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Research



# 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.

**Key words:** house fly, insecticide, resistance, behavior, neonicotinoid

The common house fly (*Musca domestica* L.) 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 yr (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

An overview of the methods described below can be found in Supp Fig. S1 (online only).

### 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 gram of 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 1 g 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 one or two (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 to 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 third 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 (3× $LC_{95}$ 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 three filial generations to complete 10 selections. Following the 5th and 10th (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 section 'Imidacloprid Susceptibility Bioassays'.

A significant difference in susceptibility to imidacloprid among behaviorally resistant and reference fly strains was determined by non-overlapping 95% CIs 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) 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 five 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 × 25 mm Petri dish that was then placed into the center of a Plexiglass observation chamber (50 × 18.25 × 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). Adult house flies (3–5 d old) were starved in their colony cage for 14 h, then sorted on a chill table into five 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.) 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.

## 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 $LC_{50}$ = 11,700 (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). Though not shown in Table 1, WT flies had a $LC_{95}$ = 1,263 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 µg/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 five selection cycles, and ultimately reaching 91.4 and 99.83% survival of male and female flies, respectively by the 10th and final selection cycle (Fig. 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 µg/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).

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 µg/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 (Fig. 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 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

**Table 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)

| Assay type[a] | Fly strain | *n* | Slope (SE) | LC50 (95% CI) (µg/g) | RR (LC50) WT[b] | RR (LC50) UCR[b] |
|---|---|---|---|---|---|---|
| No-choice | UCR | 875 | 1.4 (0.2) | 19 (10–38) A | – | – |
| No-choice | WT | 1,375 | 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) | 11,700 (10,400–12,900)[b] | – | 244 |
| Choice | BRS 1 | 750 | ND | >15,000 | ND | ND |
| Choice | BRS 2 | 750 | ND | >15,000 | ND | ND |
| Choice | BRS 3 | 750 | ND | >15,000 | ND | ND |
| Choice | BRS 4 | 750 | ND | >15,000 | ND | ND |
| Choice | BRS 5 | 750 | ND | >15,000 | ND | ND |

[a]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% CIs of the LC values and are indicated within columns by capital letters for no-choice bioassays and lower case for choice bioassays.

[b]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).

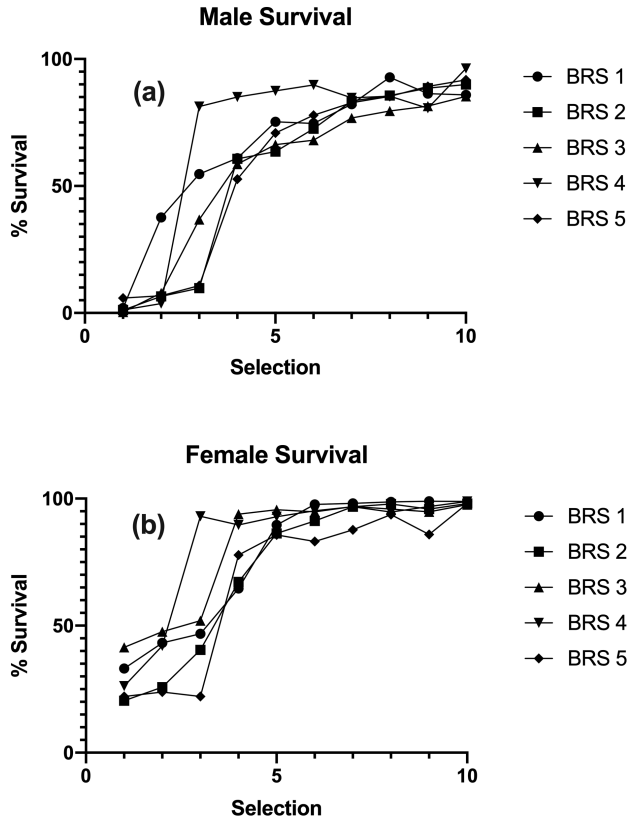


**Fig. 1.** Survival of male (a) and female (b) flies from each BRS fly strain during imidacloprid behavioral resistance selection assay over 10 selection cycles. Selection 1 indicates survival of the field-collected WT house flies during the first selection assay. Surviving offspring from each selection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

the sucrose–imidacloprid dish relative to the sucrose-only food dish ($n = 10$; $P < 0.05$). Male BRS3 flies showed nonsignificantly 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 (Fig. 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, 2014, 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).

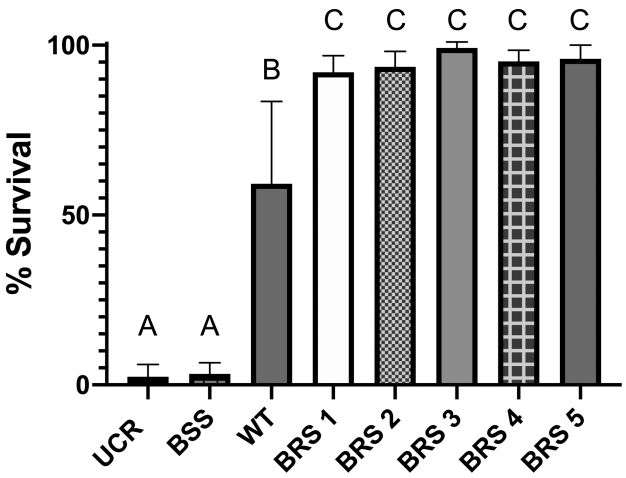


**Fig. 2.** Relative behavioral resistance to imidacloprid by fly strain as indicated by fly survival following 72-h exposure to a choice feeding assay with paired food dishes containing either sucrose alone or sucrose treated with imidacloprid at a dose of 4,000 µg/g of sucrose. Fly strains were selected 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.

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 WT 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.,

**Table 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

| Strain | *N* | Landing events (lands ± SE) | | *P*-value | Contact time (time ± SE) | | *P*-value |
|---|---|---|---|---|---|---|---|
| | | Sucrose | Imidacloprid | | Sucrose | Imidacloprid | |
| 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 | 0.30 | 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** |

*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.

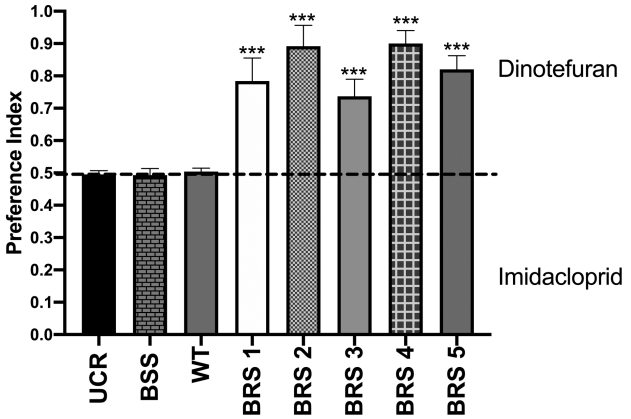


**Fig. 3.** Feeding preference index ($P_{D/I}$) for flies provided a choice to feed on either sucrose with 4,000 µg/g imidacloprid or sucrose with 4,000 µg/g dinotefuran. A $P_{D/I}$ value >0.5 indicates a greater proportion of flies feeding on the sucrose–dinotefuran, while a $P_{D/I}$ = 0.5 indicates 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 (***$P < 0.001$).

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 WT 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 (Dethier 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 WT 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) is more than 2× 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. In comparison to the modest increase in physiological resistance to imidacloprid from 2008 to 2015, the 244-fold 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 (U.S. Environmental Protection Agency 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 4-fold greater number of flies on a fly bait containing dinotefuran (QuikStrike; Wellmark International, Shaumburg, IL) 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.

## Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.

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