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Susceptibility of Adult Cat Fleas (Siphonaptera: Pulicidae) to Insecticides and Status of Insecticide Resistance Mutations at the *Rdl* and Knockdown Resistance Loci

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Abstract

The susceptibility of 12 field-collected isolates and 4 laboratory strains of cat fleas, *Ctenocephalides felis* was determined by topical application of some of the insecticides used as on-animal therapies to control them. In the tested field-collected flea isolates the LD_{50} values for fipronil and imidacloprid ranged from 0.09 to 0.35 ng/flea and 0.02 to 0.19 ng/flea, respectively, and were consistent with baseline figures published previously. The extent of variation in response to four pyrethroid insecticides differed between compounds with the LD_{50} values for deltamethrin ranging from 2.3 to 28.2 ng/flea, etofenprox ranging from 26.7 to 86.7 ng/flea, permethrin ranging from 17.5 to 85.6 ng/flea, and *d*-phenothrin ranging from 14.5 to 130 ng/flea. A comparison with earlier data for permethrin and deltamethrin implied a level of pyrethroid resistance in all isolates and strains. LD_{50} values for tetrachlorvinphos ranged from 20.0 to 420.0 ng/flea. The *rdl* mutation (conferring target-site resistance

to cyclodiene insecticides) was present in most field-collected and laboratory strains, but had no discernible effect on responses to fipronil, which acts on the same receptor protein as cyclodienes. The *kdr* and *skdr* mutations conferring target-site resistance to pyrethroids but segregated in opposition to one another, precluding the formation of genotypes homozygous for both mutations.

Introduction

The development of resistance by pest organisms poses a constant threat to the performance of on-animal applied insecticides registered as veterinary medicinal products. Monitoring for changes in response requires access to sensitive bioassays appropriate to the chemicals and species under consideration. For example, to provide scientifically sound data on responses of the cat flea (*Ctenocephalides felis*) to the insecticide imidacloprid, a larval bioassay was developed and evaluated, and field-collected isolates from three continents have been tested since 2002 (Blagburn et al. 2006; Rust et al. 2002, 2005, 2011).

Over the last 30 years, there have been a number of reports of insecticide resistance in the *C. felis*. Bossard et al. (1998) provided an extensive review of resistance studies with adult cat fleas, most studies reporting on only a single cat flea isolate and some studies lacking comparisons with susceptible reference strains. Many studies utilized a brief contact exposure test on filter paper following the recommended WHO method (WHO 1970), but subsequent studies included substrates such as nylon carpet and glass for the bioassays (Bossard et al. 2002). However, the activity of the insecticide varied greatly depending on the method used to test for resistance. A larval bioassay with imidacloprid and fipronil has provided consistent results (Rust et al. 2002, 2014), and topical application of insecticides on the cuticle of adult fleas, which enables the testing of individual fleas, has also provided reliable levels of sensitivity (Moyses 1995). Topical application bioassays of 13 insecticides to individuals of a single flea strain were reported by Moyses and Gfellar (2001).

The objective of this study was to re-examine the susceptibility of field-collected isolates of cat fleas to some of the insecticides now commonly being used to control them on pets using a topical application bioassay. We compare our results with those previously reported and discuss the possible future implications for control. We also report on the occurrence of mutations known to confer target-site resistance to cyclodiene and pyrethroid insecticides.

Materials and Methods

Origin of Field Isolates and Laboratory Strains

The 12 field-derived isolates used for this study were collected between 2006 and 2011 by veterinary clinics throughout Australia, Europe, and the United States (Table 1). Cats or dogs were placed in cages for a period of 4 to 24 h to obtain cat flea eggs. A sheet of paper was placed beneath the floor walks in each cage. At the end of the collection period, the animals were brushed or combed gently to remove hair coat debris, including any remaining flea eggs. Flea eggs and hair coat debris were then passed through a sieve and funnel and collected in a glass tube. The glass tube was packaged in a Styrofoam cooler containing several layers of insulation, moistened gauze pads, and ice packs to promote safe shipment. A questionnaire was provided to all participating clinics; information requested included household and pet information, such as the number pets on the premises, use of environmental and on-animal flea control products, level of flea infestation, and whether fleas were a recurring or first-time problem (Blagburn et al. 2006). The flea eggs were shipped overnight from the clinics to laboratories in Australia, Germany and the USA, and placed in rearing medium the following

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

Strain/isolate	Locality	Collection date [a]	Cat or dog	Previous Treatments
Auburn	Soquel, CA	1985 (2000)	Unknown	
KS1	Manhattan, KS	1990 (2000)	cat	Unknown
Monheim	Hannover, Germany	1992 (2001)	cat	Unknown
UCR	Palo Alto, CA	1978	cat	Unknown
FI1	Brisbane, Australia	2009	dog	Unknown
FI2	New Orleans, LA	4/30/2011	dog	Frontline Plus
FI3	Queensland, Australia	8/14/2007	dog	several unknown
FI4	Jefferson City, MO	8/30/2010	cat	Frontline Combo, Frontline Plus
FI5	Birmingham, AL	8/21/2006	cat	Capstar
FI6	Charleston, SC	6/3/2009	dog	Frontline
FI7	Jacksonville, FL	5/24/2006	cat	Unknown
FI8	Rochester, NY	11/2/2009	cat	none
FI9	Auburn, AL		dog	unspecified fipronil
FI10	Jacksonville, FL	1/6/2009	cat	Advantage Multi
FI11	Mount Dora, FL	12/2/2009	dog	Advantage
FI12	Houston, TX	1/9/2009	cat	none

[a] Date in parentheses is the date when the lab strain was received at UCR.

day. Isolates not initially established at University of California Riverside (UCR) were subsequently transferred to UCR where the bioassays reported here were conducted.

Since there has never been a universally-adopted 'susceptible strain of *C. felis* used for insecticide testing, the study also included four strains that have been used as long-standing reference strains in different laboratories (Table 1). The Auburn strain was originally collected in 1985 and established at UCR in 2000. The KS1 (Kansas 1) strain was started from fleas collected from cats and dogs at an animal shelter in Manhattan, Kansas in 1990 and was transferred to UCR in 2001. The Monheim strain as originally obtained from the veterinary school in Hanover, Germany in 1992. It was subsequently maintained by Bayer Animal Health at Monheim and was sent to UCR in 2001. The UCR strain was originally obtained from Stanford Research Institute in Palo Alto, California in 1978. None of these four strains had been exposed to insecticides during their history of laboratory culture.

Maintenance of Flea Isolates

Laboratory isolates of cat fleas were maintained on individual cats according to a procedure modified from Metzger and Rust (1996). The cats were housed in double cages stacked on top of one another to help prevent cross contamination of the isolates. Three different rooms were used when possible, to maintain the cats and isolates. Cats with different isolates were not exercised together. The cats were maintained under a protocol approved by the UC Riverside Institutional Animal Care and Use Committee.

Flea eggs were collected from trays underneath the 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 80% RH and 26° ± 1° C. 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 about 16 to 18 d after egg collection. To maintain the isolates on each cat, about 30 male and 30 female adult fleas were placed on each cat every 2 wks. Typically, 3 to 4 generations of rearing were required before sufficient numbers of adult fleas were present for testing.

Topical application bioassays

Topical applications of acetone solutions of the following technical grade insecticides were applied to adult fleas: deltamethrin (96.5%, AccuStandard New Haven, CT), *d*-phenothrin (97.5%, MGK Co., Minneapolis, MN), etofenprox (97.7%, Pestanal Sigma-Aldrich, St. Louis, MO), fipronil (97.8%, Pestanal D-30926 Seelze), imidacloprid (98.3% Bayer Animal Health GmbH, Monheim, Germany), permethrin (95.5% MGK Co., Minneapolis, MN) and tetrachlorvinphos (99.0% Chem Service, West Chester, PA). Droplets (0.1 µl) were deposited on the cuticle of adult fleas using a 27–gauge needle on a glass tuberculin syringe (Becton, Dickinson and Co., Rutherford, NJ). The tip of the needle was removed so that the opening was level instead of tapered as is common with hypodermic needles. This allowed solvent to bead up at the end of the syringe. Precise application was made with an Isco Model M Microapplicator (Instrumentation Specialties, Seward, NE). Acetone alone was used as a control treatment.

Adult fleas were tested when they were 18 to 20 d from the egg collection date. They were poured into test tubes (195 mm x 23 mm diam.), about 60 to 100 fleas per test tube by inverting the rearing jar and pouring them down the glass funnel, and then placed in a refrigerator at 3°C. Fleas were immobile after 40 min when the first test tube was removed and fleas poured into a plastic Petri dish (85 mm diam x 14 mm 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 chilling table was maintained at around -0.4°C. Test tubes were removed from the refrigerator as needed. We detected no detrimental effect of additional refrigeration on survival of the later-dosed fleas.

A small droplet of test solution 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 trials. 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 Petri dish with fine forceps and the flea brought to the tip of the syringe. The flea was then placed in a clean test tube. Ten fleas were dosed and placed per test tube after which a strip of filter paper (Whatman #1, approximately 18 mm wide by 90 mm long, tapered at one end) was placed inside the test tube, tapered end down, to give the treated 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°C and 56% RH. Fleas were checked 24 h later for survival. A flea was recorded as affected if it was either immobile at the bottom of the vial or if it 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 test solutions were made up fresh from a stock solution about 1 h before use. The stock solutions were refrigerated for storage and allowed to reach room temperature prior to the making of new solutions. Bioassays were done in three simultaneous trials such that the control treatments were applied first to three batches of fleas, followed by increasing order of concentration. After applying the highest

dose, the syringe (glass plunger, glass body, metal needle) was dismantled and thoroughly cleaned with acetone and allowed to soak 3 to 5 min in acetone to remove insecticide. After this soaking, the syringe was reassembled, rinsed a few more times and the three more batches of fleas were dosed with acetone alone. Typically, these later-dosed controls showed no discernible increase in mortality such that the final cleaning process was deemed successful in removing remnant insecticide. However, imidacloprid proved to adhere more tenaciously to the syringe than the other compounds, leading to higher mortality in the later control cohorts. Consequently, after the highest dose of imidacloprid, the syringe received several additional active rinsing and was soaked in the acetone approximately 10 min. This was sufficient to reduce the later control mortality to normal levels (i.e., 0 to 10 %). In addition, to further decrease potential contamination, one syringe was specifically dedicated to use only with imidacloprid.

TaqMan PCR assays

The TaqMan assay is a PCR method that uses short oligonucleotide probes that are dual labelled with a fluorescent reporter dye at the 5' end and a quencher molecule at the 3' end. Amplification of the probe-specific product during PCR causes cleavage of the probe, generating an increase in reporter fluorescence as the reporter dye is released away from the quencher. By using different reporter dyes (normally VIC and 6-FAM), cleavage of two allele-specific probes can be detected in a single PCR. Three separate assays were designed that would enable genotyping for the previously reported resistance mutations, *rdl* (Bass et al. 2004a), *kdr* and super-*kdr* (*skdr*, Bass et al. 2004b).

For genotype testing, DNA was extracted from adult cat fleas using DNAzol reagent (Life Technologies). Individual fleas were placed in 1.5ml microtubes, snap-frozen in liquid nitrogen and ground to a powder using tight-fitting plastic pestles (Burkard Scientific Ltd). DNAzol reagent (0.2 ml) was added, homogenised using the pestle and DNA recovered by ethanol precipitation. DNA pellets were dissolved in 40µl water and 1.5µl aliquots taken for each TaqMan assay.

Primer and probe sequences for the taqman PCR assays were designed against gene sequences flanking either: 1) the *rdl* mutation (A302S) within the cat flea GABA receptor and conferring target-site resistance to cyclodienes (Bass et al. 2004a) and 2) *kdr* (L1014F) or 3) *super-kdr* (T929V) mutations within the cat flea voltage-gated sodium channel, both of which confer target-site resistance to pyrethroids (Bass et al. 2004b). For each assay, forward and reverse unlabelled primers together with VIC (wild-type allele) and 6 -FAM (mutant allele) labelled probes were designed using Primer Express software v.2.0 (Life Technologies). The 5' VIC and 6–FAM labelled probes also contained a non-fluorescent quencher (NFQ) and minor groove binder (MGB) at their 3' ends. Primer and probe sequences are as follows (5' to 3'): *rdl* assay, forward primer = GTTTTGGCTGAATCGTAATGCTACA, reverse primer = CATGGTCAACACAGTGGTCACT, wild-type probe = VIC-TCGAGTCGCTCTCG-NFQ, mutant probe = 6FAM-CTCGAGTCTCTCTCG-NFQ; *kdr* assay, forward primer = GGTGATGTCAGCTGTATTCCTTTCT, reverse primer = ACTATATATTAAAATGTCGTTGGCATTAGC, wild-type probe = VIC-CTTACCACAAGATTACCA-NFQ, mutant probe = 6FAM-CTTACCACAAAATTACCA-NFQ; *skdr* assay, forward primer = CTTATATCCATTATGGGTCGAACGAT, reverse primer = CATTACGGCGAATATGAAGATGATAATAC, wild-type probe = VIC-CACAAACGTTAGATTAC-NFQ, mutant probe = 6FAM-CACAAACACTAGATTAC-NFQ.

PCR assay reactions (20 µL) contained 1.5 µL of genomic DNA, 10 µL of SensiMix Probe kit (Bioline Reagents Ltd), 800 nM of each primer and 200 nM of each probe. Reactions were run on a Rotor-Gene 6000 (Corbett Research) using temperature cycling conditions of 10 min at 95 °C, followed by 40 cycles of 95 °C for 10 s and 60 °C for 45 s. The increase in VIC and 6–FAM fluorescence was monitored in real

Table 2 The minimum lethal dose of deltamethrin (ng/flea) required to kill adult *C. felis*

Strain/isolate	n	slope ± SE	LD_{50} (95 % CL)	LD_{95} (95 % CL)	RR_{50} (isolate/ Auburn)
Auburn	210	4.13 ± 0.61	3.00 (2.40–3.58)	7.51 (6.05–10.5)	---
Monheim	145	1.91 ± 0.34	9.71 (5.41–15.0)	70.7 (34.5–633)	3.2
UCR	150	2.43 ± 0.41	7.23 (3.10–11.2)	34.4 (19.1–293)	2.4
FI1	240	2.49 ± 0.25	2.31 (1.29–3.82)	10.6 (5.87–39.2)	0.8
FI3	150	3.34 ± 0.68	6.98 (4.49–9.20)	21.7 (14.6–68.9)	2.3
FI4	150	1.49 ± 0.40	5.53 (1.89–8.88)	70.3 (34.6–553)	1.8
FI6	332	0.96 ± 0.14	2.58 (0.64–5.20)	132 (44.0–2490)	0.9
FI7	330	2.57 ± 0.28	12.5 (9.24–16.6)	54.7 (34.0–157)	4.2
FI8	420	2.63 ± 0.26	5.85 (3.01–8.80)	24.7 (14.6–111)	2.0
FI9	270	2.41 ± 0.38	4.34 (2.65–5.90)	20.9 (13.3–59.4)	1.4
FI10	150	1.76 ± 0.31	13.9 (7.71–25.9)	120 (48.4–3450)	4.6
FI11	150	2.71 ± 0.53	7.83 (4.91–10.7)	31.7 (20.2–98.4)	2.6
FI12	241	0.79 ± 0.18	28.2 (7.20–309)	3420 (not computable)	9.4

Table 3 The minimum lethal dose of etofenprox (ng/flea) required to kill adult *C. felis*

Strain/isolate	n	slope ± SE	LD_{50} (95 % CL)	LD_{95} (95 % CL)	RR_{50} (isolate/ UCR)
UCR	180	6.05 ± 1.08	67.4 (60.0–75.5)	126 (105–180)	---
FI1	238	7.35 ± 1.70	55.6 (46.7–62.0)	93.1 (78.7–147)	0.8
FI3	120	7.47 ± 1.37	74.2 (65.0–89.8)	123 (104–171)	1.0
FI4	120	1.39 ± 0.35	26.7 (8.75–55.1)	410 (144–15300)	0.4
FI6	120	5.58 ± 1.39	53.9 (42.3–63.1)	106 (85.0–187)	0.8
FI8	120	5.38 ± 1.32	63.2 (50.6–75.1)	128 (99.8–237)	0.9
FI9	331	3.26 ± 0.65	55.1 (37.3–69.2)	176 (115–915)	0.8
FI10	120	3.88 ± 1.04	68.7 (51.0–91.6)	182 (121–884)	1.1
FI11	120	4.50 ± 1.38	53.0 (33.5–64.9)	124 (92.6–354)	0.8
FI12	120	1.65 ± 0.29	58.6 (34.3–140)	584 (208–10300)	0.9

time by acquiring each cycle on the yellow channel (530 nm excitation and 555 nm emission) and green channel (470 nm excitation and 510 emission) of the Rotor-Gene, respectively. To assist with assigning genotypes, the endpoint fluorescence values for the two dyes were plotted against each other in bivariate scatter plots to give clustering of samples in three groups corresponding to wild-type homozygotes (SS), resistance homozygotes (RR) and the heterozygous genotype (RS).

Statistical Analyses

Dose-mortality lines for each strain and insecticide were determined using 'Polo' software (LeOra Software, Menlo Park, CA; Robertson and Preisler 1992). The laboratory strain with the lowest LD_{50} was selected as the baseline reference strain for each insecticide tested.

Results

Bioassays with pyrethroid insecticides

Of the laboratory strains maintained at UCR, Auburn was the most susceptible to deltamethrin (Table 2). The RR_{50} ratios of the field-collected isolates ranged from 0.8 to 9.4. The regression lines for FI4, FI6, FI10, and FI12 had very shallow slopes (< 2), requiring extremely high doses to kill 95 % of the fleas and implying that these isolates showed

Table 4 The minimum lethal dose of permethrin (ng/flea) required to kill adult *C. felis*

Strain/isolate	n	slope ± SE	LD_{50} (95 % CL)	LD_{95} (95 % CL)	RR_{50} (isolate/ UCR) [a]
Auburn	240	2.96 ± 0.42	37.2 (28.2–46.5)	134 (92.8–281)	2.1
KS1	120	2.89 ± 0.47	27.8 (20.4–41.1)	103 (61.3–347)	1.6
Monheim	150	3.47 ± 0.64	57.8 (47.0–69.7)	172 (125–324)	3.3
UCR	240	5.69 ± 0.77	17.5 (13.4–21.4)	34.1 (26.9–56.7)	---
FI1	150	5.06 ± 0.77	51.8 (40.8–62.9)	110 (84.3–215)	3.0
FI2	241	2.89 ± 0.54	54.9 (45.1–68.0)	204 (134–510)	3.1
FI3	240	1.43 ± 0.34	49.7 (24.2–99.1)	696 (231–69200)	2.8
FI4	181	1.73 ± 0.40	85.6 (61.6–151)	764 (320–7370)	4.9
FI5	90	2.23 ± 0.35	17.6 (8.43–51.8)	93.1 (37.6– 7254)	1.0
FI6	90	6.29 ± 1.43	78.0 (64.2–94.8)	142 (110–637)	4.4
FI7	370	2.27 ± 0.27	69.3 (54.3–94.4)	369 (220–965)	4.0
FI8	201	1.71 ± 0.32	68.3 (47.7–102)	623 (297–3420)	3.9
FI9	212	1.57 ± 0.29	65.6 (41.9–99.9)	727 (340–3990)	3.7
FI10	180	1.84 ± 0.35	54.6 (40.6–76.8)	429 (225–1660)	3.1
FI11	271	0.68 ± 0.14	43.9 (13.4–104)	11900 (not computable)	2.5
FI12	270	4.08 ± 0.87	41.8 (25.4–50.7)	105 (83.6–186)	2.4

the greatest internal heterogeneity in response. Responses to the non-ester pyrethroid etofenprox were more consistent with LD_{50}s ranging only three-fold from 26.7 to 74.2 ng/flea (Table 3). The field-collected isolates FI4 and FI12 had shallower slopes (< 2) than the remainder, resulting in the highest LD_{95}s of 410 and 584 ng/flea, respectively. The LD_{50}s of permethrin ranged from 17.5 to 85.6 ng/flea, representing a five-fold variation in response (Table 4). The field-collected isolates, FI3, FI4, FI8, FI9, FI10, and FI11 had shallow slopes (< 2) requiring the highest doses to kill all of the fleas. The field-collected isolates FI7, FI8, and FI9 had significantly higher LD_{50}s to *d*-phenothrin than did the laboratory strains Auburn, Monheim, and UCR, although the total range of variation was less than four-fold (Table 5). The regression equations for FI3, FI8, FI9, FI10, and FI11 had slopes < 2, requiring high doses to provide 95 % kill.

Bioassays with non-pyrethroid insecticides

All of the strains and isolates had similar responses to tetrachlorvinphos, except FI3 which showed 21-fold resistance at LD_{50} compared to Auburn (Table 6). However, the slope of the regression equation was typically < 2 for most of the strains and isolates and substantially higher doses were required to kill 95 % of fleas compared with Auburn. All of the strains and isolates responded similarly to fipronil at LD_{50} and LD_{95} (Table 7). The field-collected isolates FI3, FI4, and FI12 had regression slopes < 2. There were also very consistent responses to imidacloprid (Table 8). As little as 0.05 ng/flea killed 50 % of the adult fleas in several field-collected isolates. None of the strains or isolates had a regression slope < 2.

Genotyping assays

The isolates varied substantially in genetic composition at the *rdl* locus. All of the Auburn and FI6 insects tested were homozygous susceptible, whereas all FI1 and FI3 insects were homozygous for the resistance allele (Table 9). All the other isolates contained a mixture of genotypes.

The isolates also varied substantially in frequencies of the *kdr* and *skdr* mutations (Tables 10 and 11). Interestingly, for most isolates the distribution

Table 5 The minimum lethal dose of *d*-phenothrin (ng/flea) required to kill adult *C. felis*

Strain/isolate	n	slope ± SE	LD_{50} (95 % CL)	LD_{95} (95 % CL)	RR_{50} (isolate/ UCR)
Auburn	150	4.54 ± 0.65	35.8 (26.6–44.5)	82.3 (63.2–137)	1.0
KS1	190	6.29 ± 1.17	59.1 (51.7–65.7)	108 (91.5–149)	1.7
Monheim	150	2.99 ± 0.52	61.6 (48.8–79.7)	219 (143–565)	1.7
UCR	180	5.78 ± 0.83	35.6 (28.7–42.3)	68.5 (55.7–98.9)	---
FI1	150	2.76 ± 0.41	42.4 (28.6–59.1)	167 (103–580)	1.2
FI2	150	4.95 ± 1.45	68.0 (45.6–83.1)	146 (107–652)	1.9
FI3	240	1.45 ± 0.24	61.8 (38.0–90.2)	839 (421–3380)	1.7
FI4	150	2.35 ± 0.46	40.3 (28.2–52.4)	202 (130–507)	1.1
FI6	150	4.16 ± 0.53	32.2 (22.4–42.9)	80.0 (57.0–165)	0.9
FI7	150	3.99 ± 1.17	116 (94.5– 215)	301 (180–1910)	3.3
FI8	210	1.87 ± 0.33	102 (55.2–164)	772 (366–7610)	2.9
FI9	242	1.91 ± 0.41	130 (93.5–215)	942 (419–12300)	3.6
FI10	240	1.10 ± 0.24	14.5 (0.574–36.9)	215 (not computable)	0.4
FI11	300	1.04 ± 0.14	40.5 (20.4–76.2)	1550 (463–32300)	1.1
FI12	150	2.82 ± 0.47	36.0 (27.0–44.6)	138 (100–245)	1.0

Table 6 The minimum lethal dose of tetrachlorvinphos (ng/flea) required to kill adult *C. felis*

Strain/isolate	n	slope ± SE	LD_{50} (95 % CL)	LD_{95} (95 % CL)	RR_{50} (isolate/ Auburn)
Auburn	120	3.55 ± 0.56	20.0 (16.1–24.0)	58.3 (44.6–92.4)	---
Monheim	150	1.24 ± 0.22	28.8 (7.87–56.1)	606 (248–6090)	1.4
UCR	150	1.84 ± 0.26	63.4 (35.2–99.8)	495 (261–194)	3.2
FI1	90	1.96 ± 0.40	46.9 (27.8–79.8)	323 (27.8–79.8)	2.3
FI3	120	2.84 ± 0.60	420 (278–565)	1590 (106–3710)	21.0
FI4	180	2.86 ± 0.46	22.0 (14.1–31.8)	82.8 (53.3–205)	1.1
FI6	90	1.72 ± 0.40	50.8 (24.5–118)	461 (166–56100)	2.5
FI8	90	2.46 ± 0.46	22.9 (14.1–32.3)	107 (70.1–230)	1.1
FI9	90	2.33 ± 0.43	24.6 (8.91–43.6)	125 (64.2–1110)	1.2
FI10	150	1.20 ± 0.27	30.0 (5.93–63.4)	698 (257–14000)	1.5
FI11	150	1.52 ± 0.40	29.3 (10.8–51.8)	356 (134–26700)	1.5
FI12	270	1.09 ± 0.15	40.0 (22.7–68.0)	1310 (498–8510)	2.0

of genotypes for *kdr* was the reverse of that for *skdr*. Thus, seven FI12 insects were homozygous susceptible for *kdr* but none were homozygous resistant for this mutation. For *skdr*, the reverse was the case with seven being homozygous resistant and none being homozygous susceptible. Five insects were heterozygous for both mutations. The consistency of this pattern across isolates implies that the two mutations in the sodium channel were segregating in opposition to one another and that alleles containing both mutations are absent or very rare.

Discussion

The topical application of insecticides to the cuticle of adult *C. felis* provided a sensitive and precise method of determining the susceptibility of fleas. This method was first used for fleas by Moyses (1995) and Moyses and Gefeller (2001), who provided data for a number of insecticides against one well characterised laboratory flea strain, the Danish Pest Infestation Laboratory (DPIL) strain. Despite some minor differences in methodology, our results

Table 7 The minimum lethal dose of fipronil (ng/flea) required to kill adult *C. felis*

Strain/isolate[a]	n	slope ± SE	LD_{50} (95 % CL)	LD_{95} (95 % CL)	RR_{50} (isolate/Auburn)
Auburn	150	2.58 ± 0.39	0.18 (0.109–0.238)	0.76 (0.517–1.57)	----
KS1	330	3.00 ± 0.28	0.29 (0.235–0.358)	1.03 (0.778–1.54)	1.6
Monheim	150	3.91 ± 0.50	0.28 (0.228–0.343)	0.75 (0.586–1.10)	1.6
UCR	150	4.04 ± 0.55	0.28 (0.229–0.330)	0.72 (0.557–0.992)	1.6
FI1	120	3.38 ± 1.14	0.09 (0.072–0.261)	0.29 (0.149–6.86)	0.5
FI2	120	2.27 ± 0.76	0.13 (0.086–0.465)	0.67 (0.261–5.74)	0.7
FI3	230	1.52 ± 0.25	0.13 (0.043–0.213)	1.55 (0.845–6.90)	0.7
FI4	150	1.80 ± 0.36	0.16 (0.061–0.243)	1.28 (0.726–5.39)	0.9
FI6	150	2.55 ± 0.46	0.35 (0.167–0.530)	1.56 (0.907–9.28)	1.9
FI7	150	3.50 ± 0.45	0.24 (0.174–0.302)	0.70 (0.511–1.19)	1.3
FI8	150	2.97 ± 0.78	0.40 (0.229–0.514)	1.43 (1.01–3.79)	2.2
FI9	120	2.63 ±0.55	0.11 (0.061–0.150)	0.46 (0.324–0.923)	0.6
FI10	150	2.48 ± 0.36	0.25 (0.170–0.330)	1.15 (0.760–2.52)	1.4
FI11	270	2.00 ± 0.35	0.28 (0.154–0.382)	2.14 (1.03–31.8)	1.6
FI12	270	1.66 ± 0.31	0.22 (0.038–0.399)	2.14 (1.03–31.8)	1.2

Table 8 The minimum lethal dose of imidacloprid (ng/flea) required to kill adult *C. felis*

Strain/isolate[a]	n	slope ± SE	LD_{50} (95 % CL)	LD_{95} (95 % CL)	RR_{50} (isolate/KS1)
Auburn	120	5.19 ± 1.46	0.06 (0.047–0.078)	0.13 (0.101–0.300)	1.2
KS1	120	3.39 ± 0.562	0.05 (0.031–0.079)	0.15 (0.093–0.697)	---
Monheim	110	5.97 ± 1.36	0.08 (0.067–0.094)	0.15 (0.118–0.262)	1.6
UCR	420	2.18 ± 0.213	0.10 (0.070–0.142)	0.55 (0.298–2.08)	2.0
FI1	120	7.20 ± 1.41	0.03 (0.030–0.038)	0.06 (0.047–0.083)	0.6
FI2	120	4.68 ± 1.27	0.06 (0.041–0.081)	0.14 (0.099–0.816)	1.2
FI3	210	3.44 ± 0.850	0.07 (0.044–0.084)	0.20 (0.129–1.28)	1.4
FI4	360	8.04 ± 1.40	0.05 (0.042–0.060)	0.08 (0.069–0.147)	1.0
FI5	290	2.72 ± 0.344	0.09 (0.061–0.142)	0.37 (0.210–2.00)	1.8
FI7	245	2.91 ± 0.492	0.18 (0.071–0.291)	0.67 (0.415–1.98)	3.6
FI8	210	5.48 ± 1.09	0.07 (0.045–0.086)	0.14 (0.106–0.553)	1.4
FI9	180	4.78 ± 0.842	0.05 (0.042–0.061)	0.11 (0.092–0.18)	1.0
FI10	120	3.78 ± 0.653	0.05 (0.024–0.084)	0.13 (0.080–1.29)	1.0
FI11	120	2.18 ± 0.379	0.02 (0.015–0.031)	0.13 (0.078–0.312)	0.4
FI12	210	6.03 ± 1.29	0.05 (0.034–0.064)	0.10 (0.076–0.224)	1.0

for fipronil and imidacloprid tested against four laboratory reference strains and a diverse range of field isolates are broadly very consistent with those obtained with DPIL (Moyses and Gefeller 2001). However, most of the field-collected isolates were significantly less susceptible to deltamethrin and permethrin compared with the DPIL strain tested in 2001. When DPIL was tested against the organophosphates diazinon, fenthion, and malathion, the slopes of the regression lines were steep >3.4 and LD_{50}'s ranged from 2.6 to 12 µg/flea (Moyses and Gefeller 2001). In our study with tetrachlorvinphos, the slopes were shallow<2 and dosages >20 µg/flea required to kill 50 % of the fleas.

With the exception of the FI3 isolate (which contained only *skdr*), all of the strains and isolates possessed both the *kdr* and *skdr* mutations. Thus,

Table 9 *Rdl* genotypes of laboratory and field-collected isolates of *C. felis*.

isolate	n	S/S	S/R	R/R
Auburn	8	8	0	0
FI1	7	0	0	7
FI2	12	2	3	7
FI3	10	0	0	10
FI4	8	4	3	1
FI5	6	3	2	1
FI6	8	8	0	0
FI7	6	1	2	3
FI8	8	1	3	4
FI11	8	0	5	3
FI12	12	2	5	5

Table 10 *Kdr* genotypes of laboratory and field-collected isolates of *C. felis*.

isolate	n	S/S	S/R	R/R
Auburn	8	0	8	0
FI1	7	2	5	0
FI2	12	7	4	1
FI3	10	10	0	0
FI4	8	0	3	5
FI5	6	1	3	2
FI6	8	2	5	1
FI7	6	1	4	1
FI8	8	3	5	0
FI11	8	0	4	4
FI12	12	0	5	7

Table 11 *Skdr* genotypes of laboratory and field-collected isolates of *C. felis*.

isolate	n	S/S	S/R	R/R
Auburn	8	0	8	0
FI1	7	0	5	2
FI2	12	1	4	7
FI3	10	0	6	4
FI4	8	5	3	0
FI5	6	2	3	1
FI6	8	1	5	2
FI7	6	1	4	1
FI8	8	0	5	3
FI11	8	4	4	0
FI12	12	7	5	0

it seems unlikely that we tested a strain or isolate that was truly susceptible to pyrethroids. The amino-acid substitution underpinning *kdr* (L1014F) is thought to confer 10–30 fold resistance to a broad range of pyrethroid molecules (Farnham et al. 1987) whereas that underpinning *skdr* (T929V)

may result in very high resistance ratios (Roditakis et al. 2006). The ubiquity of *kdr* and *skdr* alleles in long-standing laboratory strains as well as field-collected isolates from Australia and the USA is surprising, given that the former have had no exposure to pyrethroids for at least 20 years, and implies that they have persisted and confer no fitness cost on their carriers under laboratory rearing conditions. The distribution of these mutations across isolates shows that fully susceptible alleles (ie. ones lacking both mutations) are uncommon. Only the FI3 isolate, which was consistently homozygous susceptible for *kdr* and contained heterozygotes for *skdr* can be implicated as possessing a completely wildtype allele, and under Mendelian rules we assume that testing more individuals from this isolate would disclose fleas homozygous susceptible for both mutations. In addition, the mutations segregate in opposition and we found no evidence of an allele containing both mutations, which is a prerequisite for generating fleas homozygous for both *kdr* and *skdr*.

The *rdl* mutation causing a conformational change to the GABA-gated chloride channel is well known to confer significant, economically damaging levels of resistance to a range of cyclodiene insecticides (Ffrench-Constant 1994). Cyclodienes share this target site with fipronil and there has been much debate over the likelihood of the *rdl* mutation, selected through prior exposure to cyclodienes, conferring cross-resistance to fipronil (Bass et al. 2004b). It can be concluded from this study, encompassing isolates in which *rdl* is evidently rare (eg. Auburn) and ones in which it appears ubiquitous (eg. FI3), yet which exhibit very similar responses to fipronil, that the extent of such cross-resistance is negligible (see also Brunet et al. 2009). This of course doesn't preclude the appearance of new alleles capable of substantial reductions in receptor binding by fipronil leading to a potent resistance phenotype (Le Goff 2005).

The susceptibility of field-collected isolates of *C. felis* from Europe, Australia and the USA to imidacloprid has been investigated since 2002 by exposing larvae to a diagnostic concentration of insecticide in a diet-impregnation bioassay (Rust et al. 2002, 2005, 2011; Schroeder et al, 2003). To date, testing of over 100,000 individuals from over 1500 isolates has disclosed no evidence for a directional shift in response or a decline is susceptibility (Rust et al, 2011; Kopp et al. 2013). However, it could be argued that over-reliance on a larval bioassay, despite its operational advantages for resistance monitoring, could disguise the emergence of a resistance phenotype expressed only in adults. The lack of significant variation in susceptibility to imidacloprid in adults through topical application is therefore reassuring and vindicates the design of the ongoing resistance monitoring programme.

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Ethical standards

The study was performed in compliance with current national laws and regulations.

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Conflict of interest

Norbert Mencke, Sabrina Foit and Kathrin Tetzner are employed by Bayer Animal Health GmbH.
Wendell Davis was, Joe Hostedler and Robert Rees are employed by Bayer Health Care.
All other authors are employed by their respective University institutions and declare no conflict of interests.

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