in the house fly (*Musca domestica* L.) (Freeman and Pinniger, 1992; Learmount et al., 1996; Darbro and Mullens, 2004; Gerry and Zhang, 2009; Mullens et al., 2010; Hubbard and Gerry, 2020).

While behavioral resistance to toxic food materials has long been documented, the underlying behavioral or molecular mechanisms that lead to behavioral resistance expression have been difficult to elucidate due to challenges associated with studying insect and animal behavior (Sparks et al.1989; Zalucki and Furlong, 2017). One major

challenge with attempting to uncover the mechanisms causing behavioral resistance to insecticides is that no traditional resistance mechanisms (target site modifications, upregulation of detoxification enzymes, cuticular thickening) likely cause behavioral resistance, meaning researchers are essentially looking for a needle in the proverbial haystack. In recent years, exciting progress has been made in further describing the phenotypic and genotypic mechanism causing behavioral resistance in the German cockroach and in the house fly.

Aversion to glucose in the German cockroach was determined to be caused by an autosomal incompletely dominant trait controlled by a single major gene on autosome 9 (Silverman and Bieman 1993, Ross and Silverman 1995). Glucose aversion was further characterized to be processed by the antennae and mouthparts. Glucose acts as a deterrent when placed onto the mouthparts and antennae of glucose averse cockroaches (Watda-Katsumata 2013). Researchers hypothesize that a bitter gustatory receptor neuron acquired sensitivity to glucose due to structural modifications of a gustatory receptor in the bitter GRN that allows for the detection of glucose, or a glucose gustatory receptor is misexpressed in the bitter GRN (Wada-Katsumata et al. 2018). To date, the gene on autosome 9 conferring glucose aversion has yet to be identified, but a functional analysis of the gustatory receptors in the German cockroach is currently being conducted (Wada-Katsumata et al. 2018).

In the house fly, behavioral resistance to imidacloprid was determined to be genetically inherited. It was rapidly selected for utilizing a novel selection protocol, all the while leaving physiological resistance to imidacloprid relatively unchanged (Hubbard

and Gerry 2020). Resistance was further characterized to be expressed as a contact-dependent avoidance behavior which reduced the length of time flies were in contact with sucrose treated with imidacloprid, and resistance was determined to be specific to imidacloprid as resistant flies preferentially fed on another commonly utilized neonicotinoid (dinotefuran) over imidacloprid in a preference assay (Hubbard and Gerry 2020). Most recently, the genetics of behavioral resistance to imidacloprid was studied and inheritance of resistance was shown to be neither fully dominant nor recessive, with resistance linked to factors on autosomes 1 and 4 (Hubbard and Gerry 2021). Interestingly, factors on autosomes 1 and 4 independently conferred contact-dependent avoidance of imidacloprid, likely indicating that behavioral resistance to imidacloprid has a polygenic resistance mechanism.

In the current study, we attempted to identify the altered genes responsible for behavioral resistance to imidacloprid in the house fly by conducting an evolve and resequence experiment in which we experimentally selected five populations of flies (BRS 1-5) to exhibit a high level of behavioral resistance for comparison with a behaviorally susceptible foundress population (WT) from which the behaviorally resistant fly lines were derived. Only flies from the WT population that did not exhibit behavioral resistance following a resistance challenge (Hubbard and Gerry 2020) were used in comparative genomic analyses.

Pooled sequencing experiments have gained popularity over the last decade as pooling individual's DNA has provided a cost-effective alternative to sequencing large numbers of individuals which would be needed to identify allele frequency differences

between populations of interest (Schlötterer et al. 2014). This approach has shown promise in both model and non-model systems (Schlötterer et al. 2014). In recent years a multitude of evolve and resequence studies have been conducted to examine biological questions in *Drosophila melanogaster,* including hypoxia tolerance (Zhou et al. 2011), body size variation (Turner et al. 2011), the genetic basis of aging (Remolina et al. 2012), parasitoid resistance (Jalvingh et al. 2014), courtship song variation (Turner et al. 2013), and resistance to *Drosophila* C virus (Martins et al. 2014). In *Drosophila simulans*, the genetic mechanisms conferring thermal adaptations to hot environments was determined (Mallard et al. 2018).

In the current study, we took special care in designing our experiment following best practices for evolve and resequence studies as outlined by Kofler et al. (2014) and Schlötterer et al. (2014). This included having multiple replicates (5), which has been experimentally shown to be sufficient for the identification of strongly selected loci, greater than 40 individuals in each pool, having a sequencing depth of greater than 50x coverage, and using a sequencing technology that has a read length of greater than 75 nucleotides with paired-end reads. The goal behind implementing each of these best practices is to improve the likelihood of identifying selected loci, reduce error (sampling error and the influence of unequal representation of individuals in the pool), and improve mapping accuracy, respectively.

Unfortunately, even with the experimental practices mentioned above, no clear set of allele frequency changes could be identified between behaviorally susceptible and behaviorally resistant house fly strains due to the amount of noise (large number of SNPs

with significant differences) that was seen in our analysis. We expected to see pronounced allele frequency changes on chromosomes 1 and 4, as factors responsible for behavioral resistance to imidacloprid are located on these two chromosomes (Hubbard and Gerry 2021).

Interestingly, when examining mean CMH p-values for SNPs within gene locus boundaries of every gene, a significant difference in 47 genes was identified between susceptible and resistant populations. Of the genes which had a chromosome assignment (28/47), chromosome 1 and 4 had 10 and 8 genes assigned to them respectively. Given behavioral resistance is associated with chromosomes 1&4, it is interesting that most genes that were different were on these chromosomes, even if none of the identified genes appears likely to be directly responsible for behavioral resistance

We currently hypothesize that behavioral resistance to imidacloprid is caused by a change to the house fly's sensory system, as resistance is contact-dependent. We would expect to identify a gene that's predicted gene function is chemosensory related. Of the 47 genes identified, none appear to have a chemosensory function. However, multiple genes were predicted that currently do not have a hypothesized gene function and should be investigated further. Interestingly, of these, 28 were identified to encode for tRNA's which seem unlikely to be functionally responsible for behavioral resistance, but no definitive conclusions can be made without further studies.

In the future, reanalysis of the current data set may be possible, which may allow for a more defined outcome. In our current study, genomic reads were mapped to the currently available reference house fly genome, Musca_domestica-2.0.2 (Scott et al.

2014), created from the sequencing of insecticide susceptible aabys house fly strain. While this reference genome has allowed for the completion of our analysis, currently, a long-read PacBio house fly genome is in the process of being published. This genome assembly will close gaps in the current reference genome sequence and provide the ability to sequence through the highly repetitive regions of the house fly genome. It may also provide the ability to identify gene isoforms or novel isoforms of annotated genes not previously detected. This PacBio genome assembly was utilized in a bulk segregant analysis to identify the genes responsible for conferring cytochrome P450 (CYP)-mediated pyrethroid resistance on chromosomes 3 and 5 with great success (Freeman et al. Unpublished). Once this new genome resource is avaiable, we can rerun our analysis mapping our reads to the new PacBio genome assembly, which may allow for the identification of genomic changes previously not identified due to the quality of the reference genome.

Alternatively, a possible approach to reduce the total number of SNPs identified and investigate only SNPs located on chromosomes 1 and 4 would be to complete pooled sequencing on fly lines A1, A4, and A1/4 isolated in Hubbard and Gerry (2021). The A1, A4, and A1/4 fly lines were selected from backcrosses of the BRS 1 behaviorally resistant fly strain and the aabys susceptible fly strain to isolate resistance factor(s) on individual chromosomes (autosomes 1, 4 or 1 and 4, respectively). Comparisons between the reference strain (aabys), behaviorally resistant parent strain (BRS 1), and the three selected fly lines that are congenic to the aabys susceptible strain except for chromosomes carrying resistance factors (1 and 4) will significantly narrow our search

window, which will significantly assist in identifying genomic changes that may contribute to behavioral resistance to imidacloprid (Reid et al. 2018).

Identifying the genetic determinants of house fly behavioral resistance to imidacloprid has proved to be challenging. Additional studies will be needed to further characterize the mechanisms conferring this novel form of resistance. Researchers may need to take a step back and address several "basic" biological questions regarding behavioral resistance before attempting to examine the molecular mechanisms conferring resistance again. Answering essential questions such as how the house fly detects imidacloprid may provide insight to guide a more targeted approach to examining the molecular mechanisms conferring behavioral resistance. Several relatively simple studies could be conducted to answer this question, including proboscis extension response assays (PER's) and tarsal ablation experiments. Electrophysiological experiments could also be conducted to examine if a difference in neuronal responses is seen between behaviorally resistant and behaviorally susceptible flies when exposed to imidacloprid. Comparative transcriptomic or proteomic approaches could also be utilized to elucidate if gene expression changes result in a fly's ability to detect imidacloprid resulting in behavioral resistance to the chemical.

In summary, while a very large number of SNPs and 47 genes were identified that varied among behaviorally susceptible and behaviorally resistant fly populations relative to a reference fly strain, additional fundamental and applied research should be conducted to understand further both the complex phenotypic and genotypic nature of behavioral resistance to imidacloprid.

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TABLES AND FIGURES

Table 3.1: Sequencing data quality statistics for DNA libraries (WT, BRS 1-5) each prepared by pooling DNA extracted from 50 female fly heads. The libraries were then sequenced on four Illumina Hiseq lanes by Novogene Corporation Inc.
† Raw bases: Raw reads*sequencing length (150 bp)

Sample	Raw data (Gb) †	Error (%)	Q20(%)	Q30(%)	GC(%)
WT (Lane 1)	100.9	0.02	95.73	91.55	36.89
WT (Lane 2)		0.02	95.86	91.77	36.89
WT (Lane 3)		0.02	95.9	91.83	36.93
WT (Lane 4)		0.02	95.9	91.84	36.92
BRS 1 (Lane 1)	76	0.02	95	90.04	35.81
BRS 1 (Lane 2)		0.02	95.16	90.31	35.8
BRS 1 (Lane 3)		0.02	95.24	90.43	35.83
BRS 1 (Lane 4)		0.02	95.22	90.39	35.82
BRS 2 (Lane 1)	87.8	0.02	95.83	91.97	37.68
BRS 2 (Lane 2)		0.01	95.95	92.17	37.69
BRS 2 (Lane 3)		0.01	95.95	92.19	37.74
BRS 2 (Lane 4)		0.01	95.97	92.22	37.71
BRS 3 (Lane 1)	99.7	0.02	95.82	91.87	37.3
BRS 3 (Lane 2)		0.01	95.95	92.09	37.3
BRS 3 (Lane 3)		0.01	95.97	92.11	37.34
BRS 3 (Lane 4)		0.01	95.97	92.13	37.33
BRS 4 (Lane 1)	113.9	0.2	95.46	90.98	37.05
BRS 4 (Lane 2)		0.02	95.6	91.22	37.05
BRS 4 (Lane 3)		0.02	95.65	91.3	37.08
BRS 4 (Lane 4)		0.02	95.65	91.29	37.07
BRS 5 (Lane 1)	91.3	0.01	96.07	92.56	37.56
BRS 5 (Lane 2)		0.01	96.16	92.71	37.57
BRS 5 (Lane 3)		0.01	96.15	92.69	37.62
BRS 5 (Lane 4)		0.01	96.18	92.75	37.59

Table 3.2: List of genes determined to contain SNP's that are significantly different between WT and behaviorally resistant fly lines (BRS 1-5) when taking the mean CMH values for the SNPs in the gene locus boundaries. "Not Placed" in Chromosome column indicates gene has not been assigned to a house fly chromosome. "uncharacterized" followed by LOC assignment indicates genehas not been named/ had function assigned

Chromosome	Scaffold	Start	End	Gene Name	$-\log_{10}$(p-value)	Gene prediction
1	NW_004758620.1	27807	28258	LOC101895870	33.49988426	dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit DAD1
1	NW_004764681.1	360916	360988	TRNAM-CAU	19.85626187	transfer RNA methionine (anticodon CAU)
1	NW_004764782.1	201491	201564	TRNAN-GUU	19.22543479	transfer RNA asparagine (anticodon GUU)
1	NW_004764782.1	201151	201224	TRNAN-GUU	16.94896601	transfer RNA asparagine (anticodon GUU)
1	NW_004756109.1	27188	28035	LOC101888920	13.45803241	uncharacterized LOC101888920
1	NW_004765715.1	89389	89462	TRNAT-AGU	11.4202228	transfer RNA threonine (anticodon AGU)
1	NW_004765283.1	53766	53893	TRNAL-CAA	11.17254685	transfer RNA leucine (anticodon CAA)
1	NW_004763718.1	29801	30678	LOC101901060	9.940410457	small nuclear ribonucleoprotein-associated protein B
1	NW_004765198.1	104444	104996	LOC101901313	9.346051797	bis(5'-nucleosyl)-tetraphosphatase [asymmetrical]
1	NW_004765868.1	40932	42053	LOC101895498	8.662118815	60S ribosomal protein L9
2	NW_004765544.1	29771	29843	TRNAA-AGC	13.81124949	transfer RNA alanine (anticodon AGC)
2	NW_004765532.1	136461	136705	LOC105261995	12.31898158	E3 ubiquitin-protein ligase RNF181 homolog
2	NW_004766450.1	71136	71378	LOC109613731	9.312068047	uncharacterized LOC109613731
2	NW_004764910.1	33350	33422	TRNAF-GAA	8.411080417	transfer RNA phenylalanine (anticodon GAA)
2	NW_004765120.1	35323	35395	TRNAV-CAC	8.265449782	transfer RNA valine (anticodon CAC)
2	NW_004765542.1	71034	71106	TRNAK-CUU	8.060359633	transfer RNA lysine (anticodon CUU)
3	NW_004764821.1	183575	183672	TRNAY-GUA	15.30523191	transfer RNA tyrosine (anticodon GUA)
3	NW_004764786.1	250687	250758	TRNAC-GCA	9.300727745	transfer RNA cysteine (anticodon GCA)
4	NW_004765661.1	817539	817611	TRNAA-AGC	17.3694987	transfer RNA alanine (anticodon AGC)
4	NW_004764916.1	130933	131004	TRNAQ-UUG	14.7725406	transfer RNA glutamine (anticodon UUG)
4	NW_004765676.1	2283	2618	LOC109613288	13.51409456	structural maintenance of chromosomes protein 3-like
4	NW_004765467.1	8191	8262	TRNAE-CUC	12.1646287	transfer RNA glutamic acid (anticodon CUC)

Table 3.2 cont. List of genes determined to contain SNP's that are significantly different between WT and behaviorally resistant fly lines (BRS 1-5) when taking the mean CMH values for the SNPs in the gene locus boundaries. "Not Placed" in Chromosome column indicates gene has not been assigned to a house fly chromosome. "uncharacterized" followed by LOC assignment indicates gene has not been named/ had function assigned.

Chromosome	Scaffold	Start	End	Gene Name	$-\log_{10}$(p-value)	Gene prediction
4	NW_004764513.1	54766	54847	TRNAS-AGA	11.58705805	transfer RNA serine (anticodon AGA)
4	NW_004769268.1	176597	177158	LOC101890795	10.90017637	protein FAM207A
4	NW_004766216.1	18235	19509	LOC105262155	10.6141284	lipase 3-like
4	NW_004764904.1	830252	830323	TRNAT-CGU	10.52777439	transfer RNA threonine (anticodon CGU)
5	NW_004765347.1	516737	516808	TRNAD-GUC	9.76286787	transfer RNA aspartic acid (anticodon GUC)
5	NW_004765347.1	485716	485788	TRNAI-AAU	8.246714769	transfer RNA isoleucine (anticodon AAU)
Not Placed	NW_004755935.1	1583	2030	LOC109611696	38.00372874	uncharacterized LOC109611696
Not Placed	NW_004756509.1	2642	3512	LOC105261519	32.82360426	uncharacterized LOC105261519
Not Placed	NW_004773638.1	41895	41967	TRNAM-CAU	28.41151473	transfer RNA methionine (anticodon CAU)
Not Placed	NW_004756959.1	858	1460	LOC101894634	20.06226456	coiled-coil domain-containing protein 51
Not Placed	NW_004756386.1	19949	20635	LOC101897718	15.2064494	muscle calcium channel subunit alpha-1-like
Not Placed	NW_004774193.1	54034	54555	LOC109614340	12.53099621	uncharacterized LOC109614340
Not Placed	NW_004768168.1	142	805	LOC101891550	12.07555336	uncharacterized LOC101891550
Not Placed	NW_004765625.1	174562	174635	TRNAT-AGU	11.67502261	transfer RNA threonine (anticodon AGU)
Not Placed	NW_004762419.1	9745	9817	TRNAK-CUU	10.36833326	transfer RNA lysine (anticodon CUU)
Not Placed	NW_004769778.1	4065	4498	LOC101895002	9.296869298	mitochondrial import receptor subunit TOM20 homolog
Not Placed	NW_004767880.1	10788	10860	TRNAA-AGC	9.243104761	transfer RNA alanine (anticodon AGC)
Not Placed	NW_004765625.1	171201	171274	TRNAT-AGU	9.048331349	transfer RNA threonine (anticodon AGU)
Not Placed	NW_004762419.1	9598	9671	TRNAI-AAU	8.552684255	transfer RNA isoleucine (anticodon AAU)
Not Placed	NW_004765482.1	173017	173088	TRNAQ-UUG	8.532522572	transfer RNA glutamine (anticodon UUG)
Not Placed	NW_004766996.1	6983	7669	LOC101889660	8.441986497	cystathionine gamma-lyase-like
Not Placed	NW_004768138.1	11114	11186	TRNAK-CUU	8.29786435	transfer RNA lysine (anticodon CUU)
Not Placed	NW_004760719.1	87312	87384	TRNAV-AAC	8.292607325	transfer RNA valine (anticodon AAC)
Not Placed	NW_004773994.1	24661	24733	TRNAV-UAC	8.181570669	transfer RNA valine (anticodon UAC)

Figure 3.1: Overview of the behavioral resistance evolve and resequence study. A population of house flies collected from the field (WT) is split into five fly lines (BRS 1-5) with each fly line independently selected for behavioral resistance for a total of 15 generations. Over time, the allele frequency of the causative allele(s) increases. The allele frequencies of the WT and the BRS 1-5 are subsequently examined with a pooled sequencing approach to identify causative alleles that can be visualized on a Manhattan plot. (Figure created with Biorender.com)

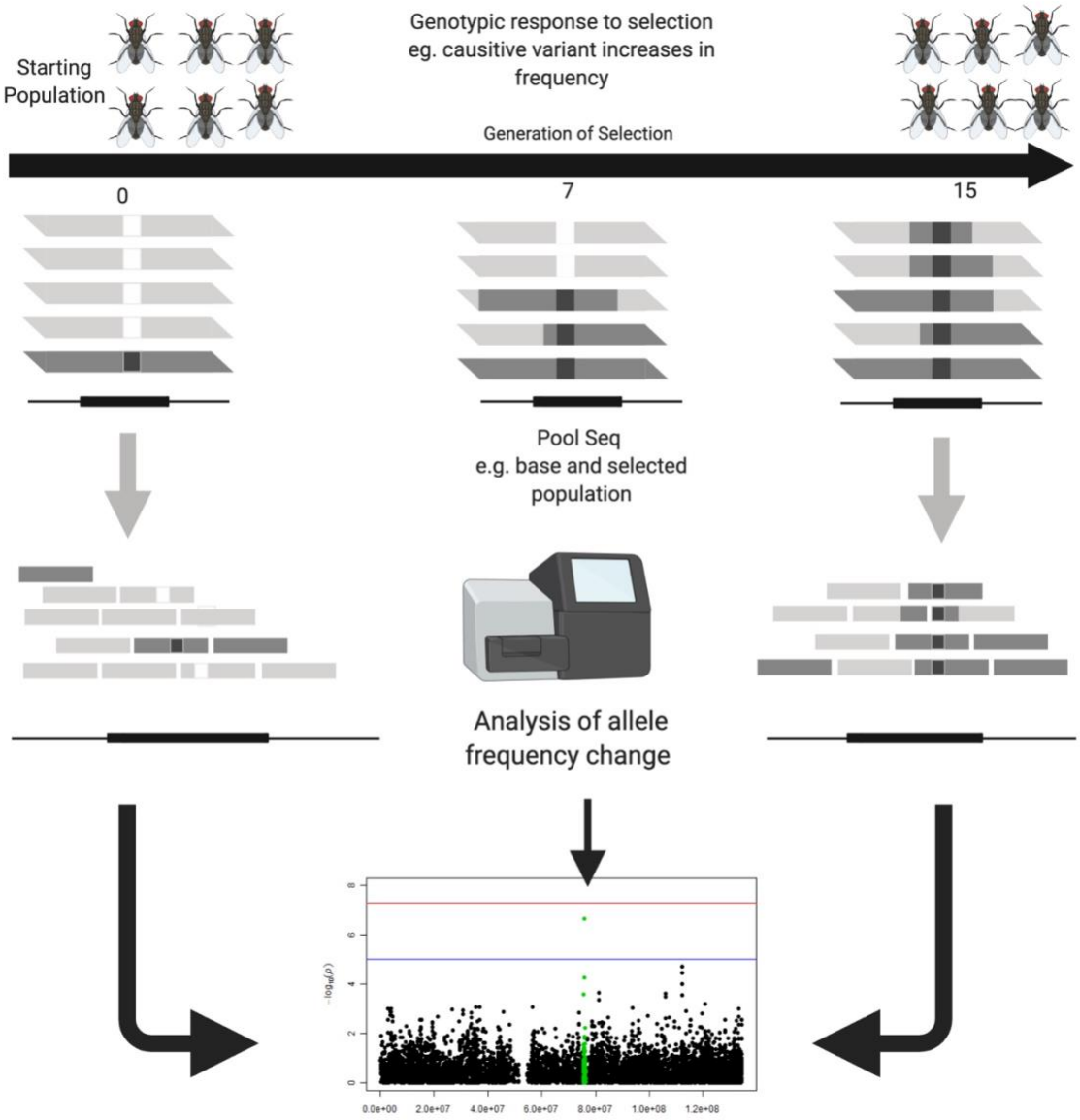


Figure 3.2: Manhattan plot of p-values associated with SNPs found on Chromosome 1 following genome wide analysis. The significance estimates (p-values) are plotted against the genomic coordinates of SNPs found on Chromosome 1. The red line indicates the significant p-value cutoff.

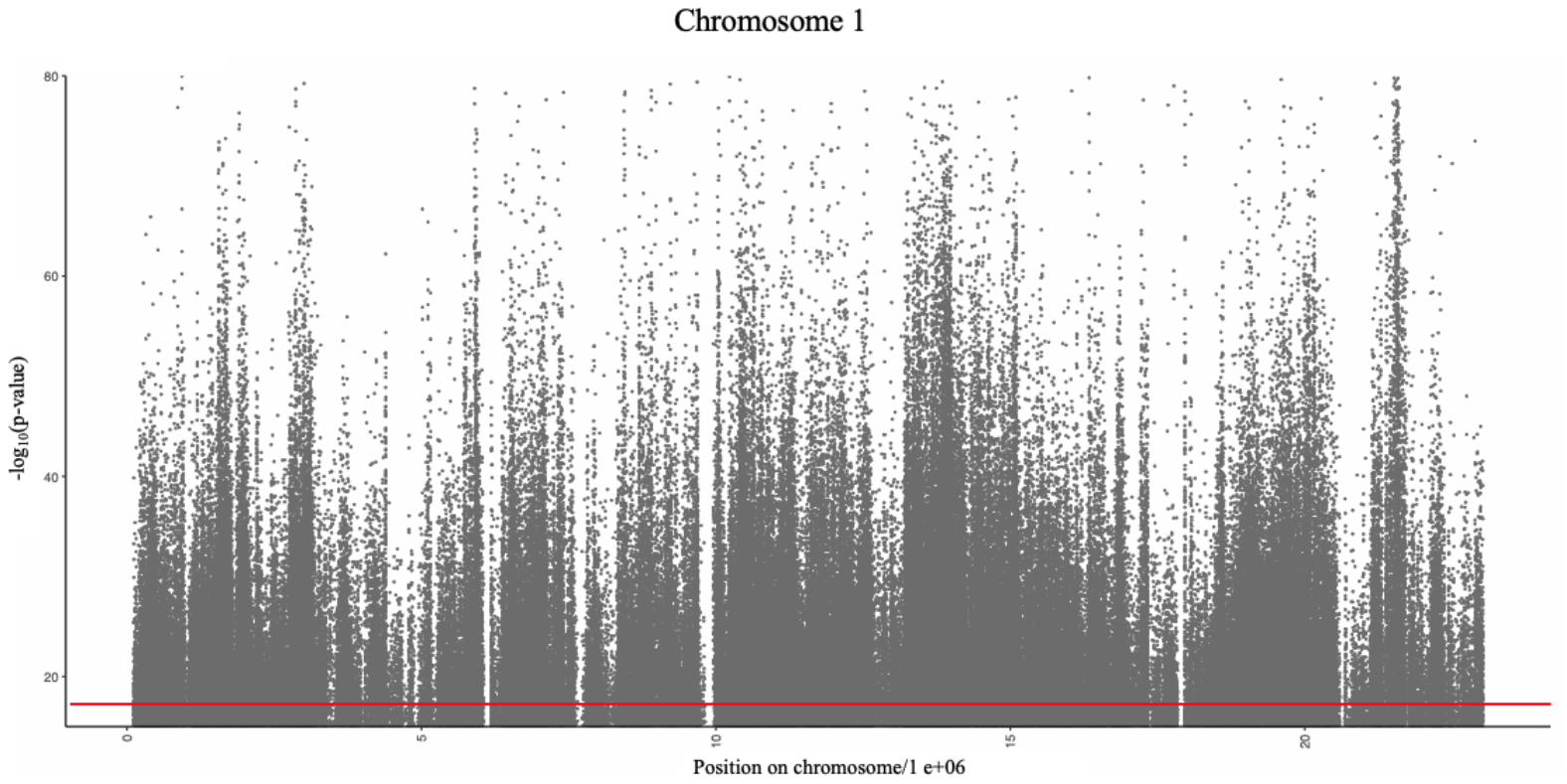


Figure 3.3: Manhattan plot of p-values associated with SNPs found on Chromosome 2 following genome wide analysis. The significance estimates (p-values) are plotted against the genomic coordinates of SNPs found on Chromosome 2. The red line indicates the significant p-value cutoff.

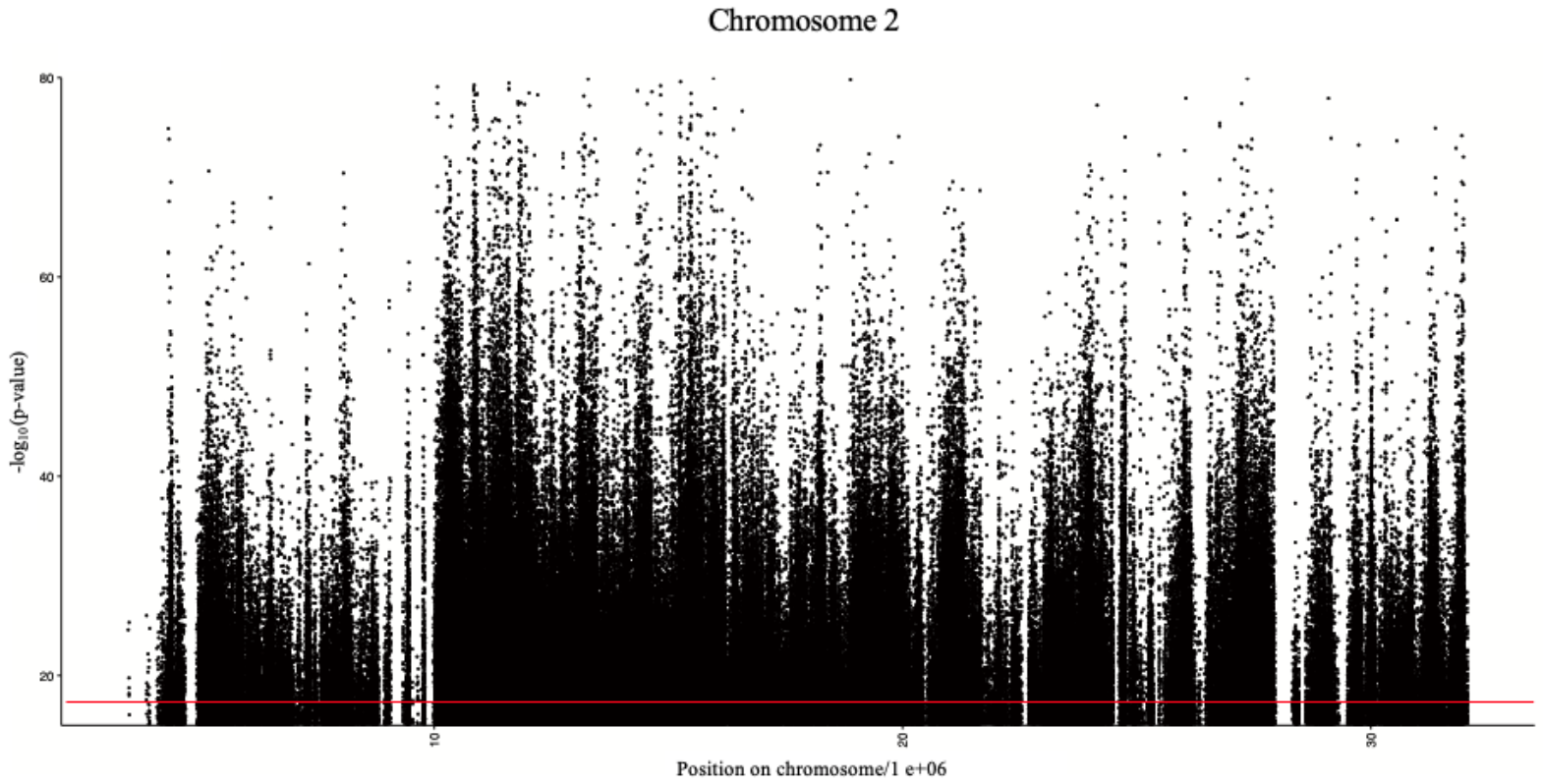


Figure 3.4: Manhattan plot of p-values associated with SNPs found on Chromosome 3 following genome wide analysis. The significance estimates (p-values) are plotted against the genomic coordinates of SNPs found on Chromosome 3. The red line indicates the significant p-value cutoff.

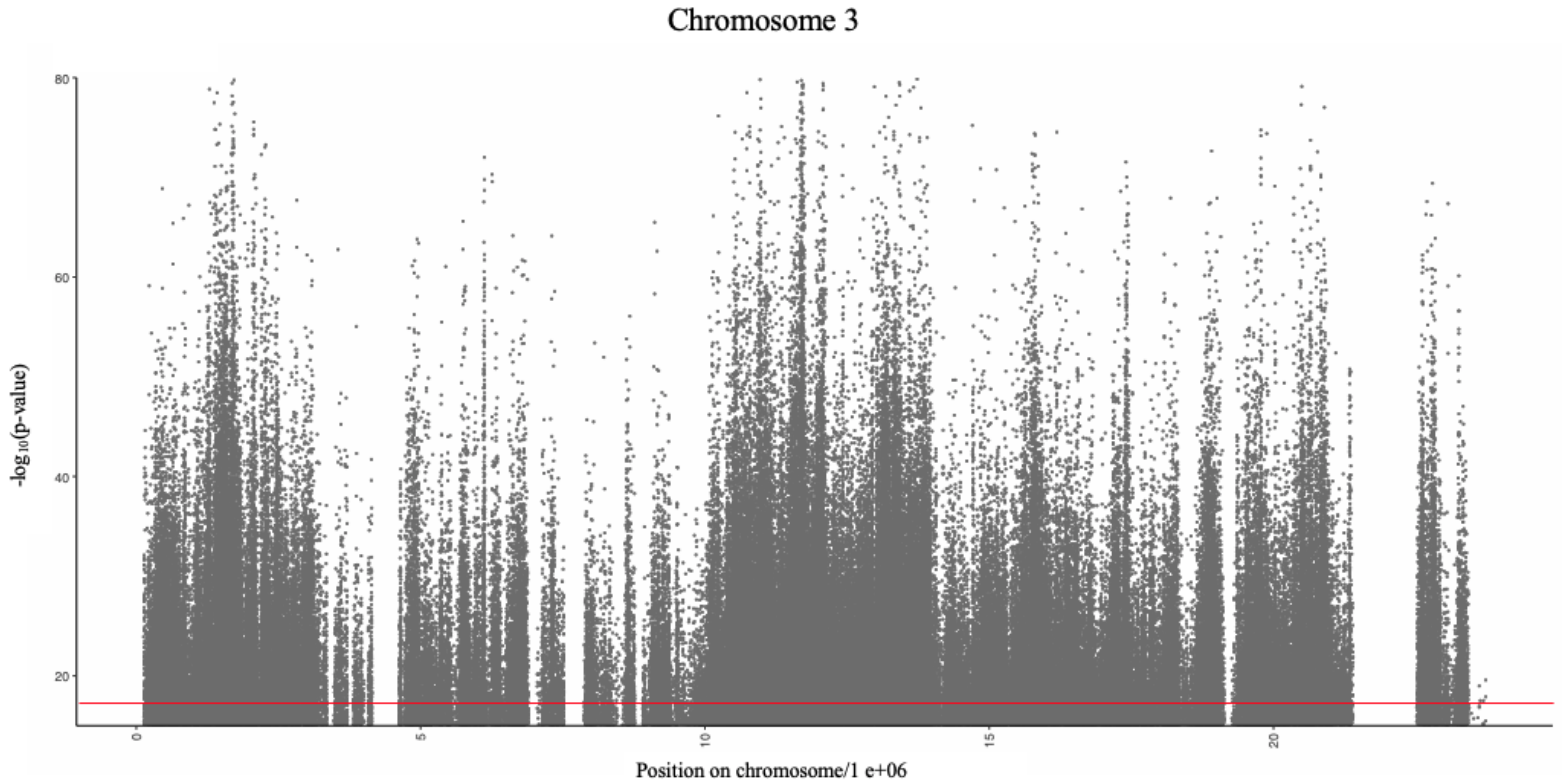


Figure 3.5: Manhattan plot of p-values associated with SNPs found on Chromosome 4 following genome wide analysis. The significance estimates (p-values) are plotted against the genomic coordinates of SNPs found on Chromosome 4. The red line indicates the significant p-value cutoff.

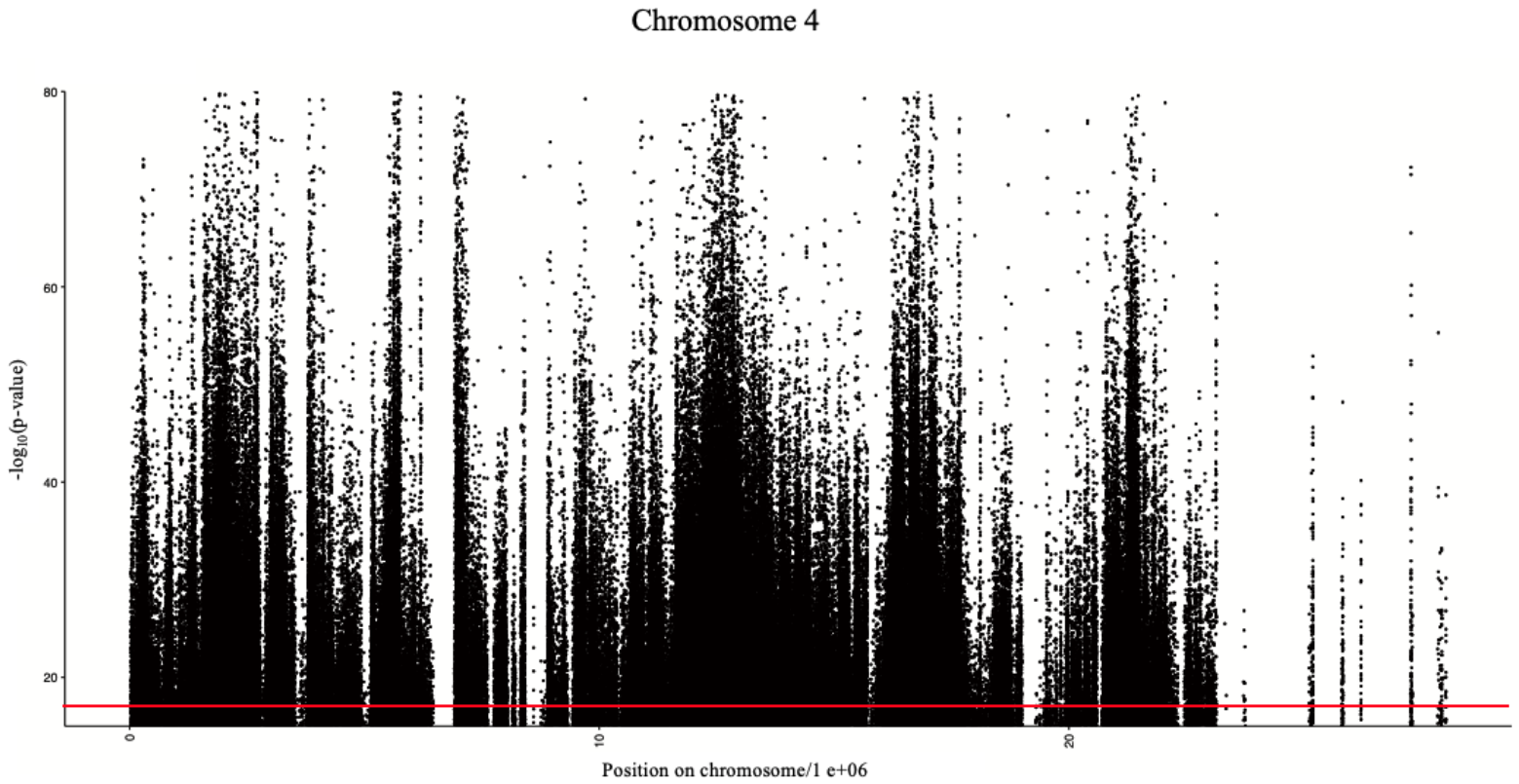


Figure 3.6: Manhattan plot of p-values associated with SNPs found on Chromosome 5 following genome wide analysis. The significance estimates (p-values) are plotted against the genomic coordinates of SNPs found on Chromosome 5. The red line indicates the significant p-value cutoff.

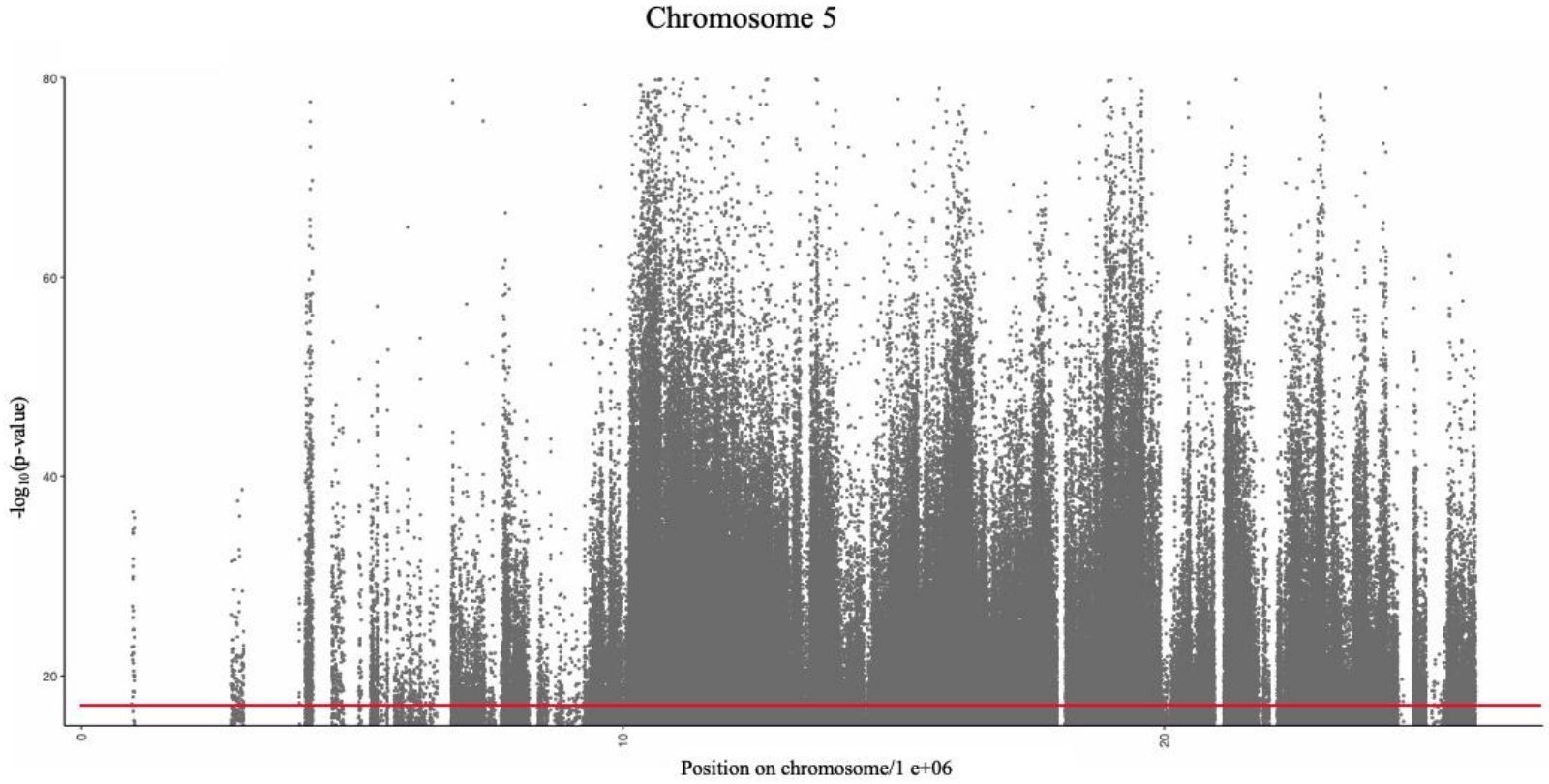
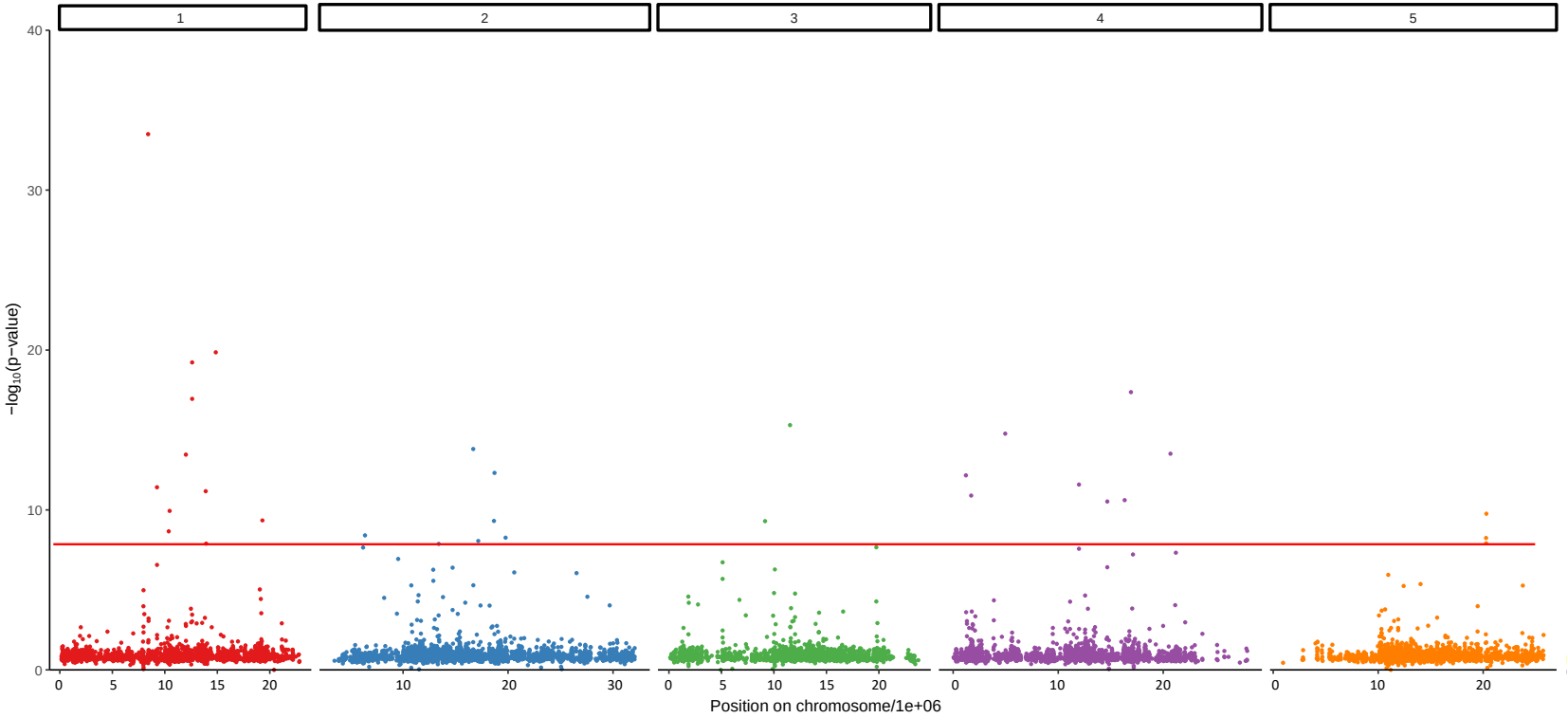


Figure 3.7: Manhattan plot of mean CMH p-values for SNPs found within gene locus boundaries found on Chromosomes 1-5. The red line indicates the significant p-value cutoff.



CONCLUSIONS

While studies of insecticide resistance have long focused on understanding physiological resistance mechanisms, behavioral resistance to insecticides in the house fly has been documented to occur for nearly 70 years (Sparks et al. 1989). This likely is because methods to describe and study the mechanisms conferring this novel form of resistance are not well developed due to the difficulty of developing rigorous protocols to explore the complex nature of insect behaviors related to resistance (Sparks et al. 1989, Zalucki and Furlong 2017).

Following the introduction of the imidacloprid containing fly bait QuickBayt® in 2002, efficacy studies in subsequent years demonstrated initial effectiveness of this bait (Butler et al. 2007), followed by rapid loss of effectiveness as a result of increasing fly resistance (physiological and behavioral) (Gerry and Zhang 2009, Mullens et al. 2010). Behavioral resistance was believed to be a contributing factor for why this insecticide rapidly failed to control flies in southern California. Without a thorough understanding of what behavioral resistance is and what mechanisms confer it, management of this form of insecticide resistance is impossible.

A series of experiments comprising this dissertation were conducted to uncover the phenotypic and genotypic mechanisms that confer behavioral resistance to imidacloprid. This included selecting for behavioral resistance to imidacloprid without increasing the physiological resistance profile, reverting a population of flies exhibiting some level of behavioral resistance back to behavioral susceptibility, characterization of

the behavioral resistance phenotype via video observation assays, determination of the genetic inheritance pattern of behavioral resistance, identification of the chromosome(s) carrying factors conferring behavioral resistance to imidacloprid, and lastly using a pooled sequencing approach to identify molecular/ genetic changes in behaviorally resistant house fly populations that may contribute to behavioral resistance to imidacloprid.

These experiments and research outcomes build a foundation to study the genetic control of behavioral resistance to insecticides in the house fly, open new avenues for research to understand aspects of inherited behavior in the house fly and may assist in the development of insecticide chemistries that limit or delay the selection for behavioral resistance by house flies or other pests.

We first tested to confirm that behavioral resistance was present in field-collected flies in southern California, as was previously documented by Gerry and Zhang (2009). We collected house flies by sweep net from the same southern California dairy where Gerry and Zhang 2009 had previously collected flies and assessed them for physiological and behavioral resistance to imidacloprid. An increase in physiological resistance to imidacloprid was seen to have occurred between 2008 and 2015. However, the LC_{95} is nearly 2x less than the imidacloprid concentration (5,000 µg/g bait) currently formulated in the commercial fly bait QuickBayt® (Bayer Healthcare LLC, Shawnee Mission, KS, U.S.A.), suggesting that QuickBayt® would still be sufficient to kill flies if physiological resistance were the only mechanism contributing to imidacloprid resistance providing

additional evidence that behavioral resistance to imidacloprid plays a significant role in the failure of this insecticide.

House fly behavioral resistance to imidacloprid was determined to be present in the fpopulation of flies collected from the dairy. However, fly survival in choice-bioassays was variable, indicating that not all individuals in the population of flies collected exhibited the behavioral resistance phenotype. This high level of variability would make it extremely difficult to study the phenotypic or genotypic mechanisms conferring resistance as non-behaviorally resistant individuals in the population would skew observational and genomic results.

A clear and deliberate approach to laboratory selection for behavioral resistance had not been previously reported. We developed a methodology that we believed would selectively increase behavioral resistance to imidacloprid without increasing the physiological resistance level. This method entailed exposing flies to a choice assay where flies were provided sucrose with and without imidacloprid at a "selection dose" of imidacloprid (3x LC_{95} for the WT colony in a no-choice bioassay). This methodology provided us with confidence that if flies were to consume imidacloprid contain sucrose, they would get a lethal dose of the insecticide and not select physiological resistance.

Resistance selection was performed independently for five fly colonies to evaluate whether more than one behavioral resistance mechanism might be selected using our protocol. It would prove critical in providing replicates for our genomic analysis. Behavioral resistance in all fly strains was rapidly selected for as flies, with fly survival being >90% following the 10^{th} selection cycle.

We also examined if flies could be selected for behavioral susceptibility to imidacloprid. By choosing the offspring of flies that died following short exposure to the two food choices populated the next generation. Behavioral susceptibility was achieved within just seven selection cycles with selected flies exhibiting a similar mortality pattern to that of the insecticide susceptible fly colony (UCR strain).

The selection of fly populations for increasing behavioral resistance or susceptibility indicates that behavioral resistance to imidacloprid is a heritable trait that can be rapidly selected for, just as physiological resistance to imidacloprid had been selected for in previous studies with house flies (Kaufman et al. 2010, Kavi et al. 2014).

Behavioral observation assays were completed in which flies were placed into a Plexiglass observation chamber provisioned with two weigh dishes placed on opposite ends of the chambers. One weigh dish contained sucrose treated with imidacloprid, and the other contained sucrose alone. Flies were allowed to forage freely for two hours while being recorded via GoPro camera.

Videos were analyzed, and the number of times a fly landed on each food dish and the amount of time each fly spent on the food dish was recorded. Behavioral observation assay results indicated that behavioral resistance to imidacloprid was contact dependent as behaviorally resistant flies equally contacted food dishes containing sucrose treated with and without imidacloprid, but flies would spend significantly less time on the sucrose treated with imidacloprid. While it was determined that behavioral resistance to imidacloprid was contact-dependent, one downside to the system we used was that the GoPro cameras did not provide fine visual detail. Unfortunately, this meant that we could

not determine if flies were extending their proboscis to taste the imidacloprid treated sugar or if the aversion was mediated by tarsal contact alone. Future experiments should be conducted to determine if behavioral resistance is mediated by tarsal or proboscis contact with imidacloprid.

We next wanted to examine if behavioral resistance to imidacloprid was specific to imidacloprid or if behavioral cross-resistance to another neonicotinoid had occurred. We evaluated this by using a preference assay in which we provisioned groups of flies with sucrose treated with imidacloprid colored with blue food coloring and sucrose treated with dinotefuran colored with red food coloring. Flies were allowed to feed for 24 hours, after which the color of the fly abdomens were inspected, and a preference index was calculated. We chose to evaluate dinotefuran as it is currently available as a toxicant in fly bait for control of house flies and has been observed to be used on dairies in southern California. Behavioral resistance was determined to be specific to imidacloprid as flies preferentially fed on dinotefuran over imidacloprid. While we determined that behavioral resistance was specific to imidacloprid when flies were provided dinotefuran as an alternative food source, it is currently unknown if flies would respond in the same way if exposed to others neonicotinoid insecticides. Dinotefuran uniquely possesses a non-aromatic ring, one oxygen capable of forming hydrogen bonds, and an asymmetric carbon (Kiriyama et al. 2003, Matsuda et al. 2020) may result in differential binding to the target site. However, this has yet to be determined. While testing dinotefuran as our alternative neonicotinoid made the most practical sense, as we know the dairy behaviorally resistant flies were collected from utilized dinotefuran containing baits,

future experiments should be conducted examine if behavioral resistance exists to other neonicotinoid insecticides with similar chemical structures to imidacloprid.

As we now possessed fly strains exhibiting a high level of behavioral resistance to imidacloprid, which was determined to be contact-dependent and specific to imidacloprid, our next objective was to understand the genetics of behavioral resistance. As the house fly is not a model system like *Drosophila melanogaster*, limiting usefulness of gene knock out experiments, we began our investigation of the genetics of behavioral resistance utilizing the Tsukamoto method for chromosomal linkage to a phenotypic trait, a technique developed in the 1960s (Tsukamoto 1964). This method allows for the identification of chromosome carrying factors conferring an expressed phenotype (behavioral resistance in these studies). This type of analysis prior to our study had only been completed to determine the location of physiological resistance factors, which meant that some small modifications to the methods were needed. In the traditional method, flies were treated topically with an insecticide or were provided with a no-choice test in which flies were provisioned with sugar treated with a set concentration of insecticide. Our study aimed to investigate behavioral resistance. Instead, we exposed flies to a choice assay in which flies were provided sucrose treated with and without imidacloprid. The slight modification to the previously described methods allowed for the first successful linkage analysis completed on a behavioral trait in the house fly. We determined that behavioral resistance was linked to factors on autosomes 1 and 4 in all five behaviorally resistant fly strains.

This likely indicates that the genetic or molecular resistance mechanisms resulting in behavioral resistance are similar if not the same in all five fly strains. Behavioral resistance in the selected house fly strains was also shown to be neither fully dominant nor recessive (Tsukamoto 1983) as indicated by an intermediate level of behavioral resistance in the F1 flies relative to the susceptible (aabys) and resistant (BRS) parent fly strains. Unfortunately, a degree of dominance (Stone 1968) for behavioral resistance could not be calculated since a single high dose of insecticide was used instead of varying insecticide concentrations. Ideally, in future studies, modifications could be made to the Stone equation to calculate the degree of dominance of a behavioral resistance trait.

After determining that autosomes 1 and 4 were carrying resistance factors, we decided to isolate fly strains that carried either autosome 1, 4, or 1 and 4 from our behaviorally resistant fly strain. This allowed us to individually examine the influence of resistance factors on each autosome on the fly's behavior and if fly behavior was modified by an interaction between factors on autosomes 1 and 4. We examined this by using previously described assays, including choice assays, behavioral observation assays, and preference assays. We determined that there is likely an additive interaction between resistance factors on chromosome 1 & 4 as flies carrying both resistance factors had the highest survival rate when exposed to a choice assay. Interestingly though, all fly lines exhibited lower survival than the behaviorally resistant parent strain they originated from, suggesting there may be trans regulation of resistance factors or the presence of minor resistance factors on other autosomes not inherited by the selected fly lines.

Flies were then exposed to behavioral observation and preference assays to determine if the isolated fly line exhibited the same contact-dependent avoidance and preference for dinotefuran over imidacloprid that our behaviorally resistant fly strains did. Each fly line expressed contact-dependent avoidance and preference for dinotefuran over imidacloprid. This indicated that autosomes 1 and 4 independently confer contact-dependent avoidance of imidacloprid and aversion to imidacloprid instead of the broader neonicotinoid class. This is a fascinating result as it may indicate that multiple molecular mechanisms have independently evolved and resulted in the same phenotypic response. Alternatively, the observational assay may simply not have the resolution to pick up subtle phenotypic differences expressed between fly lines. For instance, factors on autosome 1 and 4 could code for changes in gustatory receptor neurons located on different body regions of the fly (tarsi vs. proboscis), resulting in the fly eliciting behavioral aversion to imidacloprid that would not be able to be teased apart with our current assays. This hypothesis could also explain why survival was higher in flies with behavioral resistance factors on both autosomes 1 and 4. Future work should investigate this hypothesis by completing fine-scale behavioral analysis experiments, which may include examining behaviorally resistant flies that had tarsal hairs ablated or removed to determine if imidacloprid was detected by the tarsi or conduct proboscis extension response experiments on individual flies to examine if the tarsi or proboscis detected imidacloprid in fly lines carrying resistance factors from chromosome 1, 4 or both.

As we now understood that resistance was linked to factors on autosomes 1 and 4 in the house fly utilizing an autosomal linkage analysis, the next step was to investigate

the molecular mechanisms that conferred behavioral resistance to imidacloprid. We employed a pooled sequencing approach in which we combine multiple individuals' DNA from a population of interest and pool them together and sequence (pool-seq). In this study, we sequenced our foundress house fly population WT and each of our five behaviorally resistant fly strains (BRS 1-5), intending to identify putative selected sites or candidate loci that may be responsible for our selected phenotype by comparing house flies that did not exhibit the behavioral resistance phenotype to house flies that showed a high level of behavioral resistance—unfortunately following the analysis utilizing the PoPoolation 2 program (Kofler et al. 2011, no clear genomic signal was seen between behaviorally susceptible and behaviorally resistant house fly strains. While 47 genes were identified to have significant differences between our susceptible and resistant populations, following manual inspection of the predicted gene function, there were no identified genes that would be expected to have a role in resistance. Future studies should be conducted to examine the molecular mechanisms causing behavioral resistance to imidacloprid in the house fly. Comparative transcriptomic or proteomic approaches could be utilized to elucidate if gene expression changes result in a fly's ability to detect imidacloprid resulting in behavioral resistance to the chemical.

Further "basic" biological studies should also be conducted to determine how the house fly is detecting imidacloprid. Proboscis extension response assays (PER's) and tarsal ablation experiments could provide additional detailed behavioral information that could provide insight into guiding a more targeted approach to examining the molecular mechanisms conferring behavioral resistance. Additionally, while at a small geographic

scale (southern California), we have determined that behavioral resistance is a major contributing factor for why imidacloprid containing fly baits failed to control house flies soon after implementation; it is essential to understand if behavioral resistance is contributing to the failure of imidacloprid baits at a regional or multistate level. Monitoring of physiological susceptibility to insecticides has long been conducted. To date, no comprehensive surveys have been conducted to determine the presence of behavioral resistance/susceptibility to commonly utilized insecticides used for fly control.

House fly behavioral resistance to imidacloprid has proved to be much more complicated than initially thought. While we did not completely unravel the complex nature of behavioral resistance to imidacloprid, we did peel back many layers of the behavioral resistance onion throughout this dissertation. We determined that behavioral resistance to imidacloprid has a genetic component as it could be rapidly selected for. It was determined resistance was contact-dependent and specific to imidacloprid and was linked to factors on autosome 1 and 4. While behavioral resistance to insecticides has long been documented, we are in the infancy of truly understanding what mechanisms cause this novel form of insecticide resistance. It is anticipated that as we move further into the -omics and digital era that the complex question we currently have regarding insect behavior will be elucidated.

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