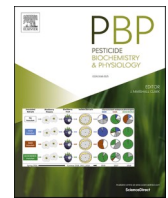




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Genetic evaluation and characterization of behavioral resistance to imidacloprid in the house fly

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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.

1. 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 and Burrus, 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,

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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., 2019). 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 (QuickBay; Bayer Healthcare LLC, Shawnee Mission, KS) with an imidacloprid concentration that is 3× 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.

2. Materials and methods

2.1. 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.

2.2. 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 (4000 µg/g sucrose; 3× 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 (Zahn and Gerry, 2018).

2.3. Linkage analysis of behavioral resistance to imidacloprid

The F₁ 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 (Fig. 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 F₁ offspring. The F₁ offspring express dominant phenotypes, including normal house fly morphology. Males from F₁ offspring were then backcrossed with aabys females to give backcross (BC) offspring displaying 2⁵ = 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 (Tables 1 and 2).

To determine the level of behavioral resistance to imidacloprid inherited by the heterozygous F₁ flies, five replicates of 25 female F₁ 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.

2.4. 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 F₁ 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 (Murvosh et al., 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.

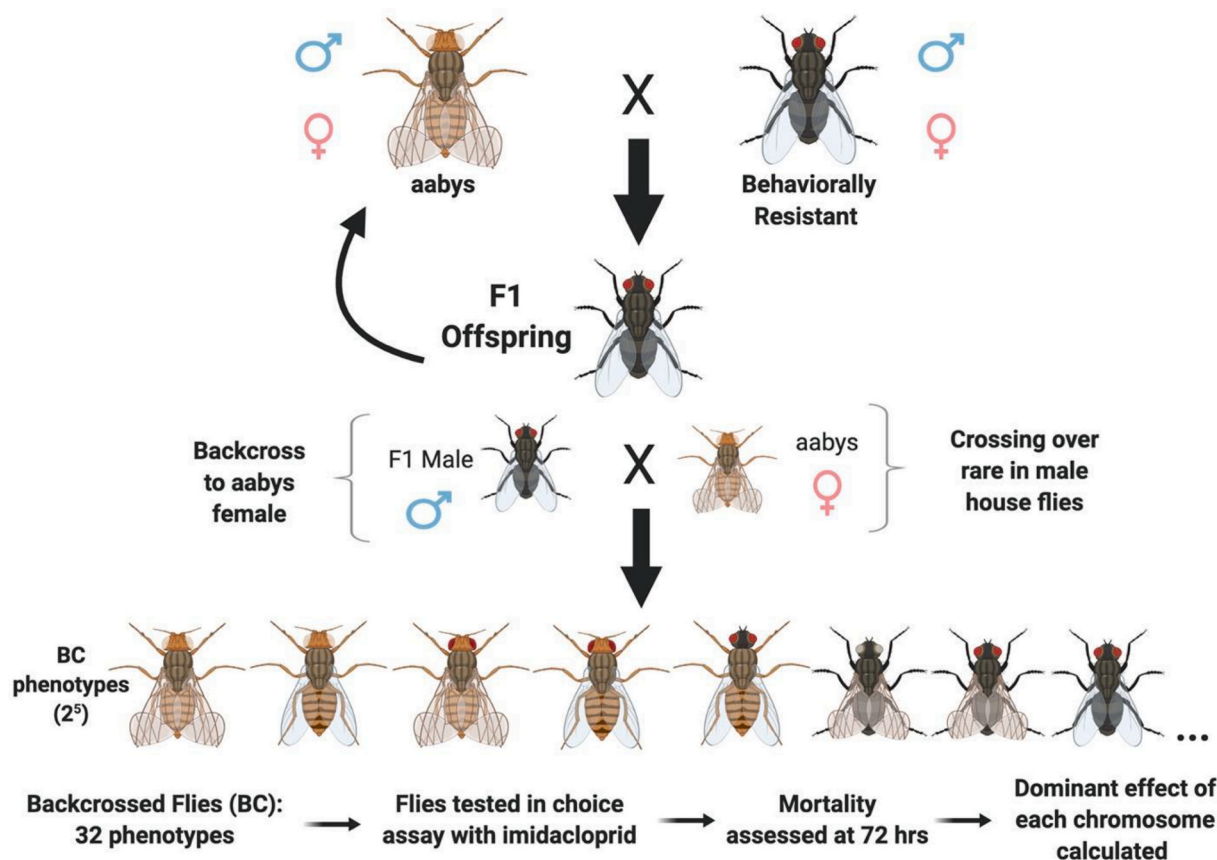


Fig. 1. Pictorial representation of the modified F₁ male backcross method of Tsukamoto (1964) for each behaviorally resistant (BRS) fly strain crossed with the insecticide susceptible (aabys) fly strain to determine which house fly chromosomes carry factors in the BRS fly strain conferring behavioral resistance to the insecticide imidacloprid. (Created with BioRender.com).

2.5. 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).

2.6. 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.

2.7. 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 × 18.25 × 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 (4000 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.

2.8. Specificity of behavioral resistance to imidacloprid

Feeding preference assays were performed for each isolated fly line

Table 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	0.27
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
Autosomes contributing statistically significant ($p < 0.01$) effects to behavioral resistance to imidacloprid in BRS 2–5 house fly strains. Full autosomal linkage analysis for BRS 2–5 available in Supplemental Tables (S1–4).

Strain	Autosome (s)	Effect	Mean square	F Value
BRS 2	1	-167.92	1762.29	7.64
BRS 2	4	-274.16	4697.71	20.37
BRS 3	1	-126.05	993.03	15.28
BRS 3	4	-127.77	1020.26	15.70
BRS 4	1	-234.47	3435.95	15.09
BRS 4	4	-392.11	9609.55	42.21
BRS 5	1	-212.01	2809.37	12.51
BRS 5	4	-404.33	10,217.65	45.49

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 (4000 $\mu\text{g/g}$ sucrose), and the second containing sucrose treated with dinotefuran at the same concentration (4000 $\mu\text{g/g}$ 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.

3. Results

3.1. 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 (Tables 1, 2, S1–S4). 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 (Fig. 2).

3.2. Evaluating behavioral resistance to imidacloprid of selected fly lines

Female F_1 offspring exposed to imidacloprid averaged $22.7 \pm 3.7\%$ survival across all F_1 reciprocal crosses in comparison to an average of $1.6 \pm 0.9\%$ for the susceptible (aabys) parent strain and $96.0 \pm 0.7\%$ for the behaviorally resistant (BRS 1–5) parent strain (Fig. 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 ($64.2 \pm 4.2\%$) nearly three times that of male survival ($23.8 \pm 4.9\%$). 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 $43.4 \pm 4.1\%$ and $56.0 \pm 6.6\%$, respectively and for A1/4 males and females of $66.4 \pm 11.4\%$ and $84.2 \pm 8.6\%$, respectively (Fig. 4).

3.3. 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 ($n = 8$; $z < 1.26$; $p > 0.23$) (Fig. 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 ($n = 8$; $z < 2.24$; $p < 0.02$) (Fig. 5b).

3.4. 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 (Fig. 6). The feeding preference for all BC fly lines was not

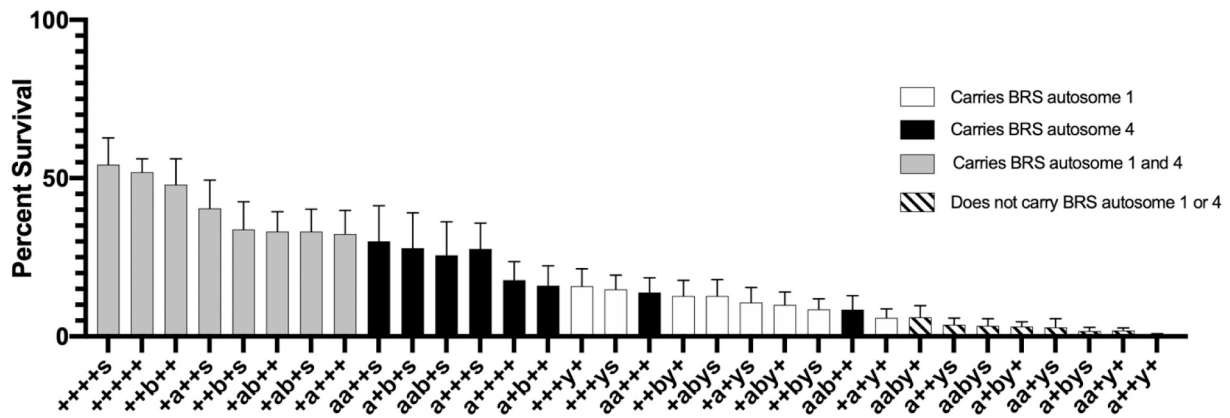


Fig. 2. Mean percent survival \pm SE of backcross flies (5 BRS fly strains \times 2 reciprocal crosses) by phenotype (chromosomal combination) following a 72 h choice feeding assay with flies provided both a food dish containing sucrose alone and a second food dish containing sucrose with a high concentration of imidacloprid (4000 μ g/g sucrose). Choice feeding assay was performed to determine the “dominant effect” of each house fly autosome (linkage analysis).

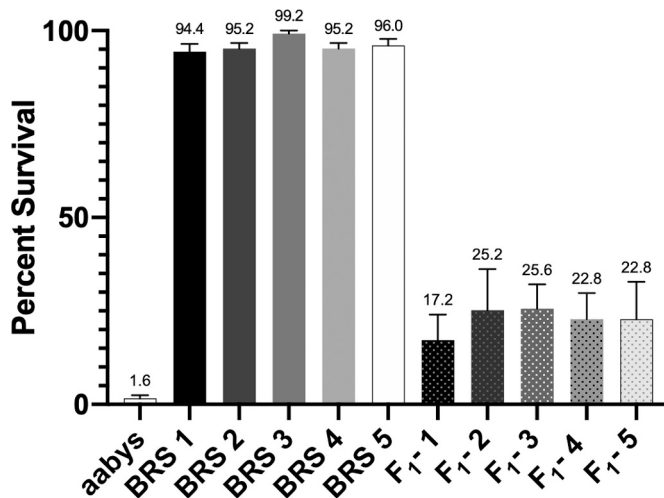


Fig. 3. Mean percent survival \pm SE of female aabys (susceptible), BRS 1–5 (behaviorally resistant), and each F_1 cross of aabys \times BRS strain flies following a 72 h choice feeding assay with flies provided both a food dish containing sucrose alone and a second food dish containing sucrose mixed with a high concentration of imidacloprid (4000 μ g/g sucrose). Data for BRS 1–5 survival from Hubbard and Gerry (2020) and shown here for comparison.

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.

4. Discussion

Behavioral resistance by insects to food baits containing insecticides has been documented in the German cockroach (*Blattella germanica* (L.)) (Silverman and Bieman, 1993, 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).

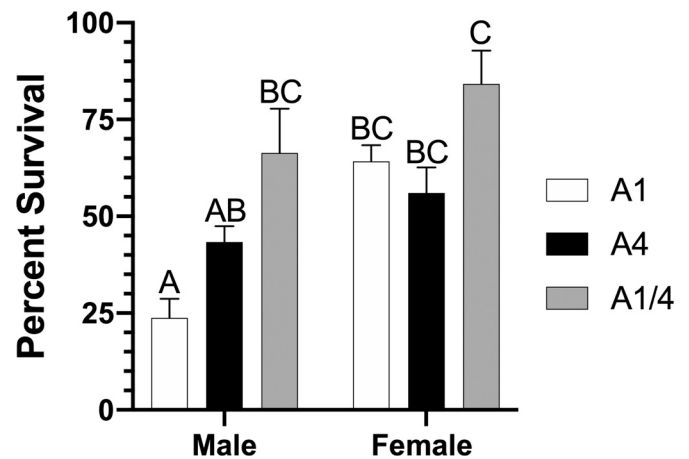


Fig. 4. Mean percent survival \pm SE of house flies carrying autosomes shown by linkage analysis to be associated with behavioral resistance when flies are subjected to a choice feeding assay with paired food dishes containing either sucrose or sucrose mixed with imidacloprid at 4000 μ g/g sucrose. Different letters indicate significance ($p < 0.05$).

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 et al., 1997; Shono et al., 2004; Tian et al., 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, 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).

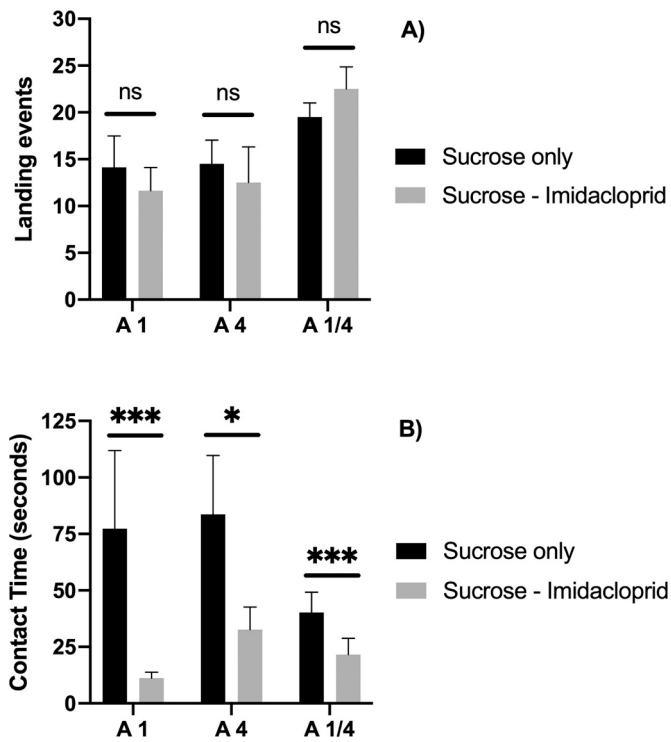


Fig. 5. Mean \pm SE landing events (a) and contact time (b) on paired food dishes containing either sucrose alone or sucrose with imidacloprid (4000 μ g/g sucrose) over a 2-h observation window. Differences between food dish treatments within fly lines were determined by Wilcoxon matched-pairs test (ns = not significant, * = $p < 0.05$, *** = $p < 0.001$).

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).

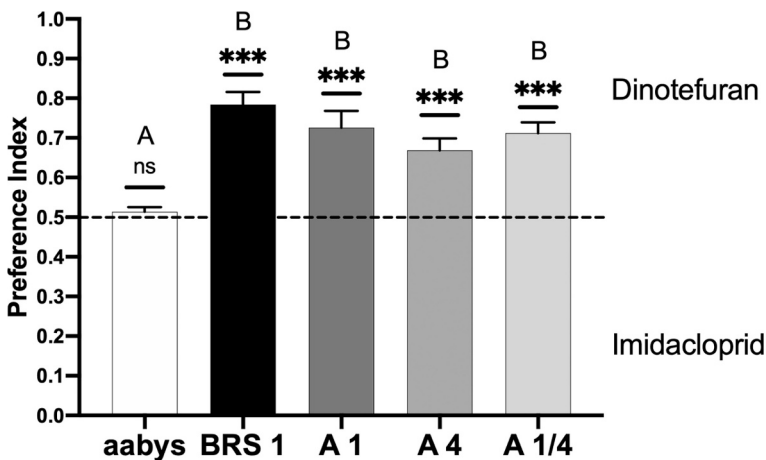


Fig. 6. Fly feeding preference index (PI) with fly lines/strains provided a choice to feed on either sucrose with dinotefuran or sucrose with imidacloprid at the same concentration of 4000 μ g/g sucrose. For comparison, data for parental fly strain BRS 1 is also shown (from Hubbard and Gerry, 2020) in this fig. A significant feeding preference for any single fly line/strain is indicated by *** ($p < 0.001$) following one-sample t-test for $PI \neq 0.5$. Different letters above each column indicates significant difference in feeding preference among fly lines/strains.

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 Pinterova, 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 cyclo-dienes (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 selected fly lines spent significantly less time in contact with the dish containing sucrose mixed with imidacloprid relative to the dish with sucrose alone. And, 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 (Kiryama 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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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.pestbp.2020.104741>.

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