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Large-scale monitoring of imidacloprid susceptibility in the cat flea, *Ctenocephalides felis*

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Abstract. Although on-animal topical treatment with compounds such as imidacloprid has revolutionized the control of the cat flea, *Ctenocephalides felis* (Bouché) (Siphonaptera: Pulicidae), the development of insecticide resistance is a continuing threat. As part of a highly co-ordinated and unprecedented resistance monitoring programme for *C. felis*, 1437 flea isolates were collected by veterinary clinics in Australia, Germany, France, the U.K. and 29 states in the U.S.A. from 2002 to 2009. About 65% of the collections were made from June to October each year and 71% of the collections were from cats. Collections of flea eggs were sent to one of five different laboratories, where they were tested with a diagnostic dose of imidacloprid (3 p.p.m.) applied to larval flea-rearing medium. Of the 1437 collections received, 1064 contained adequate numbers of eggs for testing. Of these isolates, untreated eggs failed to hatch in 22.7% and were not considered valid bioassays. Survival rates >5% and development of adult fleas (a threshold for further testing) occurred in only 22 isolates. They were re-tested with the same diagnostic dose and none produced >5% adult emergence. Complete dose–response bioassays were performed on three of the isolates that had triggered a second test and produced slopes, intercepts and LC_{50} values similar to those for existing susceptible laboratory strains. Results confirmed sustained susceptibility of *C. felis* to imidacloprid, despite its widespread use for over a decade.

Key words. *Ctenocephalides felis*, cat flea, diagnostic dose, imidacloprid, insecticide resistance, resistance monitoring.

Introduction

The introduction of insecticides for veterinary use, such as dinotefuran, fipronil, imidacloprid, lufenuron, metaflumizone, nitenpyram, pyriprole, selamectin and spinosad, has revolutionized the control of the cat flea *Ctenocephalides felis* (Bouché) on cats and dogs (Rust, 2005, 2010; Blagburn & Dryden, 2009). Previous strategies employing carbamates, organophosphates, pyrethroids and pyrethrins led to documented cases of insecticide resistance in flea populations (Bossard *et al.*, 1998, 2002). Biotic factors, such as the flea's rapid lifecycle (18–20 days), the high fecundity of female fleas (up to

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400 eggs per female during her lifetime) and the limited movement of adult fleas, and operational factors, such as the persistence of chemical treatments and repetitive applications, are known to contribute to the rapid development of resistance (Georghiou, 1983; Denholm & Rowland, 1992). Although on-animal topical treatments such as imidacloprid and fipronil have been widely used for a decade, there have been only a few sporadic and undocumented reports of the development of perceived product failures. Bardt & Schein (1996) reported that a field-collected isolate (Cottontail) was resistant to lufenuron and had reduced susceptibility to fipronil. Payne *et al.* (2001) reported reduced activity of fipronil to another flea isolate (R6) after residues had aged for 3 weeks. Molecular analyses of several flea isolates showed that alleles conferring cyclodiene resistance and potential cross-resistance to fipronil (*Rdl*) (Bass *et al.*, 2004a; Daborn *et al.*, 2004) and knock-down resistance (*kdr*) to pyrethroids (Bass *et al.*, 2004b) occur in *C. felis*. The Cottontail isolate possessed the *Rdl* allele (Bass *et al.*, 2004a), suggesting a possible link between this allele and insensitivity to fipronil. By contrast, Brunet *et al.* (2009) reported that six isolates of *C. felis* homozygous for *Rdl*, tested 3–6 years before the *Rdl* diagnosis, were fully susceptible to topical applications of fipronil. The prevalence of *Rdl* in *C. felis* has not been investigated systematically and its impact on fipronil treatments is still unresolved. The survival of some individuals of a laboratory strain of *C. felis* when exposed to cats 28 days after treatment with fipronil or imidacloprid raised concerns over resistance to these compounds (Dryden *et al.*, 2005). However, there was no evidence of the strain showing resistance in larval bioassays with imidacloprid [Rust *et al.*, 2005 (in which the strain is referred to as KSU)]. Repeated exposure of KS1 for nine generations to larval medium treated with an LC_{35} (35% lethal concentration) dose of imidacloprid failed to cause any shift in response (M. K. Rust, unpublished data, 2003).

In the crop protection sector, cases of resistance to neonicotinoid compounds such as imidacloprid have been shown to compromise control of some insect pests (Nauen & Denholm, 2005; Karunker *et al.*, 2008). There have been no confirmed cases of resistance to imidacloprid in the veterinary sector, but careful vigilance is required. For this reason, the susceptibility of *C. felis* to imidacloprid has been monitored extensively in Europe and the U.S.A., and is now being monitored in Australia, in a highly co-ordinated programme in which flea samples are collected in veterinary clinics, sent to one of five participating research laboratories, and tested for their response to a diagnostic concentration (3 p.p.m.) of imidacloprid using a larval diet incorporation bioassay (Rust *et al.*, 2002, 2005; Blagburn *et al.*, 2006). The advantage of this approach is that larvae hatching from eggs collected in the field can be tested without the need to rear isolates to adulthood in the laboratory. Isolates that are obviously fully susceptible to imidacloprid can be discarded and only potentially interesting isolates need to be propagated on host animals for further examination using full dose–response bioassays.

The current study reports results of this monitoring programme obtained between 2002 and 2009 for fleas collected from veterinary clinics in Australia, Germany, France, the U.K. and the U.S.A. The work exemplifies the challenges and benefits of large-scale resistance monitoring and demonstrates the continuing sensitivity of *C. felis* to imidacloprid.

Materials and methods

Collection of field samples

Veterinary clinics throughout the U.S.A. ($n = 131$), the U.K. ($n = 36$), France ($n = 8$), Australia ($n = 4$) and Germany ($n = 16$) were recruited to collect and send flea eggs to one of six designated research laboratories in California, Kansas and Alabama (U.S.A.), London (U.K.), Brisbane (Australia) and Monheim (Germany). Each clinic was provided with a kit and a detailed protocol for collection and shipment. An accompanying questionnaire requested information concerning each cat and dog, treatment history and details of other pets in the household (Rust *et al.*, 2005; Blagburn *et al.*, 2006).

To collect flea eggs, blank newsprint or cardboard was placed on a table or floor. A cage with an open-grate floor and an animal infested with fleas were placed over the paper. Food, water and litter pans were provided as needed. After 4–24 h, the animal was gently brushed or combed to dislodge flea eggs. The debris and eggs were brushed to the centre of the paper and poured through a sieve and funnel into a glass. The tube was covered with filter paper and sealed with white tape or parafilm.

In the U.S.A. and Australia, tubes were placed in a styrofoam cooler with insulation adequate to protect them and sent overnight to one of the laboratories. In Germany, France and the U.K., where climatic conditions were often less extreme and transportation distances much shorter, tubes were wrapped in bubblewrap and posted in a special delivery envelope (Rust *et al.*, 2005).

Diagnostic dose bioassays

Each laboratory determined the activity of imidacloprid against the field-collected cat flea isolates according to the protocol reported by Rust *et al.* (2005). For the initial phase of testing, larval rearing medium was treated with technical grade imidacloprid in acetone to provide a 3-p.p.m. concentration (w/w) of imidacloprid. This concentration had been determined previously to be optimal for disclosing potential cases of resistance to imidacloprid (Rust *et al.*, 2005). Treated medium was placed into glass Petri dishes (5.0 cm diameter, 1.5 cm depth) and 20 eggs were placed into each Petri dish using a fine camel hair brush (size 0000).

Depending on sample size, up to six replicates were set up using treated medium and up to three replicates using untreated medium were run as controls. The glass Petri dishes and flea eggs were placed in incubators maintained at $26 \pm 2\ ^{\circ}C$, 80–85% relative humidity (RH) and an LD 12 : 12 h photoperiod. In addition, 20 eggs of standard reference strains of *C. felis* maintained by each laboratory were placed on untreated medium as an internal check on environmental conditions affecting egg hatch.

Twelve days after the eggs had been added to the medium, pupae and larvae were counted and returned to the incubator. The number of adults that emerged was counted on day 28 in both the treated and untreated replicates.

Second diagnostic dose bioassays

If >5% of the eggs exposed to the 3-p.p.m. concentration of imidacloprid in the first diagnostic dose bioassay emerged as adults, the survivors of the untreated controls were collected for rearing. In the case of the participating laboratory at Auburn, AL, these were sent overnight to the laboratory at the University of California Riverside, Riverside, CA. The adult fleas were placed on cats and the isolate was propagated using standard rearing procedures (Rust *et al*., 2002). Cat flea eggs were collected from trays underneath cats supporting each isolate. The eggs and debris were passed through a series of four sieves (10-, 16-, 20- and 60-mesh) and eggs were retained on the 60-mesh screen.

The larval bioassay was repeated with three replicates of 20 flea eggs in medium treated with 3 p.p.m. imidacloprid. Twelve days after the eggs had been added to the Petri dishes, pupae and larvae were counted and returned to the incubator. The number of adults that emerged was counted on day 28 in both the treated and untreated medium.

Full dose–response bioassays

This phase of the monitoring programme applied only to isolates whose survival exceeded 5% when exposed to the 3-p.p.m. dose in both the initial and second diagnostic dose bioassays. However, a small number of field isolates that exceeded the threshold in the initial diagnostic dose screen were also used to establish dose–response relationships for comparison with susceptible reference strains. Adults of these isolates emerging from untreated controls were collected and propagated on cats using standard rearing procedures (Rust *et al.*, 2002). When sufficient numbers of eggs were collected, full dose–response bioassays were initiated.

Larval rearing medium was treated with technical grade imidacloprid to provide the following concentrations in p.p.m.: 30, 15, 10, 5, 3, 1, 0.5, 0.1 and 0.05. Treated medium was placed into glass Petri dishes (5.0 cm diameter, 1.5 cm depth) and eggs added as before. To determine the number of flea eggs that hatched, eggs were glued to the upper inner surface of the Petri dish. A thin streak of glue (UHU®Stic; Saunders Manufacturing Co., Winthrop, ME, U.S.A.) was applied to the glass with a moistened paint brush. Eggs were carefully placed onto the Petri dish lid and rolled onto the tacky surface with a fine camel hair brush (size 0000). Once the glue dried, the eggs remained attached to the Petri dish lid. As the eggs hatched, the larvae fell into the medium, preventing larvae from consuming flea eggs and providing an accurate count of larvae entering the test. The design also minimized the impact of cannibalism on larval mortality. The glass Petri dishes and flea eggs were placed in incubators and maintained at $26 \pm 2\ ^{\circ}C$ and 80% RH. Larvae and pupae were counted on day 12 as before, returned to incubators, and the number of adults that emerged or developed in the cocoons was counted on day 28.

Baseline data for standard susceptible laboratory strains maintained at four of the laboratories were compared with data for field-collected isolates. The adult emergence data were analysed by probit analysis (Robertson & Preisler, 1992) using POLO Version 1.0 (LeOra Software, Menlo Park, CA, U.S.A.).

This study was conducted according to the 'Guide for the Care and Use of Laboratory Animals' promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, 1996 and protocols adopted by each institution.

Results

A total of 1437 samples of *C. felis* were collected from clinics and sent to testing laboratories between 2002 and 2009 (Table 1). About 71% of the samples were collected from cats and 28% from dogs (Table 1). This probably reflects the fact that cats are much easier to hold overnight in cages. Between 6% and 9% of the samples collected from 2003 to 2007 came from animals with a previous history of imidacloprid use. In 2008 and 2009, these proportions increased to 13% and 16%, respectively.

Diagnostic dose bioassays

Of the 1437 samples received, 822 (57%) yielded data that contributed to the monitoring programme (Table 2). This discrepancy arose for two reasons. Firstly, numbers of eggs in a percentage of samples received each year were insufficient to conduct bioassays, highlighting a difficulty in training staff in clinics to assess the severity of flea infestation and to adhere to collection protocols. However, this problem decreased in importance over successive years. Secondly, a proportion of samples used for the bioassays showed no egg hatch or an inadequate hatch rate, even in Petri dishes containing untreated larval medium. This problem appears to have increased over time, possibly as a result of prior contamination from treatment of animals with insect growth regulators (IGRs). In 2002, 77% of the eggs received hatched; this declined to 48% in 2009. Adults emerging from untreated larval medium were checked for stout bristles on the dorsal margin of the hind tibia, which

Table 1. Flea egg samples collected from cats and dogs during 2002 to 2009.

	Samples collected and shipped, *n*								
Host	2002	2003	2004	2005	2006	2007	2008	2009	Total
Cats	112	110	194	163	94	41	126	184	1024
Dogs	25	38	51	33	23	19	77	135	401
Other*	0	2	2	2	0	1	3	2	12
Total	137	150	247	198	117	61	206	321	1437

*Host not specified.

Table 2. Summary of the diagnostic dose bioassays with imidacloprid conducted between 2002 and 2009.

Year	Samples received, *n*	Valid bioassays, *n*	Eggs tested at 3 p.p.m., *n*	Adults emerging, *n*	Percentage emergence
2002	137	106	9714	22	0.2%
2003	150	106	9657	50	0.5%
2004	247	171	16 336	30	0.2%
2005	198	130	13 342	28	0.2%
2006	117	51	6480	105	1.6%
2007	61	34	2660	1	<0.1%
2008	206	70	5620	68	1.2%
2009	321	154	8680	68	0.8%
Total	1437	822	72 489	372	0.5%

are characteristic of the closely related *Ctenocephalides canis* (Hopkins & Rothschild, 1953), but none were found.

Out of a total of 72 489 eggs in the 822 samples tested with 3 p.p.m. imidacloprid, 372 individuals (0.5%) survived to adulthood from 22 different isolates (Table 2). There was no evidence of an increasing trend in survival from year to year. Annual survival rates varied from a minimum of <0.1% in 2007 to a maximum of 1.6% in 2006. Although numbers of survivors were too low to allow detailed statistical analysis, visual inspection of the data suggests no consistent association of survival with the testing laboratory, host species, season of collection or prior treatment with insecticide.

The diagnostic dose test was repeated for isolates that exceeded the 5% survival threshold when sufficient eggs were available for that particular isolate. In the second diagnostic dose test, none of these isolates exceeded the 5% survival threshold, implying that survival was a chance effect rather than indicating a systematic shift in susceptibility.

Full dose–response bioassays

The full dose exposure test was conducted on three of the field isolates, AUS 1, USA 1 and USA 2 that allowed for >5% survival of adult fleas in the first diagnostic dose screen, although these proved fully susceptible in the second screen (Table 3). These were compared with three reference susceptible strains, AUB, KS1 and UCR, that had been reared in one or more of the testing laboratories for ≥10 years without exposure to insecticides. None of the LC_{50} or LC_{95} values for the field isolates lay outside the range of values for the laboratory strains (based on the lack of overlap of 95% confidence intervals).

Discussion

Field isolates of *C. felis* were obtained from clinics in Europe, Australia and the U.S.A., corroborating previous findings on the year-round occurrence of this species (Lyon, 1915; Osbrink & Rust, 1985; Clark, 1999). However, more isolates were received in the summer ($n = 518$) and autumn ($n = 575$) than either the spring ($n = 184$) or winter ($n = 144$). Clark (1999) reported that about 25% of adult female fleas collected from cats and dogs in the U.K. during the winter had mature oocytes; our experience suggests that, although the number of flea eggs during winter months is comparatively low, their presence represents an ongoing infestation and the nucleus for the large increase in flea populations in June and July, when indoor conditions are favourable for larval development. This supports the use of topical treatments through the winter and before the primary flea season, although the concept of preventive applications of flea products during the winter and early spring warrants some additional research.

Of the 822 valid diagnostic dose bioassays conducted between 2002 and 2009, only 22 isolates enabled >5% flea survival when exposed to 3 p.p.m. imidacloprid. This dose had previously been established as the optimal dose for diagnosing possible cases of reduced susceptibility on the basis of ring-testing of several field isolates and laboratory strains (Rust *et al.*, 2005). The second diagnostic dose assay was intended to verify the results of the first and none of the isolates that triggered a survival rate >5% in the first test gave survivors in the second. Thus, the few 'positive' results from the monitoring programme so far do not appear to have reflected the presence of individuals resistant to imidacloprid. This was supported by full dose–response bioassays on a limited number of these isolates, none of which exhibited dose–response relationships that differed significantly from those of reference laboratory susceptible strains. The emergence of some adults must, therefore, represent chance survival or extremes in the normal range of susceptibility rather than physiological resistance. The diagnostic dose used in the programme effectively eliminated 99% of the isolates from being maintained on hosts

Table 3. Dose–response bioassays of three field isolates and laboratory strains of cat fleas to imidacloprid.

Isolates/strains* (testing laboratory)		*n*	Slope ± SE	LC_{50} (95% CI)	LC_{95} (95% CI)
AUS 1	(AUS)	268	3.8 ± 0.80	0.48 (0.322–0.611)	1.29 (0.577–2.240)
USA 1	(UCR)	245	2.9 ± 0.49	0.18 (0.071–0.291)	0.67 (0.415–1.981)
USA 2	(AUB)	206	4.7 ± 1.39	0.48 (0.225–0.658)	1.06 (0.749–3.501)
UCR	(UCR)	505	6.5 ± 1.16	1.25 (1.038–1.427)	2.24 (1.838–3.756)
KS1	(KSU)	1208	4.3 ± 0.62	0.73 (0.566–0.851)	1.75 (1.405–2.638)
AUB	(AUB)	394	5.5 ± 1.06	0.48 (0.395–0.564)	0.97 (0.802–1.383)

*AUB, Auburn University; AUS, University of Queensland; KSU, Kansas State University; UCR, University of California Riverside.
SE, standard error; LC_{50}, 50% lethal concentration; 95% CI, 95% confidence interval.

and tested further, resulting in the saving of considerable time and other resources.

In recent years, the number of bioassays showing little or no egg hatch in untreated controls has increased dramatically. Weather conditions, especially in hot dry summer months, can affect the viability of flea eggs collected from the pans. However, the eggs were not dented as they are when they desiccate. If weather conditions were a factor, then we would have expected them to represent a relatively consistent factor from year to year in the late summer. A more likely explanation is that an increasing number of veterinarians and pet owners are using combination products that include an IGR. In 2004, three pet owners reported the use of products such as Frontline® Plus (Merial Ltd, Duluth, GA, U.S.A.) [9.8% fipronil and 8.8% (S)-methoprene]. In 2009, this had increased to eight pet owners. The IGR fenoxycarb affects early blastoderm formation, blastkinesis and advanced larval development up to hatching (Marchiondo *et al.*, 1990). Use of methoprene on cats can greatly reduce the hatching success of eggs of *C. felis* for up to 50 days after treatment (Olsen, 1985). The IGR pyriproxyfen affects yolk deposition, causing the egg to collapse after being laid (Palma *et al.*, 1993). However, eggs from methoprene-treated fleas show no visual signs and remain turgid, but fail to hatch or the larvae die quickly after hatching (Palma *et al.*, 1993). Combination treatments of fipronil and methoprene provided >90% ovicidal activity for ≥8 weeks (Young *et al.*, 2004). In 2009, we investigated several cases in which no control eggs hatched by retaining flea debris that accompanied the egg collection and adding dried beef blood and flea eggs from our laboratory colonies. None of the laboratory eggs hatched, suggesting that the debris was possibly contaminated with an IGR. Further studies are warranted to determine why the eggs in an increasing number of isolates have not been hatching. This may have an important impact on future resistance monitoring of eggs and larval stages.

Insecticide resistance monitoring is a key step in developing a comprehensive strategy to delay the development of resistance in *C. felis* to insecticides and to conserve important chemistries for the future. In addition to demonstrating an ongoing lack of insecticide resistance of *C. felis* to imidacloprid, the work described here demonstrates that the considerable logistical challenges involved in monitoring for shifts in susceptibility on an international scale and for a long period can be met through careful standardization and co-ordination of collecting and testing protocols. Similar programmes could be adopted for other active ingredients provided that satisfactory bioassays specifically designed for the relevant active ingredient are developed and validated.

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