Laboratory Evaluation of Diflubenzuron as a Feed-Through for Control of Immature Sand Flies (Diptera: Psychodidae)

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ABSTRACT The benzoylurea chitin synthesis inhibitor diflubenzuron was evaluated as a rodent feed-through for the control of immature stages of *Phlebotomus papatasi* Scopoli (Diptera: Psychodidae). The development and survival of second instars of *P. papatasi* larvae that were fed feces from Syrian hamsters, *Mesocricetus auratus*, that had been fed a diet containing 0, 8.97, 89.7, or 897 ppm diflubenzuron was evaluated. No pupation or adult emergence occurred when larvae were fed feces from hamsters that were fed diets containing diflubenzuron. The mortality of sand flies fed feces from treated hamsters was coincident with pupation of the controls, suggesting a specific effect on the larval-to-pupal molt. The results of this study suggest that a control strategy using rodent baits containing diflubenzuron for phlebotomine sand flies and zoonotic cutaneous leishmaniasis may be possible.

KEY WORDS *Phlebotomus papatasi*, diflubenzuron, sand fly control

Phlebotomine sand flies are the vectors of the protozoan parasites that cause leishmaniasis. Sand flies also are vectors of the disease agents *Bartonella bacilliformis* and sandfly fever virus, and they are notorious pests of humans. Worldwide, there are an estimated 400,000 cases of leishmaniasis annually, and a population of almost 350 million at risk of infection (Ashford et al. 1991). Throughout North Africa, the Middle East, and Asia, *Phlebotomus papatasi* Scopoli (Diptera: Psychodidae) is the primary vector of *Leishmania major*, which is the causative agent of zoonotic cutaneous leishmaniasis (ZCL).

In arid and semiarid foci, *P. papatasi* exhibits a close association with the semifossorial rodents that serve as the reservoirs of *L. major* (Neronov and Gunin 1971). The temperatures within rodent burrows in arid environments are both cooler in the summer and warmer in the winter than outside the burrow, and the relative humidity is near saturation, creating conditions that are ideal for survival of all life stages of sand flies (Kay and Whitfield 1978). In ZCL foci in the Old World, rodent burrows are considered the primary immature habitats for *P. papatasi*, and larvae have been consistently recovered from organic detritus within burrow chambers (Artemiev et al. 1972, Morsy et al. 1993).

The chemical control of sand flies in ZCL foci has rarely been successful due to the difficulty of delivering insecticides to their precise microhabitats (Seyedi-Rashti and Nadim 1973, Karapet'ian et al. 1983). Introducing an insecticide into the burrows is generally precluded by the length and complexity of the tunnels that make up the burrows. Additionally, even successful treatments are short-lived and would require frequent reapplication (Seyedi-Rashti and Nadim 1973, Karapet'ian et al. 1983). Therefore, the development of new methods for the control of the vectors of ZCL is considered a priority for endemic countries.

Diflubenzuron is a benzoylurea that has an arthropod-specific inhibitory effect on chitin formation and deposition in the cuticle. It has pathological effects on the terrestrial larvae of several species of Diptera, including house flies, *Musca domestica* L.; face flies, *Musca autumnalis* De Geer; stable flies, *Stomoxys calcitrans* L.; and horn flies, *Hematobia irritans* L. (Miller 1974, Wright 1974, Kunz et al. 1977). Diflubenzuron also prevents the development of immature stages of *Psychoda alternata*, which is in the same family as *P. papatasi* (Ali and Kok-Yokomi 1990).

Phlebotomine sand fly larvae have been observed feeding on the feces of rodents (WHO 1968), and incorporating larvicides into rodent bait as a method for sand fly larval control has been suggested (M. J. Perich, personal communication). The objective of this study was to assess diflubenzuron as a rodent feed-through. Thus, the development and survival of *P. papatasi* larvae fed feces from Syrian hamsters, *Me-*

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socricetus auratus, that had been fed a diet containing diflubenzuron were evaluated.

Materials and Methods

Feeding Protocol. Adult Syrian hamsters were housed individually in micro-isolator cages and maintained and used as described in Animal Care and Use Protocol 05-074, which was approved by the Institutional Animal Care and Use Committee at Louisiana State University, Baton Rouge, LA. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition. Diflubenzuron (89.7% [AI]; Crompton Corporation, Middlebury, CT) was added to a meal form rodent food (5001 Rodent Diet, LabDiet, PMI Nutrition International, Brentwood, MO). A stock of 100 g of food was prepared daily in a glass beaker for each diet group, and diflubenzuron was added to achieve three diet concentrations: 8.97, 89.7, and 897 ppm. The treated food was thoroughly mixed.

To avoid the inclusion of hamsters that were refractory to eating a powdered diet in this study, the daily food intake of 43 hamsters was recorded for 3 d. The hamsters were ranked by mean daily food intake, and the 12 hamsters with the highest daily food intake were included in this study. Three hamsters were then randomly assigned to each of four diet groups (0, 8.97, 89.7, and 897 ppm diflubenzuron). The body weight of each hamster was recorded once on the day before treated diets were administered.

All hamsters were provided 25 g of their respective diets in a ceramic bowl daily for 9 d. Remaining food was removed every 24 h, and food intake was calculated. Daily dosages of diflubenzuron were calculated by multiplying the daily food intake by the diet concentration. Body weight and daily food intake of hamsters in different diet groups were compared using a repeated measures analysis of variance (ANOVA), performed with the general linear model (GLM) procedure of SAS (SAS Institute 2001). The Tukey multiple comparison procedure was used to separate significantly different means. Within the four hamster diet groups, the ingested dosages of diflubenzuron also were compared with a repeated measures ANOVA performed with the GLM procedure (SAS Institute 2001).

All fecal pellets were removed from the hamster cages each day for 9 d, placed in uncovered plastic cups, and dried at room temperature for 1 wk. The samples of hamster feces were stored at −70°C until used.

Bioassay. A colony of *P. papatasi* was established from larvae obtained from a long-standing colony at the Department of Entomology at Walter Reed Army Institute of Research (Silver Spring, MD). The colony originated from specimens collected in Jordan. Immature sand flies were reared using a standard larval diet comprised of equal parts by weight of dried, decomposed rabbit chow (5321 Rabbit Diet, LabDiet, PMI Nutrition International) and rabbit feces (Young et al. 1981). Hamster feces collected from each diet group after the ninth day of treatment was used in these assays. Fecal pellets from the three hamsters in each diet group were pooled, ground with a pestle, and thoroughly mixed. A portion of the feces (≈0.1 g) was then placed in the vials. A second control group was provided with 0.1 g of the rabbit feces–rabbit chow standard larval diet to allow comparisons between the survival of sand fly larvae fed the two control diets. Bioassays of the five larval groups (three treated and two control groups) were conducted in 26-ml (7-dram) polystyrene vials with a 1-cm-thick basal layer of plaster of Paris extending through a hole drilled in the bottom. The plaster was saturated with distilled water before the experiment, and was blotted with filter paper to remove standing water immediately before use.

Ten second instars (13 ± 1-d-old) larvae were transferred to each vial using a moistened wooden applicator stick. Each vial was closed with a polyethylene cap that was pierced 10 times with an 18-gauge needle. There were six replications (60 larvae total) for each larval diet group. The vials were placed in an environmental chamber at 28°C, 90% RH, and a photoperiod of 14:10 (L:D) h.

Larval mortality was recorded daily; larvae were considered dead if they did not respond within 15 s to prodding with a blunt probe. Alimentation was noted by observation of the presence of frass in the vials and dark material in the guts of the larvae. All larvae were observed for abnormal behavioral and morphological characteristics.

The percentage of survival of sand flies and the age of the sand flies at death in each larval diet group were compared with a repeated measures ANOVA performed with the GLM procedure (SAS Institute 2001). The Tukey multiple comparison procedure was used to separate significantly different means. The mean number of days until adult emergence for larvae fed each larval diet was compared using Student's *t*-test (SAS Institute 2001). The percentages of survival of sand flies fed feces from untreated hamsters and the rabbit feces–rabbit chow standard larval diet also were compared using Student's *t*-test (SAS Institute 2001).

Results

The mean body weight of the Syrian hamsters was 102.6 ± 6.2 g, and there were no significant differences in mean body weight among diet groups. The mean daily food intake was 7.68 ± 1.04, 8.38 ± 1.24, 7.67 ± 0.99, and 7.36 ± 0.82 g for hamsters receiving diets containing 0, 8.97, 89.7, and 897 ppm diflubenzuron, respectively. The mean daily food intake of hamsters was not significantly different between diet groups ($F = 1.27$, df = 3, $P = 0.29$). The estimated mean daily dosages of diflubenzuron for hamsters were 0.68 ± 0.09, 6.26 ± 0.66, and 62.28 ± 7.03 mg/kg body weight for hamsters receiving 8.97, 89.7, and 897 ppm diflubenzuron, respectively.

Table 1. Percentage of mortality and age at death of second instar (13 ± 1-d-old) *P. papatasi* fed feces from three treatment groups of Syrian hamsters receiving different oral dosages of diflubenzuron, feces from untreated Syrian hamsters, or an untreated laboratory larval diet (a 1:1 rabbit feces-rabbit chow diet)

Treatment group	% sand fly mortality	Sand fly age (d) at death
Diflubenzuron		
0.68 ± 0.09 mg/kg b.wt.	100a	30.4 ± 3.6a
6.26 ± 0.66 mg/kg b.wt.	100a	30.0 ± 2.5a
62.28 ± 7.03 mg/kg b.wt.	100a	27.6 ± 2.5a
Control		
Hamster feces	5.0 ± 5.5b	32.0 ± 1.4a
Laboratory larval diet	3.3 ± 5.2b	30.3 ± 3.1a

Values (mean ± SE) within a column followed by the same letter are not significantly different ($P > 0.05$; six replicates, 10 larvae per replicate).

Bioassay. Evidence of food ingestion was found for all larvae in each larval diet group. The mean percentage of survival from second instar to adult was 95 ± 5.48% for the control hamster feces larval group and 96.67 ± 5.16% for the rabbit feces–rabbit chow larval group. Mean percentage of survival was not significantly different between sand flies fed feces from untreated hamsters and the rabbit feces–rabbit chow standard larval diet groups ($t = 0.54$, df = 10, $P = 0.5995$; Table 1). Similarly, the time to adult emergence was not significantly different between the two control groups (larval diet: 21.48 ± 2.73 d; feces 22.19 ± 3.14 d; $t = 1.30$, df = 113, $P = 0.20$).

All sand fly larvae that were fed feces from hamsters fed diets containing diflubenzuron failed to emerge as adults. Larvae fed feces from hamsters fed diflubenzuron began to die around the same time as the first appearance of pupae in the sand flies fed either untreated hamster feces or the standard larval diet (13 d after treatment). Larvae fed feces from hamsters that had been fed diflubenzuron had malformed exoskeletons (translucent and fragile), were ataxic, and did not feed. None of the larvae in the groups fed feces from hamsters fed diets containing diflubenzuron successfully pupated (Table 1; Fig. 1). The mean age at death was 30.4 ± 3.6, 30.0 ± 2.5, and 27.6 ± 2.5 d for larvae reared on feces from hamsters fed 8.97, 89.7, and 897 ppm diflubenzuron, respectively (Table 1). There was no significant difference in the age at death of the sand flies in the three diflubenzuron treatment groups (Table 1).

Discussion

Sand fly larvae fed feces from hamsters fed a diet containing diflubenzuron began to show morphological abnormalities and began to die at nearly the same time that control sand flies began to pupate, suggesting a specific effect of diflubenzuron on the pupation of sand flies. Wright (1974) observed that larvae of *M. domestica* and *S. calcitrans* that had been treated with diflubenzuron died during the transformation from larvae to pupae. Wright (1974) reported that the larvae of *M. domestica* and *S. calcitrans* that had been exposed to diflubenzuron also possessed malformed cuticles that seemed very thin and delicate.

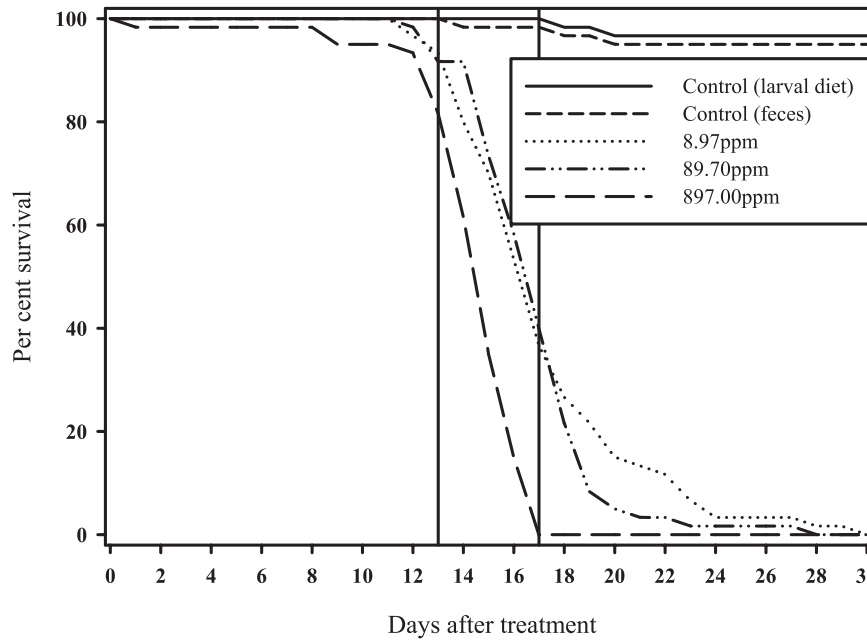

Fig. 1. Cumulative percentage of survival of second instars (13 ± 1-d-old) of *P. papatasi* fed feces from three treatment groups of Syrian hamsters receiving diets containing different concentrations of diflubenzuron, feces from untreated control Syrian hamsters, or an untreated control laboratory larval diet (a 1:1 rabbit feces–rabbit chow diet). Vertical reference lines indicate the first appearance of pupae and adults in control vials.

The food intake of the tested hamsters was not affected by the diflubenzuron treatments, suggesting that diflubenzuron treated diets are palatable to hamsters. The bait preferences of some of the rodent reservoirs of *L. major* are known. *Rhombomys opimus* and *Meriones libycus*, important reservoirs of *L. major* in parts of the Middle East and Asia, are commonly baited with grains (Yaghoobi-Ershadi et al. 2000, 2005). Reservoirs of *L. major* in Sub-Saharan Africa, such as *Arvicanthis* spp., *Mastomys* spp., and *Tatera* spp., are granivorous and could be targeted with treated baits.

The results of this study indicate that rodents could effectively be used as a vehicle to deliver insecticides to the larval habitats of sand flies which are otherwise difficult to locate and reach by conventional means. Diflubenzuron has pharmacokinetic characteristics that make it an appropriate feed additive to control immature flies that live in and feed on feces. Diflubenzuron has low mammalian toxicity, and the majority of the compound is excreted from mammalian systems unchanged in the feces (FAO 1981). It has been used successfully as a feed additive for cattle and chickens (Miller 1974, 1975; Cook and Gerhardt 1977). Diflubenzuron also is relatively persistent in the environment. Miller et al. (1976) found that more than half of the original amount of diflubenzuron was present after 45 d in the feces of cattle fed 16 mg/kg body weight.

The results of this study provide the proof of concept for the future development of feed-through rodent baits containing diflubenzuron for field use for sand fly control. If shown to be effective in field trials, this new method of controlling sand fly larvae also may play a vital role in the prevention of ZCL.

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