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Concentration Dependent Feeding on Imidacloprid by Behaviorally Resistant House Flies, *Musca domestica* L. (Diptera: Muscidae)

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Abstract

The house fly (*Musca domestica* L.) is a cosmopolitan and synanthropic pest fly commonly associated with confined animal facilities, known to mechanically vector numerous disease-causing pathogens. Control of adult house flies often relies on insecticides formulated into insecticidal baits, though many baits have failed due to insecticide resistance. House fly resistance to imidacloprid, the most widely used neonicotinoid insecticide available for fly control, has evolved through physiological and behavioral mechanisms in field populations. Behavioral resistance to imidacloprid was documented in field populations of flies from southern California dairies. Lab colonies of these flies were established and behavioral resistance to imidacloprid was selected over several generations. The current study examined the ability of these lab-selected flies to feed on varying concentrations of imidacloprid formulated in sucrose, and if these flies would demonstrate a feeding preference for different concentrations of imidacloprid when exposed in bioassays. Behaviorally resistant flies preferred to feed on untreated sucrose as opposed to treated sucrose at concentrations greater than 25 μg/g imidacloprid when provided sucrose treated with and without imidacloprid. When provisioned with only sucrose treated with a low and high imidacloprid concentration, flies fed on the low concentrations (≤100 μg/g) imidacloprid but reduced feeding on either treatment when concentrations were >100 μg/g imidacloprid. The current study extends the body of knowledge on house fly behavioral resistance to imidacloprid, which could provide insights into future failures of granular fly baits.

Key words: neonicotinoid, insecticide, feeding preference, aversion

The house fly (*Musca domestica* L.) is a cosmopolitan and synanthropic pest fly species commonly associated with confined animal facilities and urban waste storage (Geden et al. 2021). House flies are considerable nuisance pests and have been implicated in the mechanical transmission of over 200 pathogens (Nayduch and Burrus 2017, Geden et al. 2021). Failure to control these flies has resulted in litigation against animal producers (Wall Street Journal 2018). One of the most common methods for the control of adult house flies is the use of insecticides formulated into insecticidal baits. Insecticidal fly baits are easy to use, low cost, and have a low risk of off-target effects (Keiding 1975, Chapman et al. 1998, Darbro and Mullens 2004). These baits are manufactured to contain a toxicant and a phagostimulant (e.g., sucrose) to induce fly feeding. Baits are placed in bait stations or scattered on the ground in areas with high fly activity (Darbro and Mullens 2004). However, overapplication and a lack of rotation of insecticidal products have led to insecticidal bait resistance among a multitude of insecticidal chemical classes (Georghiou 1972, Darbro and Mullens 2004, Kaufman et al. 2006, Zhu et al. 2016).

House fly resistance to imidacloprid, the most widely used neonicotinoid insecticide available for fly control, has evolved in field populations through physiological and behavioral mechanisms (Kaufman et al. 2006, Gerry and Zhang 2009, Hubbard and Gerry 2020). Physiological resistance to insecticides in the house fly is associated with well-characterized physiological changes in the fly population including change to the structure of insecticide target sites (target site insensitivity) or increased production of toxin-metabolizing enzymes (Rinkevich et al. 2006, Ma et al. 2019) resulting from metabolic activity of several classes of enzymes. Physiological resistance to imidacloprid has been postulated to be caused by an overexpression of a microsomal glutathione S-transferase gene 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 is characterized by a behavioral change of an organism to avoid a toxicant, which can be categorized as either a stimulus-independent or stimulus-dependent response (Georghiou 1972, Fouet et al. 2018). Stimulus-dependent behavioral resistance to imidacloprid was documented in field populations of house flies from southern California dairies, though the resistance was not uniform among individuals in the population (Gerry and Zhang 2009, Murillo et al. 2014, Hubbard and Gerry 2020). Hubbard and Gerry (2020) selectively bred field-collected flies for behavioral resistance to imidacloprid without increasing the physiological resistance profile of the selected flies, and behavioral, genetic, and genomic analyses were completed to examine the mechanisms conferring behavioral resistance. (Hubbard and Gerry 2020, 2021).

In previous studies, house fly behavioral resistance to the insecticide imidacloprid was selected for and evaluated utilizing choice assays where flies were provided sucrose with or without a high concentration of imidacloprid, (4,000 µg/g; 3x LC95) to ensure that surviving flies had not fed on the imidacloprid-treated sucrose (Hubbard and Gerry 2020). In the current study, we determined the concentration of imidacloprid that will deter feeding by behaviorally resistant house flies and determined whether behaviorally resistant flies demonstrated a feeding preference among sucrose treatments formulated with different concentrations of imidacloprid.

Materials and Methods

Reference Fly Colonies

Two house fly strains were utilized in this study: an imidacloprid-behaviorally susceptible house fly strain (WT) (collected from a Southern California Dairy in 2015), and a strain previously selected for behavioral resistance to imidacloprid over 10 generations of selection (BRS - 1) (Hubbard and Gerry 2020). Fly strains were maintained in an insectary room at 27°C, 35% RH, and a photoperiod of 14:10 (L:D) h reared following standard practices (Zahn and Gerry 2018).

Concentration Dependent Feeding on Imidacloprid

To determine if behaviorally resistant house flies were willing to feed on varying concentrations of imidacloprid, feeding preference assays were performed with the WT and BRS - 1 fly strains (Wisotsky et al. 2011, Bantel and Tessier 2016). Three-to-five-day old adult female house flies were starved overnight for 14 h, sorted into groups of 25 on an electronic chill plate (Catalog #1431, BioQuip Products Inc., Compton, CA) and placed into assay chambers (inverted 947-ml polypropylene deli containers with a removable plastic lid and a bottom modified by adding a fiberglass screen) (Hubbard and Gerry 2020). Each experimental chamber was provisioned with water and two 37-ml soufflé cups, one containing 1g sucrose pretreated with a specified imidacloprid concentration in acetone (5, 10, 25, 100, 125, 500, 1 000, 2 000, 4 000 µg/g) (Sigma-Aldrich Chemical Co., St. Louis, Mo) and the other containing 1g sucrose treated with acetone only (control) (Hubbard and Gerry 2020). For all treatments, the acetone was allowed to volatilize from the sucrose by placement within a fume hood for 14–20 h prior to each trial. Each sucrose treatment within an experimental chamber was colored either blue or red using food grade coloring solution (McCormick & Co., Inc., Hunt Valley, MD), with colors rotated between treated and untreated sucrose. Flies placed into each experimental chamber were allowed to feed for 24 h under dark conditions within the laboratory, before being killed by freezing and sorted via abdomen color; red, blue, purple (fed on both treatments), or blank (did not feed) (Fig. 1A). If the color of the fly abdomen was in question, dissection of the abdomen to extract the alimentary canal was performed to confirm feeding preference. Two assay chambers were paired and run concurrently for each imidacloprid concentration (total of 20 experimental chambers per replicate trial) with food color assigned to the treatment or control food cup alternating among the two paired experimental chambers to account for any possible color effects. Additionally, two control assay chambers were set up per replicate where 25 female flies were provisioned with water, and two 37 ml soufflé cups with 1g sucrose treated with acetone only (one colored blue and the other red).

The preference index for each concentration and fly strain was calculated using the following:

$$P_{S/I} = \frac{N_r - N_b}{N_r + N_b + N_p} \text{ or } \frac{N_b - N_r}{N_r + N_b + N_p} \text{ and } P_{F/NF} = \frac{N_r + N_b + N_p}{N_r + N_b + N_p + N_B}$$

where $P_{S/I}$ is the preference of flies to feed on sucrose control over imidacloprid treated sucrose at the test concentration and $P_{F/NF}$ is the preference for flies to feed on any concentration imidacloprid over not feeding (NF) at all. N_r, N_b, N_p, and N_B are the number of flies with red, blue, purple, and blank abdomens respectively. Five replicates were performed with each fly strain.

Concentration Dependent Preference of Imidacloprid

To determine if behaviorally resistant flies demonstrate a preference to feed on sucrose containing imidacloprid at a lower concentration relative to sucrose with imidacloprid at a higher concentration, feeding preference assays were performed with both the WT and BRS - 1 fly strain as described above (Bantel and Tessier 2016), but with the following modifications: each experimental chamber (as described above) was provisioned with water and two 37 ml soufflé cups, one containing 1 g sucrose with a specified imidacloprid concentration (5, 10, 25, 100, 500, 1,000, 2,000 µg/g) and the other containing 1 g sucrose with 4,000 µg/g imidacloprid (Fig. 1B). 4,000 µg/g imidacloprid was selected as the high concentration of imidacloprid because in previous studies, house fly behavioral resistance to the insecticide imidacloprid was selected for and evaluated utilizing this concentration (Hubbard and Gerry 2020, 2021). Preference index for each concentration and fly strain were calculated using the above formulas, except the index was calculated to determine the preference of flies to feed on the low concentration imidacloprid (ImL) over the high concentration imidacloprid (ImH) ($P_{ImL/ImH}$). Only trials that had at least 50% response rate were included in $P_{ImL/ImH}$ calculations. Three replicates were performed per fly strain and imidacloprid concentration. Colored sucrose was used as above and rotated between paired treatments.

Statistics

All statistical analyses were performed in GraphPad Prism version 9.3.1 for macOS (GraphPad Software, La Jolla California USA, www.graphpad.com). Paired t-tests were performed on each reciprocal treatment first to determine if feeding differences between food coloring were observed. Replicates within each fly strain and concentration were combined if no differences were observed. For each

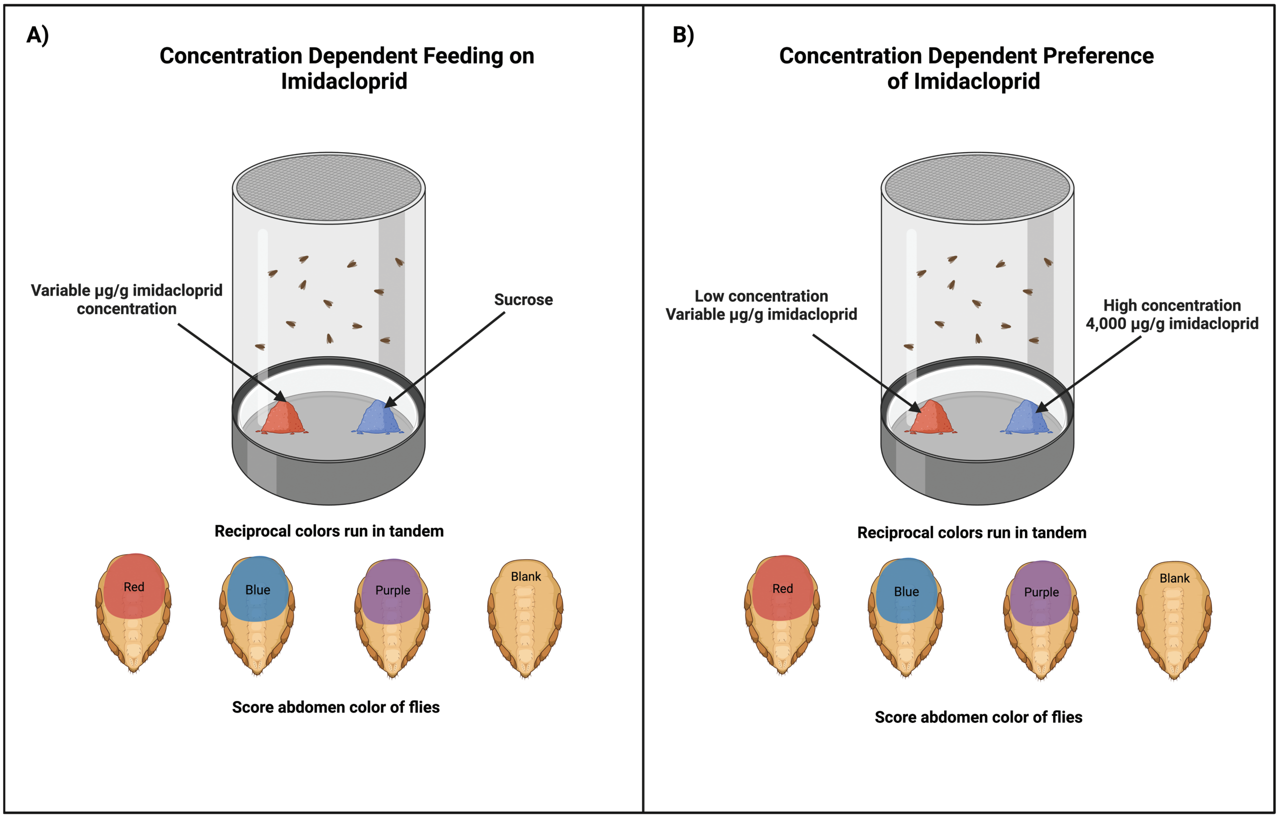

Fig. 1. Graphical representation of the experimental setup for Objective 1 (A), and Objective 2 (B) created with BioRender.com. Treatments in Objective 1 were sucrose treated with a variable concentration of imidacloprid and sucrose treated only with acetone and treatments in Objective 2 were sucrose treated with a variable concentration of imidacloprid and sucrose treated only with acetone. Fly abdomens were scored as either red or blue (fed on one treatment only), purple (fed on both treatments), or clear (no feeding).

fly strain and imidacloprid concentration, one-sample *t*-tests were performed to detect differences between treatment feeding preference or willingness to feed as compared to an expected no preference value ($P_{S/I} = 0$; $P_{F/NF} = 0$)

Results

Concentration Dependent Feeding on Imidacloprid

No significant differences were found between paired treatments with different food coloring (all $t < 2.5$; df = 4; $P > 0.0673$), so replicates were combined within fly strain and concentration. WT flies preferred to feed on sucrose treated with imidacloprid over untreated sucrose at the concentrations of 5 µg/g ($t = 4.606$; df = 9; $P = 0.0013$) and 125 µg/g ($t = 2.678$; df = 9; $P = 0.0253$) imidacloprid and showed no significant preference for either sucrose treated with or without imidacloprid at the other concentrations tested (all $t \le 2.182$; df = 9; $P > 0.0570$) (Fig. 2A). WT flies fed on either of the two treatments at concentrations less than 1,000 µg/g imidacloprid (all $t \ge 3.826$; df = 9; $P < 0.0041$) (Fig. 2B). BRS - 1 flies preferred to feed on untreated sucrose over treated sucrose at a concentration greater than 25 µg/g (all $t \ge 6.185$; df = 9; $P < 0.0002$) (Fig. 2C) and fed on either of the two treatments at all concentrations (all $t \ge 20.60$; df = 9; $P < 0.0001$) (Fig. 2D).

Concentration Dependent Preference for Imidacloprid

No significant differences between paired treatments with different food coloring were observed (all $t \le 1.99$; df = 2; $P > 0.1842$), so replicates were combined within fly strain and imidacloprid concentration. WT flies did not exhibit a feeding preference for sucrose with a lower concentration of imidacloprid relative to sucrose with the high concentration of imidacloprid (4,000 ug/g) (all $t \le 1.308$; df = 5; $P > 0.2477$), except for imidacloprid at 1,000 µg/g which the WT flies preferred to feed on over the 4,000 µg/g concentration ($t = 3.177$; df = 5; $P = 0.0246$) (Fig. 3A). WT flies fed on either of the two treatments at all concentrations tested (all $t \ge 4.964$; df = 5; $P < 0.0042$) (Fig. 3B). BRS - 1 flies preferred to feed on low concentrations of imidacloprid (≤100 µg imidacloprid) over the high concentration (all $t \ge 19.81$; df = 5; $P < 0.0001$) (Fig. 3C). When the low concentration of imidacloprid was greater than 100 µg/g imidacloprid, less than 50% of flies responded to the assay (i.e., fed at all), thus no preference analyses between low and high concentrations could be calculated. No differences were detected for BRS - 1 flies to feed or not feed on either of two treatments when exposed to the low concentration of 100 µg/g imidacloprid and the high concentration of 4,000 µg/g imidacloprid ($t = 2.457$; df = 5, $P = 0.0574$). BRS - 1 preferred not to feed when exposed to a low dose >500 µg/g imidacloprid and the high concentration of 4,000 µg/g imidacloprid (Fig. 3D).

Discussion

Behavioral resistance to imidacloprid in the house fly is expressed as a contact dependent avoidance behavior that reduces the length of time a fly is in contact with and feeding on sucrose treated with the insecticide (Hubbard and Gerry 2020, 2021).In the aforementioned study, behavioral resistance to imidacloprid was selected for and evaluated using a high concentration of imidacloprid (4,000

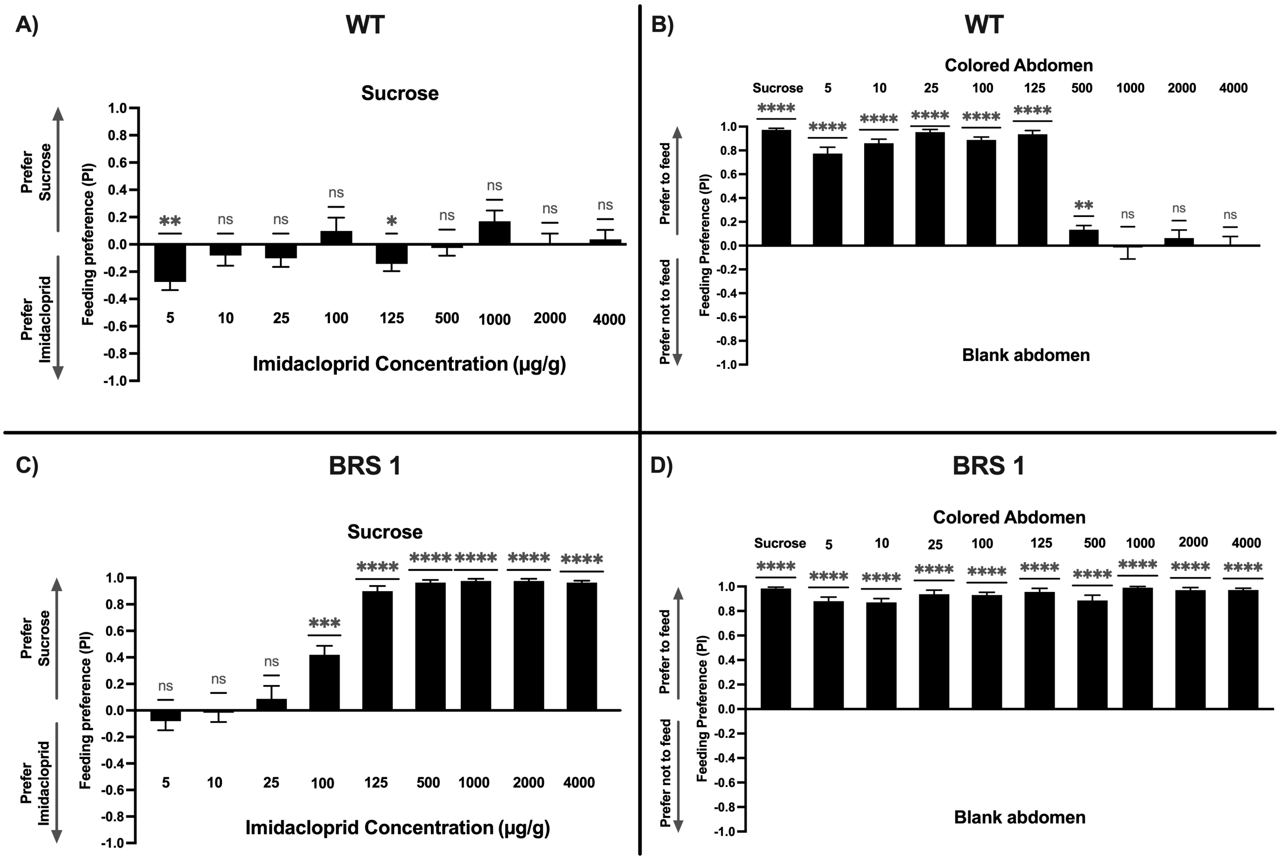

Fig. 2. Fly feeding preference index for two strains of house flies (n = 25/trt) provided a choice to feed on either sucrose with or without imidacloprid at a specified concentration ($P_{S/I}$: Box A and C) and the feeding preference for flies to respond to the assay and feed on either of the two treatments ($P_{F/NF}$: Box B and D). A $P_{S/I}$ > 0 indicates a greater proportion of flies fed on sucrose without imidacloprid, a $P_{S/I}$ < 0 indicates a greater proportion of flies fed on sucrose with imidacloprid, and a $P_{S/I}$ = 0 indicates that flies fed equally on sucrose with or without imidacloprid. A $P_{F/NF}$ value > 0 indicates that a greater proportion of flies fed on sucrose with or without imidacloprid than did not feed, a $P_{S/I}$ < 0 indicates a greater proportion of flies did not feed, and a $P_{S/I}$ = 0 indicates an equal proportion of flies fed or did not feed. A significant preference for a treatment was determined by one-sample *t*-test (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$).

µg/g), but it was unknown if behaviorally resistant house flies could detect or exhibit feeding aversion to lower concentrations of imidacloprid.

Wild type imidacloprid susceptible flies showed no preference for feeding on sucrose alone relative to sucrose with imidacloprid at concentrations of 5–4,000 µg imidacloprid per g sucrose, except at imidacloprid concentrations of 5 and 125 µg/g. Although this was likely a statistical artifact and not biologically significant as no significant feeding preference was observed at concentrations of 5 and 125 µg/g. Wild type flies also readily fed on sucrose containing even the highest concentrations of imidacloprid tested (up to 2,000 ug/g). In contrast to the wild type flies, the BRS - 1 flies that were previously selected for strong behavioral resistance exhibit a strong preference for sucrose alone over all sucrose with imidacloprid at concentrations >25 µg/g imidacloprid, but exhibited no preference for sucrose alone relative to sucrose with imidacloprid at concentrations <25 ug/g. This suggests that behaviorally resistant flies either cannot detect imidacloprid at concentrations less than 25 µg/g or those concentrations do not elicit an aversive response.

A concentration dependent preference for untreated sucrose (or an inverse concentration dependent avoidance of imidacloprid treated sucrose) was observed as BRS - 1 flies exhibited low or no preference for untreated sucrose when also provisioned with sucrose treated with low concentrations of imidacloprid (5, 10, 25 µg/g). BRS - 1 flies exhibited a greater preference for untreated sucrose when provisioned with sucrose treated with 100, 125, and 500 µg/g imidacloprid. At imidacloprid concentrations greater than 125 µg/g, preference for untreated sucrose was approximately the same (~0.96) in BRS - 1 flies. In glucose averse (GA) German cockroaches a true inverse dose-dependent response curve (high acceptance of glucose at low concentrations with a decreasing acceptance rate the higher the glucose concentration) is observed when providing cockroaches varying concentrations of glucose (Wada-Katsumata and Schal 2021). While behaviorally resistant flies exhibit a concentration dependent response like that observed in GA cockroaches, the lack of preference to feed on sucrose at low concentrations in the current study indicates behaviorally resistant flies do not have the ability to discern varying concentrations of imidacloprid in a similar way GA cockroaches detect varying glucose concentrations.

BRS - 1 flies were shown to respond (feed on either of the two treatments), at all concentrations which is consistent with previous work that determined behavioral resistance in the house fly was to the insecticide itself (imidacloprid) and not the phagostimulant (sucrose), as behaviorally resistant flies would readily feed on sucrose food bait when imidacloprid is not present (Hubbard and Gerry 2021). The lack of preference to feed at high concentrations of imidacloprid may be due to physiological effects (morbidity or death) the flies experience after contacting the sucrose treated with the insecticide, though future work should be conducted to confirm this hypothesis.

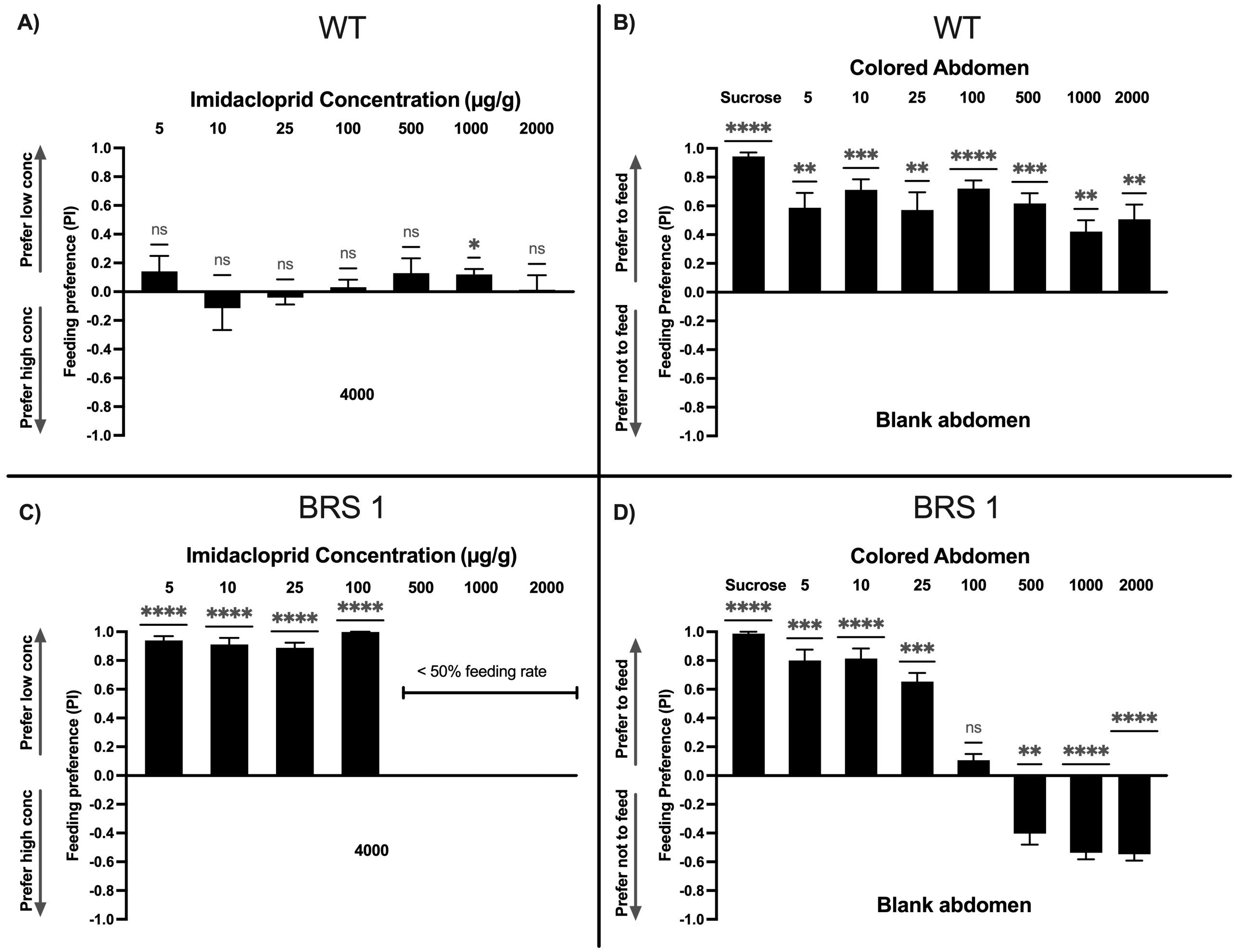

Fig. 3. Fly feeding preference index for flies provided a choice to feed on either sucrose treated with a lower concentration or a higher concentration of imidacloprid ($P_{Il/Ih}$: Box A and C) and the feeding preference for flies to respond to the assay and feed on either of the two treatments ($P_{F/NF}$: Box B and D). A $P_{ImL/ImH}$ > 0 indicates a greater proportion of flies fed on sucrose with a lower imidacloprid concentration, a $P_{ImL/ImH}$ < 0 indicates a greater proportion of flies fed on sucrose with a higher imidacloprid concentration, and a $P_{ImL/ImH}$ = 0 indicates that flies fed equally on sucrose treated with either concentration of imidacloprid. A $P_{F/NF}$ value >0 indicates that a greater proportion of flies responded to the treatments (fed) than did not respond (not fed), a $P_{F/NF}$ < 0 indicates a greater proportion of flies did not respond to either treatment, and a $P_{F/NF}$ = 0 indicates an equal proportion of flies responded and did not respond. A significant preference for a treatment was determined by one-sample t-test (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$).

When challenging flies with sucrose treated with two concentrations of imidacloprid (a variable low concentration and a fixed high concentration), BRS - 1 flies preferred to feed on low concentrations of imidacloprid (≤100 μg imidacloprid) over the high concentration, but >50% of flies did not feed on either of the sucrose formulations when the low concentration of imidacloprid was >100 μg imidacloprid. This supports the results described in Objective 1 as behaviorally resistant flies exhibited a high preference (>0.96) to feed on untreated sucrose over treated sucrose at imidacloprid concentrations of 500–4,000 μg/g. In Objective 1 and in Objective 2 BRS - 1 flies preferred to not feed on either treated sucrose option when the imidacloprid concentration was >100 μg imidacloprid.

The results presented from Objectives 1 and 2 indicate that BRS - 1 house flies will choose to feed and discern between varying imidacloprid concentrations, which allows the flies to avoid feeding on a lethal concentration of imidacloprid. Considering the current result, when flies have access to alternative food sources, such as on an agricultural facility, behavioral resistance to imidacloprid may have a protective effect for flies as they can detect concentrations of imidacloprid, allowing them the opportunity to avoid feeding on a lethal insecticide dose. Behavioral resistance to imidacloprid potentially threatens future imidacloprid-containing bait efficacies, as in this study resistant flies could detect and avoid the high concentration of insecticide formulated in sucrose. We would expect these results to be the same with commercially formulated fly baits containing imidacloprid, though that has not yet been tested with the BRS - 1 fly strain. The detection of imidacloprid by house flies may contribute to imidacloprid baits failing on some southern California dairies soon after they were implemented for use (Hubbard and Gerry 2020).

It is unknown if different populations of behaviorally resistant flies would exhibit the same threshold for detecting imidacloprid as observed in the BRS - 1 colony, or if other factors play a role in the threshold for detecting imidacloprid such as physiological resistance to imidacloprid or other toxicants. In the behaviorally resistant fly strain utilized in the current study, the imidacloprid concentration at which flies avoid feeding is less than the current LC_{50} of 779.058 (709.431–855.519) μg/g for this fly strain (Hubbard et al. in preparation) though additional research should be conducted to determine if a correlation is observed.

Future studies should address how behaviorally resistant house flies detect imidacloprid. Imidacloprid may stimulate bitter gustatory receptor neurons, as was shown in glucose averse German cockroaches (Wada-Katsumata et al. 2013), which would explain why a similar concentration dependent aversion is observed in both the cockroaches and house flies.

The current study provides insight into the ability of the house fly to detect and avoid imidacloprid and may suggest mechanisms by which house flies similarly avoid other noxious or dangerous compounds. This work provides a foundation for future studies to examine the mechanisms contributing to imidacloprid detection and can be utilized as a framework to screen additional fly populations for imidacloprid behavioral resistance.

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