Selection for Resistance to Imidacloprid in the House Fly (Diptera: Muscidae)

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ABSTRACT The house fly, *Musca domestica* L. (Diptera: Muscidae), continues to be a primary pest of livestock facilities worldwide. This pest also has shown a propensity for pesticide resistance development when under high selection pressures. In this study the house fly strain FDm was created by a 20% contribution from each of five colonies collected from dairies in Florida with known imidacloprid resistance. The FDm strain was used to evaluate the level of imidacloprid resistance after five selections near the LC_{70} value of each selected generation. Overall, the mean selection mortality was 72.7, with males being considerably more susceptible than females. The unselected (F_0) FDm strain showed considerable susceptibility to imidacloprid after its creation, compared with the five parental strains. Between 9,500 and 14,000 virgin house flies were used in each selection. After the fifth and final selection, a 331-fold increase in imidacloprid resistance at the LC_{70} was observed over the parental FDm strain. In parallel studies, the FDm strain showed increasing tolerance of the commercial imidacloprid product QuickBayt. These results suggest that livestock producers should use caution when choosing pesticides and consider rotating fly baits, as is encouraged with other pesticide treatment regimes on farms.

KEY WORDS *Musca domestica*, insecticide bait, integrated pest management, dairy pest management, insecticide resistance

The house fly, *Musca domestica* L. (Diptera: Muscidae), continues to be a primary pest of confined livestock. New insecticide chemistries for use on livestock facilities have been released only infrequently, making the preservation of efficacy among the existing active ingredients a priority. However, when a new active ingredient is introduced to a commodity, the high efficacy of the product for the producer often takes precedence over the managing of its use in ways that mitigate resistance development.

The house fly has repeatedly developed resistance to insecticides in a variety of classes (Pap and Farkas 1994, Scott et al. 2000, Deacutis et al. 2006, Butler et al. 2007, Kaufman et al. 2010). This resistance development is due to several issues, including the flies' widespread distribution, high population densities on livestock facilities, rapid developmental time, cross-resistance among insecticide classes, and public health and nuisance problems that encourage livestock producers to apply insecticides for fly control.

In contrast to those insecticides that are applied as residual premise treatments, resistance development to chemistries presented only as baits has been slow, and many of these products are highly effective (Scott et al. 2000, Butler et al. 2007, White et al. 2007, Ahmad and Zurek 2009). Deacutis et al. (2006) and Kaufman et al. (2006) conducted resistance profiling before and after the initial use of spinosad and imidacloprid, respectively. In both studies, resistance was not readily apparent in the short time that these products were used, suggesting baits are not as susceptible to resistance evolution as other formulations. The predominant fly baits used before the release of the imidacloprid-containing bait QuickBayt were those containing methomyl. Resistance to methomyl-containing baits has been documented, but it has often been attributed to behavioral rather than physiological mechanisms (Pap and Farkas 1994, Learmount et al. 1996, Kaufman et al. 2001, Learmount et al. 2002, Darbro and Mullens 2004, Butler et al. 2007). Freeman and Pinniger (1992) demonstrated that behavioral resistance to an azamethiphos spray-on bait was due to inert components or contaminants in the formulation, rather than the insecticide.

The livestock industry has a history of rapidly adopting new fly-targeting insecticides; the adoption of imidacloprid-containing QuickBayt after its registration in the United States in 2004 was no exception. The product continues to be used regularly on a wide range of animal production facilities, including poultry, dairy, and confined beef. The house fly is the primary target of this product and initial use and adoption by

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producers attest to its effectiveness. However, house flies have a diverse array of resistance mechanisms to every chemical that is commonly used to control them. In fact, previous studies documented monooxygenase-mediated cross-resistance to imidacloprid by an avermectin-resistant house fly strain (Wen and Scott 1997). However, these same authors and others (Liu and Yue 2000) determined that mechanisms other than monooxygenases were not a major mechanism of cross-resistance to imidacloprid in permethrin-resistant house flies. Studies conducted several years after the commercial release of imidacloprid for use against house flies suggest that imidacloprid may be losing its effectiveness in fly populations (Gerry and Zhang 2009, Kaufman et al. 2010). Studies performed by Kaufman et al. (2006) documented both an increase and decrease in house fly susceptibility, with overall similar results to initial evaluations 1 and 2 yr earlier. This study established susceptibility in field populations across the United States in 2004, the year of the product's initial use as a fly bait, in an attempt to provide a baseline for future studies. Although development of avoidance behavior-based resistance seems to be evident in the Gerry and Zhang (2009) study, to date no studies have documented the progression of physiological tolerance or resistance to imidacloprid when provided in a feeding assay.

The objective of this research was to record house fly resistance development to imidacloprid after selection of adult flies. This has been accomplished for other chemistries, including abamectin (Scott et al. 1991) and azamethiphos (Kristensen et al. 2000). The perception that imidacloprid baits would have long-lasting efficacy on livestock facilities due to limited overall exposure has been shown to be false (Gerry and Zhang 2009, Kaufman et al. 2010). Newer formulations that allow for wider use and exposure may only accelerate resistance selection. In this study, we selected progressive generations of a hybrid laboratory house fly colony with imidacloprid and measured the subsequent resistance development through 1) standard LC measures and 2) in choice and no-choice feeding assays with formulated product that contained a much higher concentration of imidacloprid.

Materials and Methods

House fly selections were performed on a colony resulting from the mixture of several Florida field populations. This strain (FDm) was created in November 2009 by combining pupae from each of five laboratory-reared house fly strains previously colonized from wild individuals collected from dairies in Gilchrist, Lafayette, Okeechobee, and Alachua counties as described in Kaufman et al. (2010). House flies from these colonies were previously evaluated for pesticide resistance by Kaufman et al. (2010). The FDm colony was started from a total of 300 ml of pupae (60 ml from each strain) placed in each of two rearing cages and maintained as described by Scott et al. (2000).

House fly selections with imidacloprid began using the F_{11} generation (parental generation, F_0) of the FDm strain. House fly adults were selected with technical grade imidacloprid (99.5%; Chem Service, West Chester, PA). A total of five selections were conducted using imidacloprid-treated sugar cubes, with a final evaluation of imidacloprid susceptibility after the fifth selection.

Preliminary evaluation of imidacloprid susceptibility for the parental house fly strain was carried out using a feeding bioassay to determine the necessary LC for the desired selection (70% mortality). In addition, each successive generation was similarly evaluated to obtain a new concentration value before each subsequent selection to maintain a consistent selection pressure of ≈70% mortality. Imidacloprid serial dilutions were prepared 1 d before the feeding assays, using acetone as the solvent. Sugar cubes (3.5 g) (Dixie Crystals, Imperial-Savannah LP, Sugar Land, TX) were treated with 0.5 ml of one of eight diluted imidacloprid solutions and allowed to dry for 1 h; cubes treated with 0.5 ml of acetone only (no imidacloprid) were used as controls. Twenty adult female house flies (3–5 d old) were placed inside a 500-ml plastic container (17 cm in diameter, 11 cm in height) and provided three treated sugar cubes and water ad libitum via a 120-ml soufflé cup fitted with dental wick (Kaufman et al. 2010). House fly mortality was assessed at 24, 48, 72, and 96 h, scoring ataxic individuals as dead. At each preselection evaluation, four sets of 20 flies were used for each dilution, and the entire experiment was replicated on three separate occasions for a total of 240 house flies tested per dilution. The accrued concentration response data from 96-h mortality was subjected to standard probit analysis using SAS version 9.2 (SAS Institute 2006).

The results from probit analysis were used to determine the LC_{30}, LC_{50}, and LC_{70} values for the FDm strain as well as subsequent offspring from each selection. These values were used as preevaluation concentrations to verify the accuracy of the desired LC_{70} to be used for the subsequent selection. House flies were separated by sex in groups of ≈250 and placed into two (one male and one female) 500-ml plastic containers for each LC test value. Mortality was assessed as previously described for 96 h.

The FDm strain was selected with imidacloprid for five generations. To prevent mating before selection and ensure that only surviving flies contributed genetic material to the next generation, emergent house flies were mechanically aspirated from a rearing cage within 8 h of eclosion, anesthetized with CO_2, and separated by sex (Hamm et al. 2005). These flies were placed in 500-ml plastic containers in groups of ≈400 to be used later in the selection process. House flies remaining in the emergent cage after the initial 8-h period were discarded, and the remaining pupae held at 14°C until the next morning to slow the eclosion process. The next morning, flies that had emerged were discarded and the cage held on the benchtop

allowing flies to emerge over the next 8 h. These flies were sorted and held as described above. This procedure was repeated until ≈5,000 house flies of each sex had been placed into treatment containers. Five 3.5-g untreated sugar cubes and water were placed in each container as described previously, until the flies were 3–5 d old.

When they had reached the appropriate age for selection, the house flies were anesthetized with CO_2, five sets of representatives of each sex were weighed on a digital balance in groups of 10 flies to determine average individual fly weights. The untreated sugar cubes were replaced with three new 3.5-g sugar cubes previously treated with 0.5 ml of the appropriate imidacloprid selection dilution. Fly mortality was assessed before and after the sugar cube transfer to account for anesthetization effect. Male and female house fly mortality was assessed every 24 h as described previously. However, mortality in containers with male house flies often reached 70% before the end of the 96-h holding period. Therefore, when mortality in these containers reached 70% or more, the flies were anesthetized and the imidacloprid-treated sugar cubes were replaced with untreated cubes for the remainder of the 96-h holding period. After the 96-h holding period, the house flies in all containers were anesthetized with CO_2 and transferred to a single rearing cage via a 120-ml plastic soufflé cup. These cups were left in place for 2 h to allow surviving house flies to exit. All dead flies remaining in the transfer cups were counted and mortality determined for each sex, for each selection. Surviving house flies were maintained in an incubator at 80°C and a photoperiod of 12:12 (L:D) h.

During the reevaluation of imidacloprid susceptibilities on the offspring of each selected generation, concurrent evaluation of the efficacy of the formulated scatter bait, QuickBayt Fly Bait (Bayer, Shawnee Mission, KS) was conducted. These feeding assays were conducted in a manner similar to those described above. In brief, 20 female house flies (3–5 d old) were placed into 500-ml plastic containers having one of three treatments. These treatments included water and one of the following treatments 1) 3.5 g of QuickBayt, 2) 3.5 g of QuickBayt and one 3.5-g untreated sugar cube, and 3) one 3.5-g untreated sugar cube only (controls). Mortality was assessed every 24 h for 96 h. Mortality observations included ataxic flies (dead) that were inside or outside cups containing QuickBayt, and flies unable to right themselves. This experiment was replicated once before each selection, and each treatment had five containers for a total of 100 female house flies per treatment. To identify whether differences between the QuickBayt-containing treatments existed within each selection generation, paired *t*-tests were conducted using SAS version 9.2 (SAS Institute 2006). Data were analyzed after an arcsine(sqrt) transformation of the percent mortality responses. Nontransformed data are presented in text and tables.

Table 1. Selection concentrations and mortality of the FDm house fly strain in no-choice imidacloprid selections on successive generations

Selected generation[a]	No. flies selected	Concn (μg[AI]/g sugar)	Wt (mg)[b]		% mortality		
			Male	Female	Male	Female	Mean
F_0	10,681	33.4	11.2	19.8	83	64	73.5
F_1	13,147	137.7	11.7	20.9	97	71	84.0
F_2	9,968	163.9	9.2	18.8	79	45	62.0
F_3	12,470	701.4	9.2	15.7	85	62	73.5
F_4	9,628	1,420.9	11.0	16.5	79	62	70.5
F_5			11.1	19.2			

[a] F_0 was the parental strain. Mortality assessed following 96-h exposure.
[b] Average fly wt as determined from five sets of 10 flies.

Results

House fly weights were relatively stable throughout the study (Table 1) and after the final selection were quite similar to the preselection weight. Between 9,500 and 13,500 house flies were selected for imidacloprid tolerance at each generation. Male mortality was always greater than female mortality within a selection generation, and in some cases imidacloprid sugar cubes were removed from male cages to balance our selection parameter with the need to have sufficient numbers of males to sustain the fly colony (Table 1). Although fly mortality was targeted at 70%, actual mortality across the study ranged from 45 to 97% (Table 1). To ensure that enough flies were available for the subsequent selection, the LC dilution used for each selection differed at each selection generation but was between LC_{30} and LC_{70} (Table 2). The concentration of imidacloprid needed to obtain the targeted 70% mortality increased from 33.43 μg of imi-

Table 2. Estimated imidacloprid LC-values generated in sugar-imidacloprid feeding assays for each house fly strain prior to imidacloprid selection of that generation

Generation[a]	LC	LC (CI) (μg/g)	Slope (SE)	χ^2	RR
F_0	30	11.6 (10.1–13.2)	2.29 (0.13)	164.86	
	50	19.7 (17.6–22.1)			
	70*	33.4 (29.7–38.1)			
F_1	30	57.8 (47.4–68.9)	1.39 (0.08)	185.81	5.0
	50*	138 (117–161)			7.0
	70	328 (276–399)			9.8
F_2	30*	164 (135–195)	1.25 (0.07)	168.19	14.1
	50	432 (367–512)			21.9
	70	1,140 (933–1439)			34.1
F_3	30	247 (207–290)	1.16 (0.06)	116.17	21.3
	50*	701 (608–811)			35.6
	70	1,990 (1,670–2,430)			59.6
F_4	30	363 (278–457)	0.89 (0.06)	138.16	31.3
	50*	1,421 (1,170–1,738)			72.1
	70	5,554 (4,292–7,582)			166.3
F_5	30	1,005 (832–1,189)	1.01 (0.05)	99.70	86.6
	50	3,337 (2,877–3,891)			169.4
	70	11,083 (9,113–13,897)			331.8

These data were used to choose the LC value (*) at which to select the same-generation flies.
[a] F_0 was the parental strain. n = 1,920 flies per evaluation. RR calculated as $RR_X = LC_{XFi}/LC_{XF0}$, where X is the LC dose and Fi is the fly generation.

Table 3. Percentage of mortality of female house flies in choice and no-choice assays with QuickBayt by using successive generations of imidacloprid-selected FDm house flies

Selected generation	RR_{50}[a]	% mortality (SE)[b]		
		Sugar only	Sugar + QuickBayt	QuickBayt only
F_0 (parental)		4 (2.9)	99 (1.0)	100 (0.0)
F_1	7.0	6 (1.9)	96 (1.9)	100 (0.0)
F_2	21.9	3 (2.0)	99 (1.0)	99 (1.0)
F_3	35.6	3 (1.2)	79 (5.6)*	94 (1.9)*
F_4	72.1	1 (1.0)	84 (4.6)	92 (1.2)
F_5	169.4	6 (1.0)	84 (5.3)	90 (2.2)

[a] RR compared with the parental generation using LC_{50} values generated in sugar-imidacloprid serial dilution bioassays.

[b] Female house flies (3 d posteclosion). Mortality was assessed 96-h after introduction. $n = 5$, with 20 flies per exposure container. The asterisk (*) within generation, treatments containing QuickBayt mortality was significantly different ($\alpha = 0.05$, $t = -2.851$, df = 4, $P < 0.0463$).

dacloprid (AI)/g sugar with the parental strain to >1,420 μg (AI)/g sugar in the fifth selection (F_4) (Table 1).

The initial LC_{70} for the parental (F_0) house flies was 33.4 μg (AI)/g sugar, which was 0.0033% imidacloprid (Table 2). This value increased to 11,083 μg (AI)/g sugar after five selections. This resulted in a resistance ratio (RR) that was 331.8-fold greater than the parental strain. This also represented a 1.11% imidacloprid concentration in the sugar bait. The formulated product QuickBayt contains 0.5% imidacloprid.

Fly mortality from the choice versus no choice assays demonstrated increased survival to the formulated QuickBayt product after three generations of selection (Table 3). Although mortality remained high, even after the fifth selection (F_4), it was apparent that increasing numbers of flies were able to survive exposure to the bait in the absence of an alternative food source. However, only during the F_3 generation were significantly more flies killed in the QuickBayt-only treatment than the choice treatment (Table 3).

Discussion

The FDm house fly strain used in this study was created from contributions of five strains whose imidacloprid resistance profiles were described previously (Kaufman et al. 2010). The Lafayette strain had imidacloprid LC_{50} and LC_{90} values of 231 and 1,550 μg (AI)/g sugar, respectively. The imidacloprid LC_{50} values of the remaining four fly strains ranged from 38 to 77 μg (AI)/g sugar. When these five strains were merged to form the FDm strain, although each strain contributed 20% to the FDm strain, the imidacloprid LC_{50} value fell to 19.7 μg (AI)/g sugar (Table 2). This was surprising as the new value was one half that of the most susceptible strain in the mixture and approached that of our laboratory susceptible strain (LC_{50} = 18 μg [AI])/g sugar) (Kaufman et al. 2010). A similar trend was observed with the pyrethroids examined in Kaufman et al. (2010).

Our preliminary screening assay allowed us to target imidacloprid concentration exposures that neared the LC_{70}. The over-exposure in the F_1 selection (84% mortality), particularly where 97% of males were killed, led to an underexposure (62% mortality) during the F_2 selection where the LC_{30} exposure was used. In addition, we used sufficient numbers of house flies that allowed for consecutive generation selections, without allowing for genetic recombination outside of insecticide pressure. Furthermore, our separation of the sexes before 8-h posteclosion ensured that no mating occurred between susceptible males and surviving females (Michelsen 1960, Murvosh et al. 1964). This technique ensured that only genes from survivors would be passed onto subsequent generations, thereby accelerating resistance selection.

Disproportionate survival between sexes also was observed by Scott et al. (1991) and Carrière (2003) who both noted that males were more susceptible than females. Carrière (2003) suggested that sexual size dimorphism and sex-dependent selection may account for sex differences in susceptibility. As noted in Table 1, male house flies weighed considerably less than female flies at each generation.

Gerry and Zhang (2009) reported 72% survival of suspected imidacloprid resistant house flies in choice studies with QuickBayt. In their no-choice assays, resistance was found to be <10-fold; however, in choice trials flies did not feed on the imidacloprid bait, suggesting a behavioral resistance. Freeman and Pinniger (1992) documented that house flies demonstrating behavioral resistance to Alfacron bait containing azamethophos were actually repelled by the formulation not the insecticide. Progressively increasing house fly survival in the choice-no choice bioassay (Table 3) along with the increasing resistance levels in the selection bioassays (Table 2) suggests that the resistance observed was not behavioral avoidance. If flies were able to detect the imidacloprid, survival in the treatments containing both the QuickBayt product and the untreated sugar would have resulted in much less mortality under choice conditions, as that observed by Gerry and Zhang (2009). Although mortality was generally higher in the no-choice assay compared with the choice treatment, especially beginning at the F_3 generation, the amount of imidacloprid that no-choice flies were imbibing would have been twice that of the choice treatment, where random feeding on sugar cubes and QuickBayt would have decreased the amount of imidacloprid consumed. Alternatively, it is also plausible that both physiological and behavioral resistance were developing in the FDm strain. Although this study was not designed to maintain selected generation strains for further evaluation, the observation of differential survival in only the F_3 generation could indicate that there may have been a behavioral resistance pattern emerging but that subsequent imidacloprid selections overrode or masked this resistance mechanism.

Scott and Georghiou (1985) and Shono and Scott (2003) documented the rapidity with which resistance to permethrin and spinosad, respectively, could

be selected in house flies. Scott and Georghiou (1985) documented 5,945-fold resistance to permethrin after 22 selections. In their study, after three 99% mortality selections, they achieved 3.2-fold resistance over the parental strain. Thereafter, their selections were predominantly <90%, with fewer than 3,000 flies per selection. Scott and Georghiou (1985) also noted that resistance to several organophosphate chemistries and DDT increased after their permethrin selection. Shono and Scott (2003) demonstrated 150-fold resistance to spinosad after 10 generations of selection, without cross-resistance to other insecticides. These results suggest that resistance evolution is variable and mechanisms can be diverse.

In our study, resistance to imidacloprid increased 331-fold over five successive generations of selection. These high resistance levels in sugar cube assays resulted in only 10% house fly survival to the formulated imidacloprid bait product. Our imidacloprid feeding assays required 96 h to complete. During this time, house flies must acquire sugar for survival and sugar was available only from the insecticide-treated cubes or the formulated product, respectively. In addition, house flies were readily observed feeding on the treated sugar cubes and the orange-pink QuickBayt product could be observed through the integument in the fly crop.

It is apparent that house flies are capable of developing imidacloprid resistance, both in the field (Kaufman et al. 2006, 2010; Gerry and Zhang 2009) and much more rapidly in the laboratory (current study). The mechanisms of imidacloprid resistance have been speculated on (Wen and Scott 1997) and may differ depending on the population exposed and specific mechanisms involved; however, resistance may not be stable as evidenced by the dramatic drop in resistance expression after the creation of the FDm colony from known-resistant strains. Reports of failures of QuickBayt on farms are increasing, and given the new imidacloprid formulations that are now available for residual premise treatments, field-level resistance reports will probably continue to increase.

From this study, it is apparent that house flies are capable of developing high levels of resistance to imidacloprid when presented as a sugar bait. Furthermore, this study documents the rapidity with which imidacloprid resistance can develop under high selection pressure, and the early appearance of that resistance in field situations. To this end, livestock producers should be informed that imidacloprid resistance is occurring and be encouraged to use a multifaceted integrated pest management program to keep house fly populations below threshold levels. However, pesticides are heavily relied upon on today's livestock facilities. Our results suggest that livestock producers must use caution when choosing their insecticide baits and consider rotating fly baits, as is encouraged with other pesticide treatment regimes on farms. This concept is often not understood, nor has it largely been needed in the past, as baits have typically been considered to generate low resistance-selection pressures. The widespread adoption of imidacloprid and its high effectiveness should signal a change in the way in which this and other bait chemistries are perceived and used.

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