Spinosad Toxicity to *Simulium* spp. Larvae and Associated Aquatic Biota in a Coffee-Growing Region of Veracruz State, Mexico

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ABSTRACT Spinosad is a naturally derived insecticide that has shown potential as a mosquito larvicide. To determine the activity of spinosad against blackflies, late-instar larvae from a community comprising *Simulium triittatum* (63.6%) and seven other species, including three known vectors of onchocerciasis in Mexico (*S. metallicum*, *S. ochraceum*, and *S. callidum*), were subjected to concentration-mortality laboratory bioassays following World Health Organization guidelines. Cephalic capsule measurements confirmed the relatively homogeneous distribution of experimental larvae. The 50% lethal concentration of spinosad was estimated at 1.48 ppm spinosad (95% confidence interval: 1.07–2.33) for a 10-min exposure period, whereas larvae treated with 0.05 ppm of the organophosphate temephos experienced 61% mortality. Immature aquatic insects were identified to genus and tested for their susceptibility to spinosad in the laboratory. After exposure to 12 ppm spinosad for 10 min, ephemeropterans, odonates, trichopterans, and hemipterans did not experience significantly increased mortality over that of untreated controls, whereas a significant increase in mortality was observed in spinosad-treated Plecoptera ($P < 0.001$). Tilapia and trout fry exposed to 12 ppm spinosad for 10 min did not experience increased mortality at 24-h postexposure over that of the controls. We conclude that spinosad is less toxic than temephos to these blackfly species, but is likely to have a low impact on nontarget members of the aquatic community.

KEY WORDS blackflies, bioassay, nontarget organisms, aquatic insect community, fish fry

A number of species of blackflies in the genus *Simulium* (Diptera: Simuliidae) transmit the filarial parasite *Onchocerca volvulus* Bickel in West Africa and Latin America (Davies 1994, Adler 2005). In the Americas, an estimated 450,000 people are at risk of onchocerciasis (Cupp et al. 2004); most of them are inhabitants of mountainous coffee-growing regions with fast-flowing streams that are ideal habitats for the development of blackfly larvae. Over 70% of the infected human population of Latin America is located in the foci of southern Mexico and Guatemala (Rodríguez-Pérez et al. 2008a).

Transmission of the disease has recently been suppressed in the states of Oaxaca and Chiapas in Mexico (Rodríguez-Pérez et al. 2008a,b) and in Guatemala (Cupp et al. 2004, Lindblade et al. 2007) by mass administration of ivermectin to the affected communities at intervals of 3 or 6 mo. However, a number of problems remain to be resolved. First, epidemiological modeling has raised concerns regarding the long-term efficiency of multiple ivermectin treatments for interrupting filarial transmission (Bottomley et al. 2008). Second, migrant workers can comprise a reservoir of infected individuals that potentially contribute to seasonal transmission in coffee-growing areas (Rodríguez-Pérez et al. 2007). Finally, evidence of resistance to ivermectin has begun to appear in West Africa, stimulating new projects aimed at identifying novel approaches to disease control (Taylor et al. 2009).

The naturally derived insecticide spinosad is a mixture of two tetracyclic macrolide molecules (spinosyns A and D) produced during fermentation of a soil actinomycete (Thompson et al. 2000). This product has a favorable ecotoxicological profile with extremely low toxicity to birds and mammals and low or moderate toxicity to fish. Spinosad is the active ingredient in a number of agricultural insecticidal products registered in numerous countries for control of phytophagous Lepidoptera, Diptera, and Thysanoptera. Recent studies have reported that spinosad is toxic to a number of important insect vectors and may provide effective control of the larval stages of *Aedes*, *Anopheles*, and *Culex* mosquitoes (Bond et al. 2004, Liu et al. 2004, Pérez et al. 2007, Jiang and Mulla 2009, Sadanandane et al. 2009).

For this study, we examined spinosad toxicity to *Simulium* spp. collected in a coffee-growing region of

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Veracruz State, Mexico. We quantified the concentration-mortality response and also examined spinosad toxicity to a selection of sympatric aquatic insects and fish fry obtained from local fish farms. We conclude that spinosad deserves further evaluation as a larvicide for control of pestiferous and vector blackflies.

Materials and Methods

Collection of Immature Blackflies. Blackflies and other aquatic insects were collected from the Pixquiac river as it passes through the Bola de Oro coffee plantation (19°28′16"N, 96°57′38.81"W) at an altitude of ≈1,200 m, close to the town of Coatepec, Veracruz State, Mexico. The river was 5–8 m wide and 30–50 cm deep during sampling and flowed over rocks of different sizes. Riparian vegetation consisted mainly of trailing reeds, grasses, coffee bushes, and mature plane trees.

On 12 occasions between June and September 2008, immature blackflies were collected by examining leaves and plant stems trailing in fast-flowing sections of the river for the presence of larvae and pupae. Colonized substrate was removed, placed in buckets of river water, sealed, and immediately transported to the laboratory, a process that took 30–60 min from start to finish. In the laboratory, buckets were aerated using aquarium air pumps and diffusers. Plant material was immediately examined, larvae were classified by size, and late instars were transferred using soft forceps to aerated plastic cups containing unchlorinated drinking water. Early instars were discarded and pupae were preserved in 70% denatured alcohol for later identification.

As a result of the difficulties with the reliable identification of larvae, pupae were identified using the keys of Vargas and Díaz-Nájera (1957) and Onishi et al. (1977), but a selection of late-instar larvae was also examined at each collection to ensure that taxonomic characteristics such as head capsule markings, the form of the ventral postgenal cleft, and anal papillae were consistent with the species that had been identified from pupal material.

Determination of Susceptibility of Blackfly Larvae to Spinosad. Bioassay procedures followed the WHOPES (1996) recommended protocol for chemical insecticides with minor modifications. Groups of 20 late-instar larvae were placed in 500-ml plastic cups containing 200 ml of aerated river water at ambient temperature (20–22°C) and allowed to acclimatize for 90 min. After acclimatization, water was poured out of the cups and replaced with a 200-ml suspension of spinosad (SpinTor 12SC, Dow Agrosciences LLC, Indianapolis, IN) at one of the following concentrations: 0.2, 0.4, 0.8, 1.6, and 3.2 ppm (mg active ingredient/liter) and an untreated river water control. The cups were then constantly agitated at 250 rpm for 10 min, after which time the insecticide was poured out, the cup was rinsed with river water, and finally larvae were incubated with 200 ml of aerated river water for 5 h. After this, larvae that were unresponsive to the touch of a toothpick were classified as dead and those that had pupated during the incubation period were eliminated. Additionally, a single concentration of 0.05 ppm temephos was included as a positive control. Temephos 50EC (emulsifiable concentrate) used by the Mexican Department of Public Health was diluted in ethanol and subsequently diluted 100-fold in distilled water. The bioassay with spinosad was performed on 12 occasions (a total of 231–239 insects/concentration) and the temephos treatment (a total of 118 insects) was included in six of them.

At the end of each bioassay, all larvae were placed in 70% ethanol. The length of cephalic capsule was individually measured dorsally from the most anterior to the most posterior point (length) and between the posterior base of the cephalic fans (width) using an Olympus SZX12 binocular microscope at ×40 objective with a Media Cybernetics PL-A642 camera with image analysis software (Image-Pro Plus version 4.5.1.22, Media Cybernetics, Bethesda, MD).

Screening Other Aquatic Insects for Susceptibility to Spinosad. Between October 2008 and February 2009, aquatic insects were collected from the same site as the blackflies using a triangular net with a 1-mm mesh size that was placed in a fast-flowing section of the river and held in place by one of us. A second person stood 4 m directly upstream and began kicking the riverbed while slowing walking toward the mouth of the net over a period of ≈1 min. The net was then examined and all aquatic insects were transferred using soft forceps to buckets containing river water, small stones, and vegetation as refugia and substrates.

Buckets containing insects were sealed and immediately transported to the laboratory (21–23°C) where they were aerated and carefully examined. Groups of 20 insects from similar orders were transferred to plastic cups containing 200 ml of spinosad suspension (12 ppm), or a water control, and agitated at 250 rpm for 10 min. The experimental suspension was then poured out and cups were rinsed for 10 s with clean water, which was discarded. Finally, each group of insects was placed in an aerated cup with 200 ml of unchlorinated drinking water, and a clean stone was taken from the river as substrate. Mortality was noted after a 5-h incubation period at ambient temperature. All insects used were placed in 70% alcohol for identification to genus following the keys of Lehmkuhl (1979), Stewart et al. (1993), Merritt and Cummins (1996), Westfall and May (1996), McCafferty et al. (1997), and Needham et al. (2000). The number of replicates was dependent on the availability of each order of insects collected and varied from six (Ephemeroptera), seven (Odonata), eight (Plecoptera), nine (Hemiptera), and a total of 12 replicates for Trichoptera. Samples of all aquatic insects identified in this study have been deposited in the entomological collection of the Instituto de Ecología AC (Xalapa, Mexico).

Susceptibility of Fish Fry to Spinosad. Fry of tilapia (*Oreochromis niloticus* L.) and rainbow trout (*Oncorhynchus mykiss* Walbaum) were obtained from El colibrí and El cielo ubicado fish farms in the vicinity of the villages La Antigua and Xico Viejo in the state

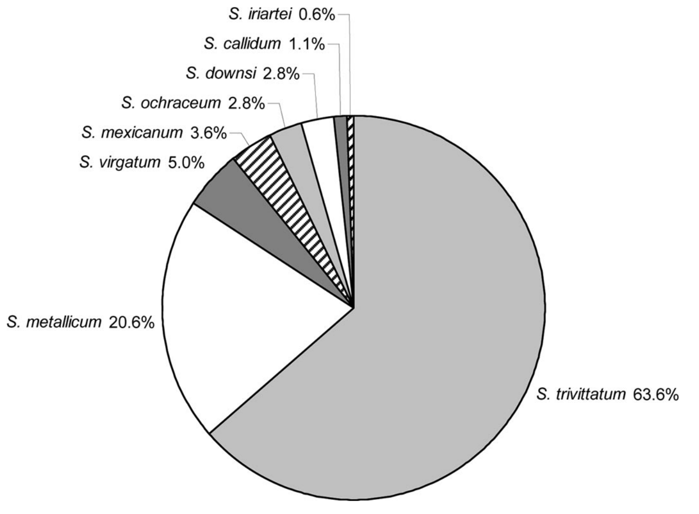

Fig. 1. Species composition of blackfly community sampled from the Pixquiac River, Veracruz State, Mexico, and used in laboratory bioassays, based on the identification of pupae collected at the same site and time (total sample size N = 360).

of Veracruz, Mexico. After several days in aerated laboratory aquaria and being maintained on a diet of aquarium fish food (Wardley tropical crumbles, Hartz Mountain, Secaucus, NJ), groups of 11–15 tilapia or 18–20 trout individuals were carefully transferred to a square net (10 × 10 cm) made from wire and the toe of a pair of women's polyamide and elastane tights. Each net was then placed in a plastic cup containing 325 ml of 12 ppm spinosad suspension or a control cup containing unchlorinated drinking water for a period of 10 min. Finally, each net was dipped in clean water for 10 s and fry were gently released into aerated cups containing 325 ml of drinking water and incubated in a bioclimatic chamber at 26 ± 0.5°C for 24 h. After this period, the number of living and dead fry was recorded, and each individual was sacrificed and measured from the anterior point of the mouth to the central point of the tail. Tilapia fry were 9.4 ± 0.97 mm (mean ± SD, N = 81) in length, whereas trout fry were 26.1 ± 1.7 (N = 231) in length. The experiment was performed four times with tilapia and six times with trout fry.

Statistical Analysis. The concentration mortality response of blackfly larvae was estimated by logit regression in generalized linear interactive modeling (Numerical Algorithms Group 1993) with a binomial error structure specified. When appropriate, moderate overdispersion was taken into account by scaling the error distribution (Crawley 1993). Similarly, mortality in control and spinosad-treated groups of aquatic insects was compared by fitting generalized linear models with a binomial error structure in generalized linear interactive modeling, except in the case of the results in Trichoptera for which the percentages of mortality were compared by Mann–Whitney U test on ranks. Length and width measurements of the larval cephalic capsule were subjected to factor analysis by the principal axis method in Statistica 8.0 (StatSoft, Tulsa, OK).

Results

Toxicity of Spinosad to Blackfly Larvae. Of a total of 360 blackfly pupae collected concurrently with larvae, the most abundant species was *Simulium trivittatum* Malloch (Fig. 1), followed by seven other species, of which three are implicated in the transmission of onchocerciasis in Mexico, namely, *S. metallicum* s.l., *S. ochraceum* s.l., and *S. callidum* Dyar and Shannon (both *S. metallicum* and *S. ochraceum* are cytospecies complexes).

Cephalic capsule measurements confirmed the relatively homogeneous distribution of larvae used in bioassays with a single continuous cluster and no evidence of stepped or distinct size classes or subgroups (Fig. 2). Principal axis analysis revealed a weak relationship between cephalic capsule width and length (eigenvalue 0.871, factor loadings 0.660) that explained 43.6% of variance.

Larvae exposed to spinosad suffered 15–74% mortality at 5-h postexposure. The slope (±SE) of the fitted logit regression was 0.8925 ± 0.1610, and the intercept was 0.3250 ± 0.1474. The 50% lethal concentration of spinosad was estimated at 1.48 ppm (active ingredient) (range, of 95% confidence interval: 1.07–2.33). The mortality of larvae treated with temephos was 61%, whereas that of control larvae was 5.1%.

Toxic Effects of Spinosad on Aquatic Insects. Appreciable levels of mortality (19–33%) were observed in control insects from the orders Ephemeroptera, Odonata, Plecoptera, and Trichoptera (Fig. 3A, and C–E), indicating that these orders were sensitive to laboratory conditions, whereas aquatic Hemiptera experienced a comparatively low level of mortality (6%; Fig. 3B). After exposure to 12 ppm spinosad, ephemeropterans, odonates, and hemipterans did not experience significantly increased mortality over that of the respective control, whereas a significant increase in mortality was observed in spinosad-treated Plecoptera

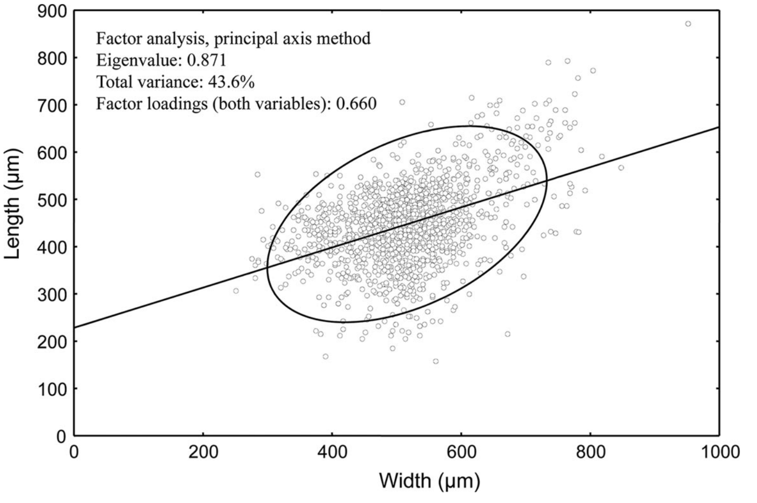

Fig. 2. Scatterplot of larval blackfly head capsule width and length for late instars used in toxicity bioassays. Measurements were subjected to factor analysis by the principal axis method. Also shown are the standard regression line and 95% interval ellipse area.

(Fig. 3D; $\chi^2 = 48.1$, df = 1, $P < 0.001$). The mortality response of Trichoptera showed high levels of overdispersion and was not suitable for fitting generalized linear models. Consequently, these results were subjected to a nonparametric Mann–Whitney *U* test on percentage of mortality. Median mortality of spinosad-treated Trichoptera was 40% compared with 25% in the control (Fig. 3E), but this difference was not significant (U = 75, $N = 23$, $P = 0.57$).

Identifications performed after the toxicity tests revealed that all orders comprised a single dominant genus (Table 1). The large majority of Ephemeroptera were members of the genus *Leptohyphes* (78%), whereas the dominant genera in the other orders were *Ambrysus* (96% of Hemiptera), *Brechmorhoga* (78% of Odonata), *Anacroneura* (100% of Plecoptera), and *Leptonema* (97% of Trichoptera).

Acute Toxicity Tests in Fish Fry. In groups of tilapia fry exposed to 12 ppm spinosad, one individual died in two of the four replicates, whereas none died in the controls, an effect that was not significant ($\chi^2 = 2.6$, df = 1, $P > 0.05$). In groups of trout fry, five of 117 individuals died after exposure to spinosad, which was not significantly different from the control in which 11 of 114 individuals died ($\chi^2 = 2.15$, df = 1, $P > 0.05$), probably because of aggressive behavior among individuals that experienced high densities during the postexposure incubation period.

Discussion

The 50% lethal concentration (LC_{50}) value of spinosad in late instars of a *Simulium* community from a coffee region near the Gulf Coast of Mexico was estimated at 1.48 ppm after a 10-min exposure period, which is considerably higher than the corresponding value (0.05 ppm) for temephos. However, spinosad may have a number of ecotoxicological advantages over temephos, particularly in riverine systems that harbor numerous vertebrate and invertebrate species, some of which are exploited directly as sources of human food. In addition, the water itself is used in the processing of harvested coffee berries and in local fish farms that are common in the region.

The LC_{50} in *Simulium* spp. was also much higher than that reported for mosquito larvae, which usually ranges between 0.01 and 0.06 ppm depending on species (Bond et al. 2004, Cetin et al. 2005, Romi et al. 2006, Darriet and Corbel 2006, Antonio et al., 2009, Jiang and Mulla 2009). However, this reflects the longer period of exposure (1–24 h) used in acute toxicity tests in mosquitoes, compared with the brief exposure used in blackfly larvicide testing.

Of the five orders of aquatic insects tested, only Plecoptera experienced increased mortality in the spinosad treatment, and this mortality was moderate in magnitude (59 versus 19% in control). It is unclear whether the sensitivity of plecopterans to spinosad would be similar in their natural habitat, as the laboratory conditions were apparently stressful to four of the five insect orders tested, as evidenced by elevated control mortalities. These results clearly require confirmation from field experiments involving the dosing of insect communities in natural rivers and streams. A separate study revealed that the abundance of the predatory mosquito *Toxorhynchites theobaldi* Dyar and Knab was markedly reduced in water containers treated with 1 or 10 ppm spinosad for control of *Aedes albopictus* (Skuse) larvae, reflecting the high activity of spinosad to nematocerous Diptera (Marina et al. 2011). In comparison, temephos treatments typically resulted in 20–30% reduction in nontarget aquatic invertebrate populations, with Baetidae and Chironomidae severely affected (Wallace and Hynes 1981).

Because of its growing use as an agricultural insecticide, most studies on spinosad toxicity on nontarget organisms have focused on natural enemies of insect pests of crops, particularly predatory insects and para-

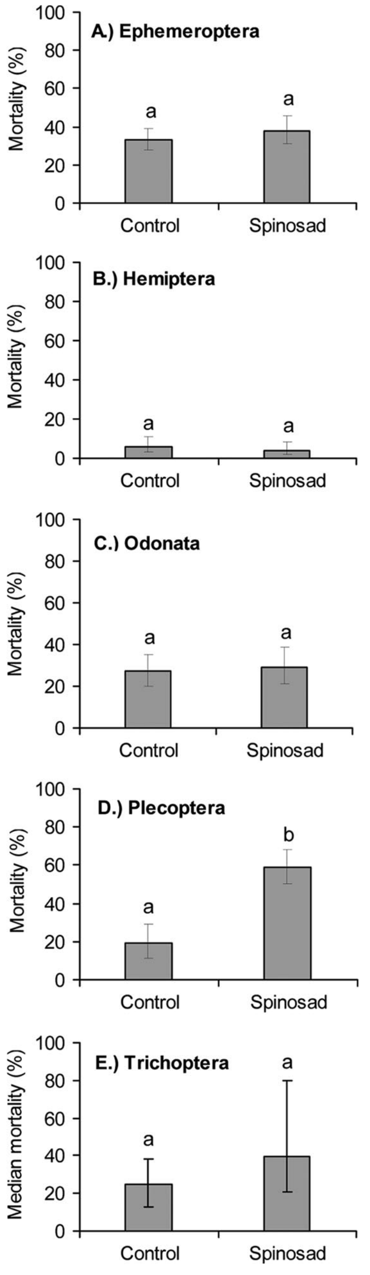

Fig. 3. Effect of 10-min exposure to 12 ppm spinosad on 5 h (A–D) mean (±SE) or (E) median (±interquartile range) percentage of mortality of different orders of aquatic insects in the laboratory. Columns headed by identical letters did not differ significantly (A–D, generalized linear model, $P > 0.05$; E, Mann–Whitney U test, $P > 0.05$). The number of replicates varied from six for Ephemeroptera to a maximum of 12 replicates for Trichoptera (see text for details).

sitoid wasps (Williams et al. 2003). Ecotoxicological studies in aquatic habitats are few in number and have mainly examined the impact of spinosad residues on a standard range of aquatic plants and animals used in product registration, such as water fleas (Cladocerca: Daphniidae). Neonate cladocercans vary markedly in their susceptibility to spinosad with 48-h LC_{50} values of 0.1290 ppm for *Daphnia pulex* Leydig, 0.0048 ppm for *Daphnia magna* Straus, and 0.0018 ppm for *Ceriodaphnia dubia* Richard (Deardorff and Stark 2009). Others have reported higher LC_{50} values on unspecified life stages of *D. magna* (Cleveland et al. 2002a). Exposure to sublethal concentrations affected the survival and reproduction of *D. pulex* in the laboratory, although spinosad was clearly less toxic than the organophosphate insecticide diazinon (Stark and Banks 2001, Stark and Vargas 2003). Populations of *D. pulex* and *D. magna* were severely affected in microcosms treated with 0.008–0.033 ppm spinosad (Duchet et al. 2008, 2009); however, these experimental concentrations exceeded the expected environmental concentration of 0.0023 ppm estimated for contamination of surface waters (Environmental Protection Agency 2005).

Table 1. Genus composition and numbers of aquatic insects used in toxicity tests

Order (Suborder)	Family	Genus	No. individuals tested
Ephemeroptera	Leptophlebidae	*Leptohyphes*	115
		Thraulodes	11
	Trycorythidae	*Tricorythodes*	16
	Baetidae	*Moribaetis*	3
	Heptagenidae	*Stenonema*	2
Odonata			
Zygoptera	Calopterygidae	*Hetaerina*	74
	Coenagrionidae	*Argia*	1
Anisoptera	Gomphidae	*Erpetogomphus*	1
	Libellulidae	*Brechmorhoga*	268
Plecoptera	Perlidae	*Anacroneura*	292
Hemiptera	Naucoridae	*Ambrysus*	252
	Belostomatidae	*Abedus*	10
Trichoptera	Hydropsychidae	*Leptonema*	478
	Polycentropodidae	*Polycentropus*	14
	Lepidostomatidae	*Lepidostoma*	1

We observed no significant increase in fish fry mortality after exposure to 12 ppm spinosad. These results agree with other studies that have reported low toxicity in carp, trout, bluegill sunfish, and minnow with LC_{50} values in the range 5–30 ppm after 96-h exposure (Thompson et al. 2000), or practically no toxicity in juvenile Coho salmon, *Oncorhynchus kisutch* (Walbaum), at rates up to 500 ppm (Deardorff and Stark 2009). Indeed, spinosyns have been patented recently for use in the control of fish ectoparasites in aquaculture, either in the water or in fish feed, underlining their low toxicity to fish (Dick et al. 2008).

A further advantage of spinosad use in aquatic environments is its degradation by photolysis. The half lives of spinosad solutions exposed to natural sunlight are usually 1–2 d (Cleveland et al. 2002b, Liu and Li 2004, Pérez et al. 2007). Spinosad is also degraded by sediment partitioning and microbial degradation, but at a rate significantly lower than that of photolysis (Saunders and Bret 1997, Cleveland et al. 2002a,b). Bioaccumulation and associated ecological problems are therefore highly unlikely.

The transmission of onchocerciasis by blackflies can be effectively controlled by mass medication of the human population with ivermectin. However, *Simulium* spp. also transmit human manssonellosis, bovine onchocerciasis, and avian leucocytozoonosis, as well as causing exsanguination and lethal toxic shock (simuliotoxicosis) in livestock in different parts of the world. In Mexico, apart from the onchocerciasis foci in Chiapas and Oaxaca, blackflies represent a considerable nuisance with biting density indices of ≈3,000 bites/person/season and may exceed 50,000 bites/person/season in some areas during some years (Rodríguez-Pérez et al. 2008a). The human population living in heavily infested areas suffers significant disruption to their daily work or school activities during periods of high biting rates (Adler 2005). In such situations, rapid blackfly control measures are required, and accordingly, spinosad merits additional studies to determine its effectiveness as a blackfly larvicide.

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