VECTOR CONTROL, PEST MANAGEMENT, RESISTANCE, REPELLENTS

Susceptibility of Cat Fleas (Siphonaptera: Pulicidae) to Fipronil and Imidacloprid Using Adult and Larval Bioassays

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ABSTRACT The monitoring of the susceptibility of fleas to insecticides has typically been conducted by exposing adults on treated surfaces. Other methods such as topical applications of insecticides to adults and larval bioassays on treated rearing media have been developed. Unfortunately, baseline responses of susceptible strains of cat flea, *Ctenocephalides felis* (Bouchè), except for imidacloprid, have not been determined for all on-animal therapies and new classes of chemistry now being used. However, the relationship between adult and larval bioassays of fleas has not been previously investigated. The adult and larval bioassays of fipronil and imidacloprid were compared for both field-collected isolates and laboratory strains. Adult topical bioassays of fipronil and imidacloprid to laboratory strains and field-collected isolates demonstrated that LD_{50}s of fipronil and imidacloprid ranged from 0.11 to 0.40 nanograms per flea and 0.02 to 0.18 nanograms per flea, respectively. Resistance ratios for fipronil and imidacloprid ranged from 0.11 to 2.21. Based on the larval bioassay published for imidacloprid, a larval bioassay was established for fipronil and reported in this article. The ranges of the LC_{50}s of fipronil and imidacloprid in the larval rearing media were 0.07–0.16 and 0.11–0.21 ppm, respectively. Resistance ratios for adult and larval bioassays ranged from 0.11 to 2.2 and 0.58 to 1.75, respectively. Both adult and larval bioassays provided similar patterns for fipronil and imidacloprid. Although the adult bioassays permitted a more precise dosage applied, the larval bioassays allowed for testing isolates without the need to maintain on synthetic or natural hosts.

KEY WORDS *Ctenocephalides felis*, insecticide resistance, resistance monitoring

The monitoring of insecticide resistance in cat fleas, *Ctenocephalides felis* (Bouchè), has been typically conducted with tests similar to a World Health Organization (WHO) procedure that exposes adults on treated filter paper (WHO 1970). Alternative methods of determining the susceptibility of cat fleas to insecticides have been conducted. Moyses (1995) found assays with topical applications of insecticides to adult fleas to be more sensitive and less susceptible to interactions between substrates and insecticides than the WHO filter paper method. A comprehensive review of insecticide resistance in cat fleas by Bossard et al. (1998) summarized the data collected on carbamate, organophosphate, and pyrethroid insecticides. Further studies revealed multiple cross-resistance to

many of these insecticides (Bossard et al. 2002). Moyses and Gfeller (2001) reported the use of topical applications to determine susceptibility of a single laboratory strain to 13 different insecticides. To monitor the susceptibility of field-collected isolates to imidacloprid, a larval bioassay using an insecticide-impregnated food medium was developed by Rust et al. (2002). This method allows for the worldwide shipment of flea eggs, permits the development of a diagnostic dose, and minimizes the need for maintaining flea isolates in the laboratory (Rust et al. 2005).

The relationship between adult and larval bioassays of fleas has not been previously investigated. The objective of this study was to compare adult and larval bioassays of fipronil and imidacloprid against both laboratory strains and field-collected isolates. The utility of each bioassay is discussed.

Materials and Methods

Maintenance of Cat Fleas. The isolates of *C. felis* used for this work were collected as part of a larger study involving the monitoring of cat flea susceptibility to imidacloprid (Kopp et al. 2013). Fleas were maintained on individual cats according to a procedure modified from Metzger and Rust (1996). The cats were housed in double cages and segregated as much

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Table 1. Information regarding the field-collected isolates and the laboratory strains tested

Strain/isolate	Locality	Collection date	Cat or dog	Previous treatments
Auburn	Soquel, CA	1985	Cat	Unknown
Monheim	Hannover, Germany	1992	Cat	Unknown
UCR	Palo Alto, CA	1978	Cat	Unknown
Clancy	Queensland, Australia	14 Aug 2007	Dog	Several unknown
Inky	Jefferson City, MO	30 Aug 2010	Cat	Frontline combo, frontline plus
Jeepers	Charleston, SC	3 June 2009	Dog	Frontline
Kiki	Jacksonville, FL	24 May 2006	Cat	Unknown
Monster	Rochester, NY	2 Nov 2009	Cat	None
Oliver	Jacksonville, FL	6 Jan 2009	Cat	Advantage multi
Sassy	Mount Dora, FL	2 Dec 2009	Dog	Advantage
Tweedle Dee	Houston, TX	9 Jan 2009	Cat	None

as possible to minimize the chance of cross-contamination between isolates. Three different rooms were used to maintain the cats and flea isolates. The maintenance of the cats and rearing of cat fleas were conducted under protocols approved by the Institutional Animal Care and Use Committee of the University of California, Riverside.

Cat flea eggs were collected from trays underneath cats supporting each field-collected or laboratory isolate. The eggs and debris were passed through a series of four sieves (10, 16, 20, and 60 mesh), with the eggs being retained on the 60-mesh screen. The eggs were placed on a larval flea-rearing medium (1 part nutritive medium [0.15 dried beef blood; America's Laboratories, # NK3027034 SD Hemoglobin Powder, Omaha, NE] to 0.75 ground dog chow by weight to 0.1 inactive baker's yeast [Red Star Bio Products-Nuttrex 55, Milwaukee, WI] to three parts 30-mesh silica sand by volume) and held at 26 ± 1°C and 80% relative humidity (RH). Larvae completed development within 11–13 d, and the cocoons and larval medium were passed through a 16-mesh sieve to separate cocoons. Adults emerged ≈16–18 d after egg collection. To maintain the isolates on each cat, ≈30 male and 30 female adult fleas were placed on each cat every 2 wk. Typically, three to four generations were required before sufficient numbers of adult fleas were present for testing.

Data on Flea Strains and Isolates. Table 1 provides collecting information regarding the strains and isolates tested. Not all isolates were tested with both imidacloprid and fipronil. Unfortunately, there has never been a standard "susceptible strain" of *C. felis* used consistently for insecticide testing and resistance monitoring in different laboratories. Consequently, as much information as possible has been collected about the laboratory strains and field-collected isolates used in this study. The Auburn strain was originally established at the University of California, Riverside (UCR) in 2000. The Monheim strain was originally obtained from the veterinary school in Hannover, Germany, and established at UCR in 2000.The UCR strain was originally obtained from Stanford Research Institute in Palo Alto, CA, in 1978. It has since then been maintained on cats at UCR without any exposure to insecticides.

Comparisons were also made with data collected previously for the Danish Pest Infestation Laboratory (DPIL) strain. DPIL was originally collected in Copenhagen, Denmark, in 1981, transferred to Switzerland in 1984, and reared on an artificial system in 1990 (Moyses 1995, Moyses and Gfeller 2001).

Insecticides. Acetone solutions of technical grade fipronil (97.8%, Pestanal D-30926, Sigma-Aldrich, Seetze, Germany) and imidacloprid (Bayer Animal Health, Monheim, Germany) were applied to the larval-rearing medium or directly to the adult cat flea.

Adult Topical Bioassays. A 0.1-μl droplet of insecticide was deposited on the cuticle of each flea using a 27-gauge needle in a glass tuberculin syringe (Becton, Dickinson and Co, Rutherford, NJ) held in an Isco model M Microapplicator (Instrumentation Specialties, Seward, NE). The tip of the needle was removed so that the opening was level instead of tapered as is common with hypodermic needles. This allowed the solvent to bead up at the end of the syringe.

Adult fleas were tested when they were 18–20 d from the egg collection date. Fleas were placed into two or three test tubes (195 by 23 mm in diameter) in groups of ≈60–100 fleas per tube by inverting the rearing jar and pouring them down a glass funnel. They were placed in a refrigerator at 3°C. Fleas were immobile after 40 min when the first test tube was removed. The fleas were lightly anesthetized with CO_2 and poured into a plastic petri dish (85 mm in diameter by 14 mm in depth) sitting on a chill plate (#1429, Bioquip, Rancho Dominguez, CA) and covered with the plastic petri dish lid. Covering the petri dishes was important because, despite the chilling, some fleas retained enough mobility to kick and propel themselves out of the petri dish. The fleas were kept under a gentle flow of CO_2. The chilling table was maintained at around −0.4°C. The second and third test tubes were removed from the refrigerator as needed. We detected no detrimental effect of additional refrigeration and CO_2 on survival of the later-dosed fleas.

A small droplet of acetone and insecticide (or acetone alone in the controls) was initially forced out of the syringe to ensure flow; despite the small aperture of the syringe opening, some evaporation occurred inside the needle shaft between applications. After the test droplet was forced out and wiped off with a Kimwipe tissue, a second droplet was forced out. An immobilized flea was removed from the covered petri dish with fine forceps and was brought to the tip of the

syringe. The flea was then placed in a clean test tube to recover. Ten fleas were dosed and placed per test tube after which a strip of filter paper (Whatman #1, ≈18-mm wide by 90-mm long, tapered at one end) was placed inside the test tube, tapered end down, to give the recovering fleas a vertical substrate onto which they could crawl. The test tube was then covered with a small square of parafilm to confine the fleas. The test tubes and fleas were placed inside an environmental chamber held at $27 \pm 1°C$ and 56% RH. Fleas were checked 24 h later for survival. A flea was considered dead (or irreversibly affected) if it was either immobile on the bottom of the vial or, if moving, could not right itself and crawl upward on the piece of filter paper.

A series of doses was administered from lowest to highest concentration with a corresponding control batch before and after each insecticide series. All insecticide doses were made up fresh from a stock solution within an hour of use, and each group of 10 fleas of the same dose received insecticide from a separate vial (i.e., three groups of 10 required three vials of prepared insecticide). The stock solutions were refrigerated for storage and allowed to reach room temperature before the making of new dilutions for testing. Tests were performed in three simultaneous trials such that the precontrol treatments were applied first and in succession, followed by the lowest insecticide doses, then the next highest doses, and so on. After applying the highest dose, the syringe (glass plunger, glass body, and metal needle) was dismantled and thoroughly cleaned with acetone and allowed to soak 3–5 min in acetone to remove insecticide. After this soaking, the syringe was reassembled, rinsed a few more times, and the postcontrol treatments were applied. Typically, the postcontrols showed no discernible increase in mortality, such that the final cleaning process was deemed successful in removing remnant insecticide. However, imidacloprid appeared to adhere more tenaciously to the syringe than fipronil (i.e., higher mortality in the postcontrol treatments). Subsequently, after the highest dose of imidacloprid, the syringe received several additional active rinsing and was soaked in the acetone for ≈10 min. This was sufficient to reduce the postcontrol mortality to normal levels (i.e., 0–10%). In addition, to further decrease potential contamination, one syringe was specifically dedicated to use only with imidacloprid.

Larval Bioassays. To determine the activity of fipronil and imidacloprid against larval cat fleas, immature fleas were exposed to larval-rearing medium treated with serial dilutions of each insecticide. The medium was placed in the bottom of plastic Sarstedt vial (2 grams per vial) and treated with 2 ml of nine serial dilutions of technical fipronil or imidacloprid in acetone (0.003, 0.0015, 0.001, 0.0005, 0.0003, 0.0001, 0.00005, 0.00001, and 0.000005%), resulting in concentrations in the medium from 30 to 0.05 ppm (Rust et al. 2002). The mixture was stirred, and the medium was allowed to dry for at least 2 h. The treated mixture was transferred to a glass petri dish (60 by 15 mm).

To permit an accurate count of the number of eggs that hatched, cat flea eggs were glued to the underneath surface of the petri dish lids. The petri dish lids were placed over the media and placed into incubators maintained at $26 \pm 2°C$ and 80% RH. The number of hatched eggs was counted on day 5.

The medium and cocoons were passed through a 16-mesh screen on day 12, and the number of cocoons was counted. The cocoons were placed in a 2.5 cm in diameter by 4.5 cm in length plastic snap cap vial, and a 5.5-cm-diameter disk of Whatman filter paper (Whatman, Hillsboro, OR) was placed over the top and secured with a snap cap lid. The vials and cocoons were returned to a chamber maintained at $26 \pm 2°C$ and 80% RH. The number of adults that emerged was counted on day 28.

Statistical Analyses. Dose–mortality lines for each strain and insecticide were determined using "Polo" software (LeOra Software, Menlo Park, CA: Robertson and Preisler 1992). Data obtained for DPIL laboratory strain by other authors, but using a comparable topical application method, were used as baseline for determining resistance ratios (RR_{50}) for the adults (Moyses and Gfeller 2001). Because larval bioassay data were not available for DPIL, the Auburn strain was selected as the baseline reference strain because of its high frequency of susceptible alleles for the *Rdl* (Bass et al. 2004b) and knockdown resistance mechanisms (Bass et al. 2004a).

Results

Adult Topical Bioassays. Fipronil was toxic to adult cat fleas, giving LD_{50} values against the three laboratory strains (Auburn, Monheim, and UCR) ranging

Table 2. The minimum lethal dose (nanograms per flea) required to kill adult *C. felis* by topical applications of fipronil

Strain/isolate	n	Slope ± SE	LD_{50} (95% CL)	LD_{95} (95% CL)	Ratio LD_{50}[a]
Auburn	150	2.79 ± 0.604	0.11 (0.059–0.149)	0.42 (0.298–0.800)	0.61
Monheim	150	3.91 ± 0.508	0.28 (0.228–0.343)	0.75 (0.586–1.097)	1.56
UCR	150	4.04 ± 0.557	0.28 (0.229–0.330)	0.72 (0.557–0.992)	1.56
Clancy	230	1.52 ± 0.256	0.13 (0.043–0.213)	1.55 (0.845–6.897)	0.7
Jeepers	150	2.55 ± 0.462	0.35 (0.167–0.530)	1.56 (0.907–9.283)	1.94
Monster	150	2.97 ± 0.781	0.40 (0.229–0.514)	1.43 (1.008–3.791)	2.22
Oliver	150	2.48 ± 0.361	0.25 (0.170–0.330)	1.15 (0.760–2.520)	1.39
Sassy	270	2.00 ± 0.354	0.28 (0.154–0.382)	2.14 (1.031–31.781)	1.56
DPIL[b]	418	2.8 ± 0.42	0.18 (0.12–0.27)	0.69 (0.40–2.3)	

[a] Ratio of isolate or strain/DPIL.
[b] DPIL = Danish Pest Infestation Laboratory strain (Moyses and Gfeller 2001).

Table 3.　The minimum lethal dose (nanograms per flea) required to kill adult *C. felis* by topical applications of imidacloprid

Strain/isolate	n	Slope ± SE	LD_{50} (95% CL)	LD_{95} (95% CL)	Ratio LD_{50}[a]
Auburn	120	5.43 ± 1.046	0.06 (0.052–0.071)	0.12 (0.105–0.178)	0.32
Monheim	110	5.97 ± 1.359	0.08 (0.067–0.094)	0.15 (0.118–0.262)	0.42
UCR	420	2.18 ± 0.213	0.10 (0.070–0.142)	0.55 (0.298–2.084)	0.56
Clancy	210	3.44 ± 0.850	0.07 (0.044–0.084)	0.20 (0.129–1.277)	0.37
Inky	360	8.04 ± 1.398	0.05 (0.042–0.060)	0.08 (0.069–0.147)	0.26
Kiki	245	2.91 ± 0.492	0.18 (0.071–0.291)	0.67 (0.415–1.981)	0.95
Monster	210	5.48 ± 1.090	0.07 (0.045–0.086)	0.14 (0.106–0.553)	0.37
Sassy	120	2.18 ± 0.379	0.02 (0.015–0.031)	0.13 (0.078–0.312)	0.11
Tweedle Dee	210	6.03 ± 1.29	0.05 (0.034–0.064)	0.10 (0.076–0.224)	0.26
DPIL[b]	560	5.2 ± 0.41	0.19 (0.15–0.22)	0.70 (0.54–1.1)	

[a] Ratio of isolate or strain/DPIL.
[b] DPIL, Danish Pest Infestation Laboratory strain (Moyses and Gfeller 2001).

from 0.11 to 0.28 nanograms per insect (Table 2). These values were close to that (0.18 nanograms per insect) obtained previously with DPIL (Moyses and Gfeller 2001). The LD_{50}s of the field-collected isolates ranged from 0.13 to 0.40 nanograms per flea, yielding RR_{50} ranging from 0.61 to 2.22 compared with DPIL.

Imidacloprid proved similarly toxic to fipronil against adult *C. felis* (Table 3). The LD_{50}s ranged from 0.02 to 0.18, and the RR_{50}, compared with the DPIL strain, ranged from 0.11 to 0.95.

Larval Bioassays. Larval medium treated with fipronil was extremely toxic with a concentration of 0.07 ppm, providing 50% kill of larvae (Table 4). RR_{50}, compared with Auburn, ranged from 0.58 to 1.33. Imidacloprid was similarly toxic against larvae, with LC_{50}s ranging from 0.09 to 0.21 ppm and RR_{50} ranging from 0.5 to 1.75 (Table 5).

Discussion

Spot-on animal therapies have been widely used to control adult cat fleas since the registration of imidacloprid (Advantage) and fipronil (Frontline) in the mid-1990s. Despite their widespread use for nearly 15 yr, there have been no scientifically documented reports of reduced susceptibility of fleas to either of these compounds. A long-term program to monitor the susceptibility of imidacloprid has not revealed any reduced susceptibility of larvae in 1,347 isolates collected from North America, Europe, and Australia (Blagburn et al. 2006, Kopp et al. 2013). There has not been a comparable monitoring program for other currently used spot-on animal therapies, including fipronil.

Unfortunately, there are no fully insecticide-susceptible strains of *C. felis* in culture. Molecular studies have shown that even existing long-standing laboratory strains such as Auburn, UCR, and Monheim contain alleles conferring resistance to pyrethroids and cyclodiene insecticides (Bass et al. 2004a,b). Brunet et al. (2009) reported that six strains thought to be susceptible to fipronil were in fact homozygous for the *Rdl* mutation conferring resistance to cyclodiene insecticides and cross-resistance to fipronil. Consequently, we included in this study the topical application data for adults collected by Moyses and Gfeller (2001) because the DPIL strain had been cultured since the 1980s and was one of the first tested with fipronil and imidacloprid. Unfortunately, this strain is no longer in existence.

None of our laboratory strains or field-collected isolates of *C. felis* were significantly less susceptible to fipronil than results obtained by Moyses and Gfeller (2001) for DPIL. Interestingly, most of our strains and isolates were significantly more susceptible to imidacloprid compared with the DPIL strain. In our study, small residues of imidacloprid appeared to remain even after normal washing and rinsing of syringes. The adult fleas were also lightly anesthetized with CO_2 and then confined on a cold plate before treating them. These procedural changes may have contributed to increased susceptibility and lower LD_{50} values.

When treating holometabolous insects such as fleas, the insecticide applications may target specific developmental stages. For example, insect growth regulators such as methoprene and pyriproxyfen typically affect eggs, larvae, or both, whereas most other insecticides target the adult fleas because they are applied

Table 4.　The lethal concentration of fipronil in larval-rearing medium (ppm) required to kill larval *C. felis*

Strain/isolate	n	Slope ± SE	LC_{50} (95% CL)	LC_{95} (95% CL)	Ratio LC_{50}[a]
Auburn	836	3.73 ± 0.600	0.12 (0.076–0.142)	0.33 (0.255–0.5567)	
Monheim	446	1.63 ± 0.229	0.07 (0.025–0.120)	0.72 (0.423–2.063)	0.58
UCR	834	2.28 ± 0.299	0.13 (0.049–0.206)	0.69 (0.438–1.952)	1.08
Clancy	329	1.38 ± 0.229	0.14 (0.014–0.331)	2.11 (0.783–49.141)	1.17
Jeepers	302	2.81 ± 0.478	0.12 (0.080–0.157)	0.46 (0.332–0.771)	1.00
Monster	333	0.87 ± 0.184	0.10 (0.006–0.282)	8.19 (2.297–654.139)	0.83
Oliver	336	1.96 ± 0.297	0.16 (0.070–0.270)	1.12 (0.654–3.013)	1.33
Sassy	354	1.11 ± 0.218	0.11 (0.010–0.279)	3.40 (1.291–57.499)	0.92

[a] Ratio of strain or isolate/Auburn.

Table 5. The lethal concentration of imidacloprid in the larval-rearing medium (ppm) required to kill larval *C. felis*

Strain/isolate	n	Slope ± SE	LC$_{50}$ (95% CL)	LC$_{95}$ (95% CL)	Ratio LC$_{50}$[a]
Auburn	410	4.64 ± 0.962	0.12 (0.094–0.158)	0.28 (0.212–0.518)	
Monheim	403	3.44 ± 0.986	0.11 (0.070–0.154)	0.34 (0.239–0.673)	0.92
UCR	534	2.68 ± 0.292	0.12 (0.039–0.214)	0.48 (0.260–2.957)	1.00
Clancy	458	3.16 ± 0.528	0.09 (0.059–0.120)	0.30 (0.207–0.608)	1.33
Inky	393	3.05 ± 0.503	0.15 (0.051–0.272)	0.52 (0.280–3.228)	1.25
Kiki	245	2.91 ± 0.492	0.18 (0.071–0.291)	0.67 (0.415–1.981)	1.50
Monster	373	3.50 ± 0.689	0.21 (0.133–0.272)	0.62 (0.473–0.968)	1.75
Sassy	438	2.81 ± 0.375	0.15 (0.100–0.198)	0.58 (0.419–0.964)	1.25
Tweedle Dee	435	4.56 ± 0.715	0.18 (0.141–0.216)	0.42 (0.341–0.560)	1.50

[a] Ratio of strain or isolate/Auburn.

to the host. The developmental stage and age of the insect can affect susceptibility to insecticides. Neonate codling moth larvae, *Cydia pommonella* (L.), were more sensitive than fifth instars to azinphosmethyl mixed in rearing media. The age of the adult moths affected the sensitivity to topical applications (Reuveny and Cohen 2007). In two strains of tobacco whitefly, *Bemisia tabaci* Gennadius, prepupal nymphs were more susceptible to imidacloprid than adults, and resistance to imidacloprid conferred by overexpression of a monooxygenase enzyme was much more potent in adults and larvae (Nauen et al. 2008). In western flower thrips, *Frankliniella occidentalis* (Pergande), larvae were more susceptible than the adults to acrinathrin, formetante, and methiocarb (Contreras et al. 2010). Higher resistance levels were found in adults, and this carried over to larvae at lower levels.

Clearly, factors such as feeding activity, developmental processes, and the route of exposure affect the susceptibility of various life stages to insecticides. However, our studies suggest a larval bioassay to be the right laboratory test system for both adulticides, fipronil and imidacloprid. This is because of the major limitation of topical bioassays of adults, as the test is that they require artificial or natural hosts to generate the large numbers of adult fleas. However, if 60 flea eggs are collected, it is possible to test for susceptibility using the larval bioassay technique, provided a diagnostic dose has been developed (Rust et al. 2005).

To date 1,347 flea isolates from the field have been tested in larval bioassay against imidacloprid, with no reports of reduced efficacy with spot-on applications (Rust et al. 2011, Kopp et al. 2013). In contrast, only a few isolates have been tested with fipronil. However, with the reported larval bioassay, a reliable study could be implemented for further investigation.

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