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Novel methods for the control of phlebotomine sand flies (Diptera: Psychodidae)

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NOVEL METHODS FOR THE CONTROL OF PHLEBOTOMINE SAND FLIES (DIPTERA: PSYCHODIDAE)

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DEDICATION

This dissertation is dedicated to my parents, Kate and Walter Mascari, and to my brother David Mascari. Their love, support, patience, and encouragement over the years made all of this possible.

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ABSTRACT

In arid and semi-arid parts of the Old World, *Phlebotomus paptasi* is a significant biting pest of man and is the primary vector of *Leishmania major*, the causative agent of zoonotic cutaneous leishmaniasis (ZCL). *Phlebotomus papatasi* exhibits a close association with the burrowing rodents that serve as the reservoirs of *L. major*. Rodent burrows are considered to be the primary habitat of immature *P. papatasi* in ZCL foci, and sand fly larvae have been observed feeding on rodent feces. In laboratory studies, five insecticides (diflubenzuron, novaluron, methoprene, pyriproxyfen, or ivermectin) were incorporated into the diet of Syrian hamsters and evaluated as feed-throughs to control immature sand flies. Feces of hamsters fed a diet containing approximately 10 mg/kg diflubenzuron, novaluron, or pyriproxyfen, or 20 mg/kg ivermectin killed 100% of sand fly larvae that consumed these feces. Feces of hamsters fed a diet containing up to 978.8 mg/kg methoprene caused significant, but not complete, mortality of sand fly larvae. Feces of novaluron-treated hamsters also were held under simulated field conditions for up to 30 d, and all larvae that consumed these feces died before pupation; a significant reduction in treated larval survival relative to control was observed when the feces were aged for up to 150 d. Novaluron also was shown to be effective as a feed-though larvicide when novaluron-treated food made up only a portion of the diet of hamsters. Ivermectin also was evaluated as a systemic insecticide; ivermectin treatment of hamsters was 100% effective against bloodfeeding sand flies for up to 7 d after hamsters were withdrawn from ivermectin-treated diets. In the final study, proof of concept was established for a novel biomarker system using a feed-through fluorescent dye. The value of this method is that it can mark rodents and their feces to establish the consumption of treated-baits, mark adult female sand flies that feed rodents for the duration of persistence of the dye in rodents, and mark adult male and female sand flies that had fed on feces of bait-fed rodents as larvae.

INTRODUCTION

Phlebotomine sand flies are major biting pests of man and are the vectors of several viruses, the bacterium *Bartonella bacilliformis*, and, most importantly, the protozoan parasites that cause leishmaniasis. Worldwide, there are an estimated 2 million new cases of leishmaniasis annually, and 12 million people are currently believed to be infected (WHO 2006). Throughout North Africa, the Middle East and Southwest Asia, *Phlebotomus papatasi* is the primary vector of *Leishmania major*, the causative agent of zoonotic cutaneous leishmaniasis (ZCL).

While larvicides are commonly used to control mosquitoes and many other flies of medical and veterinary importance, there is no current use of larvicides for phlebotomine sand fly control. In arid and semi-arid foci, *P. papatasi* exhibits a close association with several burrowing rodent reservoirs of *L. major* (Neronov and Gunin 1971). In ZCL foci in the Old World, rodent burrows are considered to be the primary immature habitats for *P. papatasi*, but introducing an insecticide into the burrows is generally precluded by the length and complexity of the tunnels which comprise the burrows (Seyedi-Rashti and Nadim 1973, Karapet'ian et al. 1983).

In Old World ZCL foci, sand fly larvae also have been observed feeding on the feces of rodents (WHO 1968). Because of this fact, rodent feed-through insecticides are a potential means of controlling sand fly larvae. Therefore, the chitin synthesis inhibitors diflubenzuron and novaluron, the juvenile hormone analogs methoprene and pyriproxyfen, and the macrocyclic lactone ivermectin were evaluated as rodent feed-through insecticides to control sand fly larvae. The development and survival of *P. papatasi* larvae fed feces of Syrian hamsters, *Mesocricetus auratus*, that had been fed a diet containing an insecticide were measured. Additional studies were conducted to determine the effectiveness of novaluron as a feed-though larvicide to control sand flies under simulated field conditions.

Because populations of *P. papatasi* that live in burrows rely upon rodent reservoirs of *L. major* as a bloodmeal source, incorporating a systemic insecticide into rodent bait could be a potential way to control this epidemiologically important group of adult sand flies. Therefore, experiments were conducted to determine whether the post-bloodmeal survival of adult sand flies would be affected by feeding their rodent hosts a diet containing ivermectin. In this study the insecticidal effect of ivermectin treatments against bloodfed sand flies was monitored for 14 d after rodents were withdrawn from their ivermectin-treated diets, and bioassays with larval sand flies were conducted using feces voided by ivermectin-treated rodents over this same time period.

Prior to or simultaneous with field evaluations of feed-through or systemic control of sand flies in the different sand fly/rodent associations that exist, establishing whether the larvae of different species of sand fly feed exclusively on the feces of rodents must be demonstrated. There are currently no available methods to directly demonstrate if the larval diet of phlebotomine sand flies is exclusively rodent feces. Although sand fly larvae have been recovered from rodent burrows and have been observed feeding on the feces of rodents, larval sampling is an impractical method to demonstrate the larval diet of sand flies. An objective of this research was to establish a fluorescent tracer technique using rhodamine B as a rodent feed-through to identify adult sand flies that had fed on the feces of rhodamine-B treated hamsters as larvae. We also evaluated rhodamine B as a biomarker of bait-fed rodents and the female flies that fed upon them.

CHAPTER 1. LITERATURE REVIEW

1.1 Taxonomy of Phlebotomine Sand Flies

1.1.1 Family Psychodidae

Phlebotomine sand flies belong to the family Psychodidae, which is among the most primitive families of Diptera (Young and Duncan 1994). The family Psychodidae is characterized by their wing venation (the presence of numerous parallel veins running to wing margin), and the presence of dense hairs on the wings and thorax (Triplehorn and Johnson 2005).

1.1.2 Subfamily Phlebotominae

Phlebotomine sand flies are classified within the subfamily Phlebotominae, and are called phlebotomine sand flies to distinguish them from other flies that are sometimes referred to as sand flies (such as members of families Simulidae or Ceratopogonidae). Phlebotomine sand flies are differentiated from other subfamilies within Psychodidae by the presence of biting mouthparts that are longer than the head, five-segmented palps, nearly cylindrical antennae, a five-branched radial vein on the wing, and the absence of an eye-bridge (Triplehorn and Johnson 2005). Some general attributes that can often be used to distinguish sand flies from other small flies include their size (1.5 to 2.5 mm in length), characteristic hopping flight, and the “V” position in which they hold their wings while resting.

1.1.3 Phlebotomine Sand Fly Genera

There are three New World genera within subfamily Phlebotominae: *Brumptomyia* França & Parrot, *Warileya* Hertig, and *Lutzomyia* França (Young and Duncan 1994). Sand flies in the genus *Brumptomyia* have not been reported feeding on humana, and are distinguished from sand flies in other genera by differences in the morphology of male external genitalia (Young and Duncan 1994). Sand flies in the genus *Warileya* are reported to be anthropophilic, but they have not been implicated in the transmission of any human pathogens (Young and

Duncan 1994). Sand flies in the genus *Lutzomyia* feed on mammals and are the only medically important genus of sand flies in the New World. *Lutzomyia* is distinguished from *Brumptomyia* by the number of rows of teeth on the cibarium (*Lutzomyia* has 1 row of transverse teeth, *Brumptomyia* has 4 horizontal rows of teeth), and from *Warileya* by the presence of episternal setae (*Lutzomyia* has episternal setae, and *Warileya* does not).

There are two Old World genera within the subfamily Phlebotominae: *Sergentomyia* França and *Phlebotomus* Rondani & Berté (Lewis 1982). Sand flies in the genus *Sergentomyia* feed primarily on lizards, and may be the vectors of the agents of saurian leishmaniasis. Sand flies of the genus *Phlebotomus* feed on mammals, and represent all of the medically important sand flies in the Old World. Sand flies of the genus *Phlebotomus* can often be distinguished from those within *Sergentomyia* by the cibarium; *Phlebotomus* does not have a row of teeth and usually does not have a patch of pigment (Lewis 1982).

1.2 Sand Fly Biology, Ecology, and Sampling

1.2.1 Immature Stages

The eggs of phlebotomine sand flies are dark brown or black and elliptical in shape. The eggs have ridges in species-specific patterns that potentially could be used for identification. The number of eggs laid by a single female at one time varies greatly by species and by factors such as species of bloodmeal host or ambient temperature, but typically is between 40 to 70 eggs (Young and Duncan 1994). Eggs are laid in batches on moist substrates, and the presence of conspecific eggs can serve as an oviposition attractant and stimulant (Elnaiem and Ward 1991, Srinivasan et al. 1995). The hatching of eggs usually occurs within 10 d after oviposition, but hatching of some eggs in a batch is sometimes delayed for as long as 30 d (Young and Duncan 1994).

Sand fly larvae have four instars. Sand fly larvae are covered in setae along the length of their bodies, and have four caudal setae by the time they reach 4th instar. Sand fly larvae feed on organic matter near the site of oviposition. The larval stage of phlebotomine sand flies is completed in as few as 18 d, but typically lasts longer and can be dependent on temperature (Young and Duncan 1994). Before pupation, sand fly larvae cease feeding and some species may travel a short distance upward to a drier location. Pupae sometimes attach to rocks or other fixed objects.

The sand fly larval habitats have been identified for only a handful of species. In the Old World, immature stages of *P. argentipes*, *P. martini*, *P. papatasi*, *P. celiae*, *P. ariasi*, *P. perfiliewi*, and *P. langeroni* have been recovered from soil taken from inside of structures housing humans or domesticated animals (Dhiman et al. 1983, Mutinga et al. 1989, Killick-Kendrick 1987, Bettini 1989, Doha et al. 1990). Larvae of *P. martini*, *P. papatasi*, and *P. duboscqi* have consistently been recovered from soil taken from inside of rodent burrows (Mutinga et al. 1986, Mutinga et al. 1989, Doha et al. 1990, Dedet et al. 1982, Perfil'ev 1968, Artemiev et al. 1972, Morsy et al. 1993). Larvae of the sand flies *P. martini* and *P. celiae* have been recovered from termite mounds in East Africa (Mutinga et al. 1989).

In the New World, structures housing livestock have been shown to be a larval habitat for *L. longipalpis*, and *L. intermedia* (Deane and Deane 1957, Forattini 1954). Larvae of other species, including many of medical importance (including, *L. trapidoi*, *L. umbratalis*, *L. anduzei*, and *L. whitmani*), have been found among soil and leaf litter on the forest floor (Rutledge and Ellenwood 1975, Arias and Freitas 1982, Casanova 2001).

For many of the species listed above, very few immature specimens have been recovered, and thus little can be stated about the importance of their larval habitats. However, for some species, enough evidence has been compiled to make more definitive conclusions about their

larval habitat. For example, the primary immature habitat of *P. papatasi* outside of urbanized areas is considered to be rodent burrows. Similarly, larvae of *P. duboscqi* have been recovered consistently from inside of rodent burrows; this is considered to be the principle larval habitat for this species.

Several methods have been employed for sampling immature sand flies. However, the process remains time consuming and frequently unproductive regardless of the method used. To illustrate this point, researchers in Central Asia processed over 6 tons of soil and recovered only around 150 immature sand flies (Petrischeva and Izyumskaya 1941). The first sand fly larva (*P. mascittii*) recovered in nature was found by direct examination of a soil sample taken from a cellar in Rome (Grassi 1908). Direct examination of soil to find sand fly larvae was the method used throughout the early 20th century and is still the preferred method of some more recent researchers (Dhiman et al. 1983). A method of extracting immature sand fly larvae from soil samples though differential flotation in salt or sugar solutions also has been used, but there is no improvement in the rate of success and it is no less labor intensive (McCombie-Young et al. 1926). This method has been modified by combining differential flotation with passing the soil samples through a series of nested sieves, but the modified method still was no simpler or productive than flotation or direct examination (Hansen 1961). The larvae of *P. papatasi* also have been extracted from soil samples through dessication with some success in Iran (Seyedi-Rashti and Nadim 1972). This method was validated in the laboratory by extracting larvae from soil samples that had been spiked with larvae from a laboratory colony (Killick-Kendrick 1987). Breeding sites also have been identified by isolating soil samples and recovering adult sand flies as they emergea either through the incubation of soil samples in the laboratory, or by placing emergence traps over suspected breeding sites in the field (Mutinga and Kamau 1986, Bettini et al. 1986).

1.2.2 Adults

Male adult sand flies typically emerge before females from the same egg batch, and they become sexually mature within 1 d (Young and Duncan 1994). Male sand flies can find potential mates through the use of pheromones, or by locating vertebrate hosts or resting sites to which female sand flies also may be attracted. Both specific pheromones and wing-beat rhythms have been identified for mate location for the sand fly *L. longipalpis* (Phillips et al. 1986, Ward and Morton 1991).

Adult male and female sand flies obtain energy by ingesting sugars. Sugar meals can be obtained from a variety of sources, including the sap of plants and honeydew from aphids (Schlein and Warburg 1986, Killick-Kendrick and Killick-Kendrick 1987, Cameron et al. 1995). In arid areas where sand flies are found, the available sources of sugar can be limited to a handful of plant species (Schlein and Yuval 1987). Female sand flies also are required to feed on the blood of vertebrate hosts for the production of eggs. Females of most species take bloodmeals only once per gonotrophic cycle, though females of some species, such as *L. shannoni*, will feed multiple times throughout the gonotrophic cycle (Young and Duncan 1994).

Because of their characteristic short, hopping flight, sand flies are often perceived as weak fliers unable to travel long distances. For many species this holds true: the longest recorded dispersal distance for a *P. papatasi* sand fly was 280 m. Sand flies in forested areas of the New World also do not have long flight ranges; in one study in Panama in which 20,000 sand flies were marked with fluorescent powder and released, the majority of re-captured sand flies were collected within about 50 m of the release site; four sand flies were recaptured 200 m away (Chaniotis et al. 1984). However, *P. ariasi* sand flies have been shown to fly as far as 2 km in southern France (Killick-Kendrick et al. 1984).

Adult sand flies of all species are active at night. During the day, adults of the majority of New World sand fly species have been found resting in tree holes or the buttresses of trees. Adults of the majority of Old World species and some New World species have been found resting in rock crevices, caves, or in man-made structures such as cellars, wells, or animal sheds. Adults of *L. anthophora*, *P. papatasi*, and *P. duboscqi* are all frequently recovered from the burrows or nests of rodents.

In addition to collecting adult sand flies through direct examination of potential resting sites, sand flies can be sampled using either interception traps or attraction traps. Trapping by interception samples the population of sand flies that is active in an area with little bias. Malaise traps (mesh, tent-like devices placed across the suspected flight paths of insects) are often used in New World forests to collect sand flies. This method collects sand flies of both sexes, but generally collects low numbers of sand flies and many non-target insects that may damage sand fly specimens (Alexander 2000). Sticky traps are the most commonly used tool in the Old World for sampling sand fly by interception. The typical design of a sticky trap is a sheet of paper dipped in castor oil and placed in an area where sand flies are thought to be active, including man-made structures, fields, rock crevices, or at the openings of animal burrows and nests (Alexander 2000). Sticky traps are used less frequently in Central and South America because the traps are less effective in areas with high humidity.

Sampling sand flies by attraction can be conducted using animal baited traps. The Disney trap is an effective and simple animal baited trap in which a small animal (often a rodent) is placed in a cage on a tray coated with castor oil (Disney 1966). As sand flies approach the caged animal in short hops, they are trapped in the castor oil. A cone trap has been developed for attracting sand flies to larger animals (Montoya-Lerma and Lane 1996). An animal, such as a

horse, is tethered inside a mesh cage with concave cones that allow sand flies to enter the cage but not exit. The trapped sand flies then can be collected off of the interior walls of the cage.

Battery-operated light traps also have been used to sample sand flies. Light traps are not attractive to sand flies over a great distance; the maximum distance was 2m for *P. ariasi*, 6 m for *L. youngi*, and about 2.5 m for *L. intermedia* and *L. whitmani* (Killick-Kendrick et al. 1985, Valenta et al. 1995, Campbell-Lendrum et al. 1999). Light traps have been shown to preferentially sample females of certain species. This sampling bias is particularly present in the some New World sand flies; for example, over 75% of adult *L. whitmani* collected by light traps were female, but females made up less than 25% of the catch when the bulbs were removed from the traps (Campbell-Lendrum et al. 1999). Using carbon dioxide in conjunction with light traps can be used to increase the number of sand flies collected as well as the range of attraction (Gillies 1980).

1.3 Disease Agents Transmitted by Sand Flies

Sand flies of more than 30 species in the genus *Lutzomyia* and 40 species in the genus *Phlebotomus* are vectors of human pathogens. Phlebotomine sand flies are the vectors of several viruses, the bacterium *Bartonella bacilliformis*, and, most importantly, nearly 20 species of protozoan parasites in the genus *Leishmania*.

1.3.1 Viruses

Sand flies have been shown to be vectors of medically important viruses in three families: Bunyaviridae, Reoviridae, and Rhabdoviridae. The most important viruses transmitted to man by sand flies are in the family Bunyaviridae and genus *Phlebovirus*. In the New World, more than 30 serotypes of the genus *Phlebovirus* have been identified, but their medical importance is not fully known (Tesh et al. 1989). However, in the Old World, two viruses in the genus *Phlebovirus* are of significant public health importance: *Sandfly fever Sicilian virus* (SFSV) and *Toscana*

virus (TOSV, species *Sandfly fever Naples virus*), and. Human infections with SFSV have been confirmed in Italy, Cyprus, Egypt, Iran, and Pakistan; SFSV antibodies have been found in humans in Israel, Jordan, Algeria, Tunisia, Sudan, and Bangladesh (Karabatsos 1985, Papa et al. 2006, Batieha et al. 2000, Cohen et al. 1999, McCarthy et al. 1996, Chastel et al. 1983, Gaidamovich 1984, Izri et al. 2008). The vector of SFSV has been shown to be the sand fly *P. papatasi*, and it is suspected that the distribution of SFSV coincides with the distribution of *P. papatasi* (Karabatsos 1985). The symptoms of infection with *SFSV* typically are pyrexia and myalgia, and cases usually resolve within a week.

Toscana virus has been found in many countries around the Mediterranean including Italy, Spain, Portugal, France, Slovenia, Cyprus, Greece, and Turkey (Hemmersbach-Miller et al. 2004, Peyrefitte et al. 2005, Mendoza-Montero et al. 1998, Echevarria et al. 2003, Eitrem et al. 1985). Two species of sand flies have been incriminated as vectors of TOSV: *P. perniciosus* and *P. perfiliewi* (Charrel et al. 2005). Unlike human infections with SFSV, infection with TOSV can be life-threatening. In Italy, TOSV is considered to be a leading etiological agent of aseptic meningitis (Charrel et al. 2005).

In the New World, a number of viruses in the genus *Orbivirus* and family Reoviridae have been shown to be transmitted to man and other mammals by sand flies (Rosa et al. 1984). In man, these little-studied viruses are believed to produce symptoms similar to infection with *Phlebovirus*.

Chandipura virus (CHPV) is in the genus *Vesiculovirus* and family Rhabdoviridae and has been isolated from sand flies in India and West Africa (Dhanda et al. 1970, Fontenille et al. 1994). Human infections with CHPV typically involve fever, but encephalopathy was reported in one fatal case. The sand fly *P. papatasi* is believed to be the vector of CHPV in India, but the vector remains unknown in West Africa. Venereal transmission of CHPV in *P. papatasi* has been

demonstrated in the laboratory, and *P. argentipes* has been shown to be a competent vector (Mavale et al. 2006, Mavale et al. 2007).

On an uninhabited island in the Atlantic Ocean off the coast of Georgia, USA, the sand fly *L. shannoni* serves as the vector of another virus in the family Rhabdoviridae, the New Jersey serotype of *Vesicular stomatitis virus* (VSV-NJ). The virus has been isolated from the sand fly vector, and from swine and other mammals; because the island is uninhabited, humans are not at risk of infection (Clarke et al. 1996).

1.3.2 *Bartonella bacilliformis*

The bacterium *Bartonella bacilliformis* is transmitted by the sand fly *L. verrucarum* in Peru and parts of Ecuador. There is no known non-human reservoir for *B. bacilliformis*. The disease resulting from infection with *B. bacilliformis* is called bartonellosis or Carrión's Disease (named after Daniel Carrión, who died in 1885 after inoculating himself with infectious material taken from a patient). There are two distinct clinical forms of disease: verruga peruana and Oroya fever. Verruga peruana, the benign form of bartonellosis, is characterized by the appearance of numerous painless nodules on the skin of patients, which, if untreated, resolve within a year. Oroya fever is characterized by fever, arthralgia, hemolytic anemia, and jaundice, and if untreated has a mortality rate of up to 90% (Grey et al. 1990). Both clinical forms of bartonellosis can be treated successfully with antibiotics, such as chloramphenicol.

1.3.3 *Leishmania* spp.

Leishmania is a genus of heteroxenous parasites in the family Trypanosomatidae. *Leishmania* parasites are the etiological agents of a complex of diseases with a broad clinical spectrum called leishmaniasis. Nearly 20 species of *Leishamania* have been shown to cause human disease (Desjeux 2004). Worldwide, 2 million new cases of leishmaniasis are believed to occur annually, and as many as 12 million people currently may be infected (WHO 2006).

Traditionally, species within the genus *Leishmania* have been categorized according to the form of leishmaniasis they cause: visceral leishmaniasis (VL) or cutaneous leishmaniasis (CL). Visceral leishmaniasis (fever, wasting, anemia, and enlargement of the liver and spleen) is often fatal if untreated, and CL, while not life-threatening, can cause long-lasting lesions that can leave disfiguring scars after they heal. The species of *Leishmania* also are further categorized according to the whether or not non-human reservoirs are important in the transmission cycle: zoonotic leishmaniasis (ZCL and ZVL) or anthroponotic leishmaniasis (ACL and AVL). *Leishmania* parasites are transmitted to humans by phlebotomine sand flies of around 30 species in the genus *Lutzomyia* in the New World, and of the genus *Phlebotomus* in the Old World (Desjeux 2004).

New World

In the New World, the main etiological agents of ZCL are *L. mexicana* and *L. amazonensis*. Infections with *L. mexicana* occur primarily among people working or living in forested areas in Central America and Mexico. Climbing rats (*Ototylomys phyllotis*) and other forest rodents serve as the primary reservoirs of *L. mexicana* parasites (Disney 1968). The vector species for *L. mexicana* in Central America and Mexico are *L. olmeca* and *L. ayacuchensis*, respectively (Eduardo 1991). Human cases of ZCL due to infection with *L. mexicana* have been reported in Texas, where the Southern Plains woodrat (*Neotoma micropus*) serves as the enzootic host (Kerr et al. 1995). The sand fly *L. anthophora* frequently is collected in and around woodrat nests and has been incriminated as the vector of *L. mexicana* in Texas.

Infections with *L. amazonensis* occur in northern South America (Bolivia, Colombia, Ecuador, Venezuela Brazil, and French Guyana) and, like *L. mexicana*, occur primarily in inhabitants of settlements that encroach into forests or in visitors to these areas. The incriminated vector of *L. amazonensis* is the sand fly *L. flaviscutellata*, and the reservoir of *L. amazonensis* is

believed to be the spiny rat (*Proechimys* spp.) and a large number of other small mammals (Dedet et al. 1989). Human infections with several other *Leishmania* species that cause ZCL have been reported, including *L. guyanensis*, *L. peruviana*, *L. lainsoni*, *L. panamensis*, *L. shawi*, *L. naiffi*, *L. colombiensis*, and *L. venezuelensis* (Young and Arias 1992).

Infection with *L. braziliensis* causes a primary lesion that occurs at the site of infection and a delayed secondary lesion that occurs in the buccal and nasal mucosa. The cartilage and surrounding tissue degenerate and often become necrotic and subject to secondary bacterial infection. This condition can last for several years and can result in severe deformity, removing the palate, lips, and nose. Infections with *L. braziliensis* occur in Brazil, Colombia, Venezuela, and Bolivia, where it is transmitted by several species of sand flies including *L. wellcomei*, *L. complexus*, *L. whitmani*, and *L. ovalessi* (De Souza et al. 1996, De Queiroz et al. 1994, Feliciangeli and Rabinovich 1998, Warburg et al. 1991, Young and Arias 1992). Nearly a dozen other sand fly species are suspected to be vectors of *L. braziliensis*. The reservoirs of *L. braziliensis* parasites are believed to be sloths and other forest-dwelling mammals (Dedet 1992).

New World ZVL is caused by *L. infantum*. Infections with *L. infantum* occur throughout Central and South America, where the sand fly *L. longipalpis* (an abundant, peridomestic species) serves as the vector (Young and Arias 1992). Many sylvatic animals, particularly foxes, are suspected as important reservoirs of *L. infantum* parasites. However, the role of dogs in the transmission cycle is well established, and they are considered to be the most important reservoir host (Dedet 1992).

Old World

In the Old World, ACL is caused by *L. tropica*. Human infections with *L. tropica* have been reported in the Middle East, Southwest Asia, and North and East Africa. Transmission generally occurs in densely populated areas, where the peridomestic sand flies *P. sergenti* and *P.*

guggisbergi serve as vectors (Lawyer et al 1991, Al-Zahrani et al. 1988, Killick-Kendrick et al. 1995). Transmission of *L. tropica* appears to be maintained indefinitely in humans without the involvement of non-human reservoirs, although some possible non-human reservoirs such as the rock hyrax have been suggested (Sang et al. 1992).

The primary etiological agent of ZCL in the Old World is *L. major*. Human infections with *L. major* have been reported throughout the arid zone stretching from North Africa through the Middle East and into Central and Southwest Asia, and also in arid areas of Sub-Saharan Africa. *Leishmania major* exists as a zoonosis among populations of burrow-dwelling rodents in the family Muridae. Humans are infected with *L. major* when they encroache into enzootic foci (for example, during development projects, urban expansion, or military movements). In Central and Southwest Asia and Iran the rodent reservoirs of *L. major* are *Rhombomys opimus* and *Meriones* spp., and the sand fly vector is *P. papatasi* (Yaghoobi-Ershadi et al. 2004). In North Africa and the Middle East, *Psammomys obesus*, *Meriones* spp., and *Gerbillus* spp. are the main rodent reservoirs, and *P. papatasi* serves as the vector (Saliba et al 1994, Rioux et al. 1982, Rioux et al. 1992, Morsy et al 2001, Morsy et al. 1996, Fichet Calvet 2003). In Sub-Saharan Africa, a number of agricultural and peridomestic rodent pests serve as the reservoirs, and *P. duboscqi* is the only incriminated vector species (Gebre-Michel et al. 1993, Githure et al. 1984, Githure et al. 1986).

Leishmania aethiopica also is an etiological agent of ZCL in the Old World. Human infections with *L. aethiopica* have been reported in Kenya and Ethiopia. Cases of ZCL due to *L. aethiopica* often present with multiple lesions, and the disease is sometimes called diffuse cutaneous leishmaniasis. Hyraxes (*Procavia* spp. and *Heterohyrax* spp.) have been implicated as reservoirs of *L. aethiopica*, and two species of sand flies have been incriminated as vectors: *P. pedifer* and *P. longipes* (Gemetchu 1990).

In the Old World, as in the New World, ZVL is caused by *L. infantum*. Human cases have been reported primarily in the Mediterranean littoral, but also in Southwest and Central Asia. As in the New World, the primary reservoir for *L. infantum* is the dog. The sand fly species that have been incriminated as vectors of *L. infantum* include *P. ariasi*, *P. langeroni*, *P. neglectus*, *P. perfiliewi*, and *P. perniciosus* (Rioux et al. 1979, Pires et al. 1984, Maroli et al. 1987, Doha and Shehata 1992).

Leishmania donovani is the causative agent of AVL in the Old World. Cases of AVL due to *L. donovani* have been reported in Kenya, Ethiopia, Sudan, and the Indian subcontinent. Infection with *L. donovani* is similar to infection with *L. infantum*, and AVL often is fatal if untreated. After treatment, a small percentage of patients develop post-kala-azar dermal leishmaniasis: a condition in which the skin is covered in large nodules that can be disfiguring. Humans are thought to be the only reservoirs for *L. donovani*, but several animals such as the mongoose have been suggested as potential non-human reservoirs (Elnaiem et al 2001). The vectors of *L. donovani* in Africa are the sand flies *P. orientalis*, *P. martini*, and *P. celiae* (Elnaiem et al. 1996, Gebre-Michel and Lane 1993), while *Phlebotomus argentipes* serves as the vector of *L. donovani* in the Indian subcontinent (Joshi et al 1986).

1.4 Rodent/Sand Fly Associations

1.4.1 Sand Flies Associated with Rodent Reservoirs of New World ZCL and Old World VL

Phlebotomine sand flies of many species are associated with rodents. The closeness of this association varies by habitat and the involvement of other (non-rodent) mammals in the transmission of a particular *Leishmania* parasite. In Central and South America, the known reservoirs of *Leishmania* parasites in ZCL foci include rodents such as the spiny rat, *Proechimys* spp., and climbing rat, *Ototylomys phyllotis*. However, many other forest mammals also are suspected to be reservoirs including rodents (*Sciurus vulgaris*, *Heteromys desmarestianus*,

Oryzomys capito, *Nyctomys sumichrasti*, *Akodon* sp., *Sigmodon hispidus*, *Rattus rattus*, *Coendu* sp., and *Agouti paca*), marsupials, edentates, carnivores, and non-human primates (Dedet 1992). The larval habitats of many of the sand fly vectors of ZCL in Central and South America have been shown to be leaf litter and other organic debris dispersed throughout the forest floor (Hanson 1961, Hanson 1968, Arias and Freitas 1982, Vieira et al. 2000, Casanova 2001, Rutledge and Ellenwood 1975). The presence of many alternative hosts and the widely dispersed habitats for immature sand flies make control measures that target reservoir hosts and sand fly larvae improbable.

In the Old World, some sand fly species that are vectors of the agents that cause VL are associated with rodents. Rodents have not been shown to be reservoirs of *L. infantum* or *L. donovani*, and adult sand flies have not been associated with rodents or the rodents' nests or burrows. However, larvae of *P. martini* and *P. langeroni*, have been recovered from inside rodent burrows (Mutinga et al. 1989; Doha et al. 1990). Each of these sand fly species also has many alternative larval habitats in a single VL focus. In Kenya, larvae of *P. martini* have been recovered with greater frequency from termite mounds, and also from inside houses and tree holes (Mutinga et al 1989). A single specimen of *P. langeroni* was recovered from soil inside of a rodent burrow; larvae of *P. langeroni* are much more commonly recovered from piles of rocks and garbage, animal sheds, and wells (Doha et al. 1990).

1.4.2 Sand Flies Associated with Rodent Reservoirs of Old World ZCL

Three species of medically important sand flies exhibit a very close association with the rodents that serve as reservoirs of *Leishmania* parasites in ZCL foci: *L. anthophora*, *P. duboscqi*, and *P. papatasi* (Table 1.1). Each of these rodent/sand fly associations occurs in arid or semi-arid habitats and involves rodents that construct burrows and sand flies that are frequently collected from rodent burrows.

Adult female sand flies require nutrients from mammalian blood for reproduction, and by sharing a burrow with rodents they have continuous access to a source of blood. This relationship creates an environment suitable for the intense transmission of *Leishmania* parasites among rodent populations.

In arid and semi-arid areas, rodents construct burrows as refuges from the high diurnal temperatures (and a number of other external stresses including predation and fire). The air temperature within the burrows of desert rodents remains relatively stable, and the burrows can serve as a heat-sink to remove the animal's excess metabolic heat (Grenot 2001). In one study the soil temperature within the burrow of *P. obesus* was shown to be 27 °C and constant throughout the day, while the temperature of the soil outside the burrow reached over 60 °C (Grenot 2001). Sand flies also benefit from the temperature moderating effects of rodent burrows; laboratory colonies of sand flies are kept between 24 and 29 °C.

The relative humidity within rodent burrows in arid environments has been found to be very high or near saturation (Grenot 2001, Shenbrot et al. 2002). The concentration of fine earth and organic matter lining the burrows of rodents increases the water-holding capacity of the soil, and on a larger scale, burrows also may affect the hydrology of the surrounding area by allowing rainfall to infiltrate the soil (Shenbrot et al. 2002). Both adult and immature sand flies benefit from the humid microhabitat created within rodent burrows; sand fly colonies are typically maintained in conditions with a relative humidity between 75 and 100%.

In arid environments, the burrows of desert rodents often are in close proximity to vegetation. The rodents benefit from constructing their burrows beneath the root systems of plants by gaining structural integrity and soil retention, which helps prevent tunnel collapse (Hole 1981). The plants also serve as a food source for the rodents, and by building their burrows nearby plants, rodents can avoid extended intervals outside foraging in high temperatures and

under threat of predation (Hole 1981). Adult sand flies also benefit from the proximity to plants, from which they obtain sugar meals (Schlein and Warburg 1986).

Table 1.1 Burrowing Rodent Reservoirs of *Leishmania* spp. and Associated Sand Fly Vectors

Reservoir species	Sand fly vector	Location
Muridae		
Murinae		
Aethomys kaiseri	*P. duboscqi*	Kenya
Arvicanthis spp	*P. duboscqi*	Kenya, Senegal, Sudan
Mastomys spp	*P. duboscqi*	Kenya, Nigeria, Senegal
Nesokia indica	*P. papatasi*	Iran, Palestine
Gerbillinae		
Gerbillus pyramidum	*P. papatasi*	Egypt
Meriones crassus	*P. papatasi*	Israel
Meriones hurriannae	*P. papatasi*	India
Meriones libycus	*P. papatasi*	Iran, Libya, Saudi Arabia, Tunisia, Uzbekistan
Meriones persicus	*P. papatasi*	Iran
Meriones rex	*P. papatasi*	Saudi Arabia
Meriones sacramenti	*P. papatasi*	Egypt
Meriones shawi	*P. papatasi*	Algeria, Morocco, Tunisia
Psammomy obesus	*P. papatasi*	Algeria, Egypt, Israel, Jordan, Libya, Palestine, Saudi Arabia, Syria, Tunisia
Rhombomys opimus	*P. papatasi*	Afghanistan, Iran, Kazakhstan, Tajikistan, Turkmenistan, Uzbekistan
Tatera gambiana	*P. duboscqi*	Nigeria, Senegal
Tatera robusta	*P. duboscqi*	Kenya
Taterillus emini	*P. duboscqi*	Kenya
Cricetidae		
Neotominae		
Neotoma micropus	*L. anthophora*	Texas

The availability of habitats and food for immature sand flies is severely limited in rural arid environments, and may be limited to rodent burrows. In nature, rodent burrows contain feces, nest material, and other organic detritus, which support sand fly larval development. In a ZCL focus in Central Asia, sand fly larvae have been observed feeding on the feces of rodents (WHO 1968). The larval diet used in laboratory colonies of sand flies typically includes the feces

of rodents or other small mammals (Young et al. 1981). For example, Mascari et al. (2007) have reared sand fly larvae using a 1:1 mixture of rabbit feces and rabbit food, or the feces of hamsters alone.

Association between *Lutzomyia anthophora* and *Neotoma micropus*

In the semi-arid ZCL foci in Southern Texas, *L. mexicana* parasites are transmitted by the sand fly *L. anthophora* among populations of the southern plains woodrat, *N. micropus* (Table 1.1; McHugh et al. 1991). Woodrat nests typically consist of subterranean tunnels beneath a small constructed pile of woody debris and cactus. The burrows are simple, with a common chamber for food storage and bedding; feces are scattered throughout the burrow. Adult *L. anthophora* were first collected from woodrat nests near San Antonio, Texas, USA in 1965 (Young 1972). Since then, adult *L. anthophora* have been collected in and around woodrat nests throughout Southern Texas (Young and Duncan 1994, McHugh et al. 2001). Bloodfed female *L. anthophora* sand flies have been found resting among the bedding inside of woodrat nests (Young 1972). Soil samples taken from woodrat nests have been examined for immature stages of *L. anthophora*, but none have been recovered (Young 1972). Because there are believed to be no alternative micro-environments appropriate for the development of sand fly larvae in these arid and semi-arid ZCL foci in Texas, woodrat nests are considered to be the likely habitat for immature *L. anthophora*.

Association between *Phlebotomus duboscqi* and Burrowing Rodents

In the arid belt south of the Sahara Desert, *P. dubsocqi* is the vector of *L. major* parasites among populations of different burrowing rodents (Table 1.1). The ecology of the sand flies, vectors, and rodents have been studied extensively in an enzootic of *L. major* focus in Baringo District, Kenya, and there is considerable evidence promoting the idea that both adult and immature *P. duboscqi* use rodent burrows as their primary habitat. Adult *P. duboscqi* sand flies

have been recovered from the burrows of rodent reservoirs of *L. major* by direct aspiration, sticky paper traps, and updraft traps (Mutero et al. 1991). During entomological surveys of potential diurnal resting sites, the majority of adult *P. duboscqi* sand flies typically are collected from rodent burrows; in one study, the number of adult *P. duboscqi* sand flies collected from rodent burrows was more than 19-times greater than the number collected from termite mounds (Basimike 1992). Larvae of *P. duboscqi* also have been collected from the burrows of rodents in ZCL foci in Kenya. The mean temperature of the soil inside of rodent burrows from which *P. duboscqi* sand flies had been recovered was 25.6 °C; the optimum temperature shown to promote the development and survival of *P. duboscqi* in laboratory colonies is 27 °C (Basimike et al. 1990, Beach et al. 1986).

Association between *Phlebotomus papatasi* and Burrowing Rodents

In North Africa and the Middle East, *Psammomys obesus*, *Nesokia indica*, *Gerbillus pyramidum*, and *Meriones* spp. have been identified as the reservoirs of *L. major* (Table 1.1; Desjeux 1991, Gunders et al. 1968, Schlein et al. 1984). In Iran and Southwest and Central Asia, *Rhombomys opimus* replaces *P. obesus* as the most ubiquitous reservoir of *L. major* (Kellina 1981, Nadim et al. 1979). In India, the rodent implicated in the enzootic cycle of *L. major* is *Meriones hurrianae* (Mohan and Suri 1975). All of these rodents are in the family Muridae, and all are within the subfamily Gerbillinae except for *Nesokia indica* (subfamily Murinae). Rodents in each of these species construct burrows: from simple burrows constructed in sand and loose soil by *Meriones crassus*, to the expansive burrow complexes constructed by *R. opimus* that are used by many generations over a period of decades (Shenbrot et al. 2002).

Adult *P. papatasi* sand flies are collected from diverse habitats. Around human settlements in arid areas, adult *P. papatasi* are recovered from animal sheds and cellars, and also from burrows of rodents. Similarly, in areas of human habitation, larvae of *P. papatasi* have been

recovered from animal sheds, stone piles, and rodent burrows (Artemiev et al. 1971, Doha et al. 1990). However, in less-developed or natural habitats, adult and immature *P. papatasi* are recovered almost exclusively from rodent burrows (Desjeux 1991). Because of the very close association between *P. papatasi* (the sole vector species for *L. major* in the region) and burrowing rodents that serve as reservoirs of *L. major*, the prevalence of infection with *L. major* parasites can be as high as 21% in *P. papatasi* and 85% in the rodent population (Wasserberg 2003, Nadim and Amini 1970). Populations of *P. papatasi* sampled in rodent burrows and in villages in an area do not appear to be genetically distinct (Parvizi et al. 2003).

1.5 Control of Leishmaniasis

1.5.1 Introduction

The World Health Organization considers leishmaniasis to be an emerging and uncontrolled disease (WHO 2005). As a vector-borne zoonosis, control of leishmaniasis could be achieved through: A) control of *Leishmania* parasites, B) control of mammalian reservoirs, C) control of sand fly vectors, D) or protection of humans against infection.

1.5.2 Control of Leishmania Parasites

Control of *Leishmania* parasites could be achieved through treatment of all infected human and non-human hosts. However, current chemotherapy for leishmaniasis is with sodium stibogluconate, meglumine antimonite, amphotericin B, or liposomal amphotericin administered by injection daily for at least 28 d (Abramowitz 2004). Therefore, this approach would not be cost effective for use in domestic animals nor practical for use in wildlife.

1.5.3 Host-Targeted Control

There are two notable cases where host-targeted control of mammalian reservoirs of leishmaniasis has brought about a reduction in the incidence of leishmaniasis: A) canine reservoirs of *L. infantum*, and B) rodent reservoirs of *L. major*. Host-targeted control methods

have played an integral part in campaigns to reduce the incidence of VL in South America, the Mediterranean littoral, and in Iran, where dogs serve as the primary non-human reservoir of *L. infantum* parasites. The cornerstone in the current approach to control of VL is the use of insecticide-impregnated dog collars. In Brazil, polyvinylchloride collars impregnated with deltamethrin reduced the feeding rates of *L. longipalpis* and *L. migonei* sand flies on treated dogs for up to eight months (David et al. 2001). Furthermore, the survival of sand flies exposed to dogs wearing deltamethrin-impregnated collars also was reduced for up to eight months (David et al. 2001). Deltamethrin-treated dog collars also had anti-feeding and insecticidal effects against *P. perniciousus* sand flies in Southern France and *P. papatasi* sand flies in Iran for up to 8 months, which could protect a dog throughout the entire annual period of sand fly activity (Killick-Kendrick et al. 1997, Halbig et al. 2000). A large scale (multiple village) evaluation of the use of deltamethrin-impregnated dog collars also was conducted in Iran; children in villages in which all domestic dogs were fitted with insecticide-treated collars had a significantly lower seroconversion rate for *L. infantum* (Gavgani et al. 2002). Topical treatment of dogs with insecticides also has been evaluated as a potential control method against VL. Spot-on treatments of imidacloprid and permethrin showed significant repellent and insecticidal effect against *P. papatasi* sand flies for up to a month after treatment (Mencke et al. 2003).

In parts of the former Soviet Union in Central Asia, attempts to control the great gerbil , *R. opimus*, (the primary reservoir of *L. major* in the area) and their burrows through plowing or crushing with heavy machinery was conducted. Zonal control of the great gerbil (eliminating all burrows within a 2 to 3 km radius of all towns) was found to be inadequate due to re-invasion of the controlled areas by great gerbils (Sergiev 1978, Eliseev 1980). On the other hand, massive campaigns to eradicate the great gerbil and their burrows were carried out over vast areas surrounded by natural borders such as mountain ranges and rivers, and yielded lasting reductions

in the sand fly population (by a factor of 270) and no cases of ZCL reported within the areas for at least 4 years afterwards (Sergiev 1978, Eliseev 1980). In Central Asia, large scale eradication of the great gerbil through poisoned baits successfully eliminated the rodent, but had no effect on the population of *P. papatasi* (Dergacheva and Zherikhina 1980).

Attempts to reduce the incidence of ZCL in Isfahan, Iran by treating the burrows of the rodent reservoirs (*R. opimus* and *M. libycus*) with dichloro-diphenyl-trichloroethane (DDT) powder were unsuccessful (Seyedi-Rashti and Nadim 1974). In a concurrent study, rodents also were poisoned with baits containing zinc phosphide, yielding a reduction in the number of rodents, but having no effect on the incidence of human infection with *L. major* (Seyedi-Rashti and Nadim 1974). A successful campaign to reduce incidence of ZCL was undertaken in Badrood, Iran. All rodent burrows within 500 m of several villages were systematically excavated (and any surviving rodents were killed with bait containing zinc phosphide). Subsequently, the incidence of *L. major* infection in humans was significantly lower than the incidence in untreated villages.

1.5.4 Control of Sand Fly Vectors

Adult Control

Control measures targeting the sand fly vectors remain a major component of control of leishmaniasis and other sand fly-borne diseases. Control measures against phlebotomine sand flies include chemical control measures (contact insecticides and larvicides) and control through environmental modification.

The earliest report of chemical control of sand flies was carried out in a bartonellosis endemic region of Peru in 1944 (Hertig and Fairchild 1948). Spraying houses with DDT protected inhabitants from sand fly bites for around one week after treatment. This approach was attempted on a small scale in Italy, Greece, and Palestine to prevent new infections of sand fly

fever and anthroponotic CL with some degree of success (Hertig 1949, Jacusiel 1947, Hertig and Fisher 1945). In India, residual spraying of houses and cattlesheds with DDT and benzene hexachloride (BHC) reduced the number of sand flies (*P. papatasi* and other medically important species); reductions lasted for up to 8 months for DDT and less than one month for BHC (Ghosh 1950). On a larger scale, ACL due to *L. tropica* was eliminated in the Central Asian republics of the former Soviet Union through the use of residual BHC and DDT (Nadzharov 1966, Nadzharov and Gasan-Zade 1980). Initially, entire villages were treated with the residual insecticides; after a few years of control, the disease was eliminated by follow-up treatment of the houses of leishmaniasis cases.

Control of sand flies and sand fly-borne diseases also was achieved in several countries as a collateral effect of intense large-scale campaigns to eradicate malaria. All successes in reducing the incidence of sand fly-borne diseases through residual insecticide spraying were with pathogens for which humans can serve as an important reservoir of infection (*L. tropica*, *L. donovani*, and Sand Fly Fever virus). This suggests that control using residual house spraying may be successful only under certain epidemiological circumstances, such as with certain species of sand fly or with certain peri-domestic populations of sand fly. In Pakistan and India in the 1950s and 1960s, visceral leishmaniasis was nearly eliminated during the anti-malaria campaign, which involved spraying all houses with DDT (Sanyal et al. 1979). However, a resurgence of the disease was observed immediately following the cessation of the campaign. In Greece, the antimalaria campaign significantly (and temporarily) reduced the incidence of sand fly fever but not of visceral leishmaniasis (Tesh and Papaevangelou 1977).

In foci of zoonotic leishmaniasis in Iran, control of malaria with DDT yielded no effect on incidence of leishmaniasis or the sand fly population (Seyedi-Rashti and Nadim 1975). Similarly, in South America, residual spraying of houses with DDT for control of malaria and

chagas disease had no detectable effect on the incidence of zoonotic leishmaniasis (Viokov 1987).

In the aftermath of the global campaign to eradicate malaria with DDT, the infrastructure to spray houses with residual insecticides to control sand flies rarely is present. Nevertheless, several residual insecticides have since been evaluated against sand flies in different foci around the world. In India, cattlesheds were treated with a single application of malathion, leading to a reduction in the sand fly population for up to 8 months (Pandya 1983). In Egypt, the residual effect of propoxur, permethrin, malathion, and BHC after 75 d was evaluated against *P. papatasi* (Morsy et al. 1993). The results were not encouraging; after sand flies were exposed to treated surfaces for 30 minutes, mortality was around 75% for propoxur, and 50% for permethrin, malathion, or BHC. In Bolivia, the effect of treating houses and animal sheds with deltamethrin differed among sand fly species. The vector of *L. infantum* (*L. longipaplis*) was eliminated for up to 10 months, while the population density of the vector of the parasites that cause CL (*L. nuneztovari*) was unchanged (Le Pont et al. 1989). The authors pointed out that this difference likely was due to the endophilic behavior of *L. longipalpis* and the exophilic behavior of *L. nuneztovari*. This point was further demonstrated by Alexander et al. (1995), who showed that treating houses in a village in a Colombian forest with deltamethrin had no effect on the number of sand flies collected in and around the houses, even though the treated surfaces of the houses were shown to be insecticidal to sand flies.

Spraying residual insecticides to form a protective barrier around a human settlement has been evaluated in sylvatic areas of Central and South America where leishmaniasis is associated with human encroachment into forests. In an early attempt at barrier spraying in the 1950s in a forested region in French Guiana, tree trunks (which were known to be resting sites of sand flies in the area) were sprayed with DDT (Floch 1957). There was no reduction in the number of sand

flies collected inside of the treated area. However, another study in French Guyana in the 1980s found that clear-cutting the forest to create a 400 m wide barrier around human settlements effectively reduced the number sand flies collected and the incidence of human cases of leishamaniasis, and eliminated all mammals, removing all potential reservoirs of *Leishmania* parasites from the area (Esterre et al. 1986). In Panama, spraying trees and vegetation bimonthly with malathion to form a 100 m diameter treated area in a forest yielded a small reduction in the number of sand flies (approximately 30%) collected off of human bait or on tree trunks within the barrier (Chaniotis et al. 1982). Perich et al. (1995) reported the successful application of barrier spraying to control sand flies in a small-scale trial conducted in Guatamala. Cyfluthrin was sprayed on vegetation, forming a 100 m treated band around a simulated human settlement, and the number of sand flies collected inside the treated area was significantly lower than outside the area for more than 80 d.

Larval Control

The larval habitat for many sand fly species is unknown, and therefore larval control has not played a large part in sand fly control. Nevertheless, larval control methods could play a role in certain situations where a larval habitat is well defined.

The possibility of sand fly larval control in houses and cattlesheds has been shown on a small-scale in India (Dhiman 1995). Crevices suspected of harboring larvae of *P. papatasi* were covered with cement, and a reduction in the number of sand flies collected inside of the buildings (up to 70% reductions) was reported. However, this species is known to have alternative larval habitats outside of human settlements (such as in rodent burrows). Therefore this control measure may have a limited impact on the transmission of ZCL.

The sporulating bacterium *Bacillus thuringiensis israelensis* was the first larvicide evaluated in the laboratory for sand flies, and it was found to cause significant mortality when

fed to larvae of *P. papatasi* and *L. longipalpis* (De Barjac et al. 1982). *Bacillus sphaericus* also has been evaluated as a control agent for sand fly larvae. A high level of mortality was observed for larvae of *P. papatasi* that had been fed a diet treated with *B. sphaericus*, and sand fly eggs treated with *B. sphaericus* were significantly less likely to hatch than control eggs (Pener and Wilamowski 1996, Robert et al. 1998). An elaborate system using adult sand flies that had ingested sugar baits containing *B. sphaericus* to deliver the insecticide to the larval habitat of sand flies also has been evaluated (Robert et al. 1997). The authors of this study reported a reduction in the sand fly population for up to 12 weeks after treatment.

1.5.5 Protection of Humans against Infection

Since adequate parasite, reservoir, and vector control measures are currently not available for many epidemiological settings, humans could be protected against sand fly bites and infection with sand fly-borne pathogens by using vaccines or personal protective measures such as insecticide treated materials (clothing, curtains, wall cloths, bed sheets, screens, and bednets) or repellents (topical and area-wide).

Vaccines

The development of an effective vaccine against any of the *Leishmania* spp. presents an ongoing challenge. The earliest attempt to induce immunity to *Leishmania* parasites was by inoculating people with infectious material taken from patients infected with *L. major*, a process called leishmanization. Leishmanization was intended to cause a single, self-healing lesion that would confer lifelong immunity against re-infection, and was carried out throughout the Middle East and Soviet Union from the 1940s until the 1990s (Palatnik-de-Sousa 2008). However, leishmanization largely has been discontinued because of the risk of complications resulting from infection with *L. major* including the development of multiple and persistent lesions, the potential migration of parasites to the spleen and liver, and ethical concerns. A live vaccine for

humans has been licensed for use in Uzbekistan and is used in some circumstances in Iran, such as the movement of troops into areas of high risk of infection (Nadim et al. 1997, Kamesipour et al. 2006). In Brazil, a killed-parasite vaccine for the agents that cause cutaneous leishmaniasis has shown some efficacy when used in conjunction with antimony chemotherapy, but it is not used as a monotherapy vaccine for the prevention of leishmaniasis (Mayrink et al. 2006). Currently there are several vaccine candidates in different phases of clinical trial, and stable multiple-gene DNA vaccines are considered to be a promising line of investigation (Palatnik-de-Sousa 2008).

Insecticide Treated Materials

Anecdotal evidence suggests that sand fly bites (and subsequently, CL lesions) occur on parts of the body where skin is exposed because sand flies, unlike some other hematophagous flies, do not bite through clothing. Evidence that clothing may provide a physical barrier against sand fly bites was provided by Dedet et al (1987) who reported the location of multiple CL lesions relative to clothed and exposed areas on patients who had travelled to French Guyana. As no lesions were present in places that had been covered by the patients' clothing at the time of infection, the authors concluded that sand flies were unable to bite through the fabric. As a short-term preventive measure Dedet et al. (1987) recommended that travelers to areas of CL transmission wear long pants and long-sleeved shirts.

Laboratory studies have been conducted to determine whether the protection against sand fly bites conferred by clothing could be enhanced by impregnating the fabric with permethrin. In one published study, permethrin-treated clothing did not cause significant "knock-down" in sand flies (*P. papatasi*) exposed to the material for short periods of time (as would occur during feeding attempts in a field setting); the majority of sand flies exposed for up to 3 minutes were unaffected and potentially able to feed (Fryauff et al. 1996).

Field trials evaluating the effectiveness of insecticide-treated clothing to prevent sand fly bites and leishmaniasis also have been conducted. In a field evaluation in a lowland tropical forest in Panama, human subjects wearing permethrin-treated uniforms received fewer sand fly bites per hour than control subjects (Schreck et al. 1982). However, the number of sand fly bites received by treated subjects was high (16 bites per hour), and the sand flies did not appear to experience quick "knock-down" after exposure to the permethrin-treated uniforms. Sand flies exposed to treated uniforms for 15 min were still able to feed to repletion (Schreck et al. 1982). A double-blind placebo controlled study of insecticide-treated clothing as preventive measure against CL was conducted with Iranian soldiers (Asilian et al. 2003). Soldiers in the intervention group were provided with permethrin-treated uniforms (shirts, undershirts, pants, socks, and a hat), and their use was strictly monitored over a period of 3 months. The attack rates of CL in soldiers in the intervention and control groups were not significantly different, and the authors concluded that permethrin-treated uniforms alone were not sufficient to prevent sand fly bites or CL (Asilian et al. 2003). While results of these two studies suggest that insecticide-treated uniforms do not effectively prevent sand fly bites, the use of insecticide-treated uniforms has been adopted by the U.S. Armed Forces, as well as other militaries around the world, as a personal protective measure against sand fly bites and leishmaniasis.

The use of insecticide-treated curtains as a means of protection against sand flies has been evaluated in diverse ecological settings with varying degrees of success. The use of insecticide-treated curtains to prevent sand flies from entering houses was first evaluated in Burkina Faso against *P. duboscqi* and several *Segentomyia* spp (Majori et al 1989). Cotton curtains impregnated with permethrin were placed in doorways and under the eaves of houses, and a nearly 100% reduction in the number of sand flies collected inside of treated houses was observed. Similarly, in Italy the number of specimens of *P. papatasi* and *P. perniciosus*

collected in light traps inside stables was significantly reduced by placing permethrin-impregnated curtains over windows (Maroli and Majori 1991). In Khartoum, the number of sand flies obtained by pyrethrum knockdown collection was significantly different between rooms with permethrin-treated curtains and rooms without curtains or with untreated curtains (Elnaiem et al. 1999).

Insecticide-treated curtains also have been evaluated in the New World. In one field trial in Colombia, the number of sand flies collected during human landing catches in rooms with deltamethrin-treated or untreated curtains was not significantly different (Alexander et al. 1995). However, a study in Venezuela found that using curtains treated with lambdacyhalothrin provided significant reductions in the number of sand flies collected inside of houses as well as a reduction in the incidence of cutaneous leishmaniasis (Kroeger et al. 2002).

The use of insecticide-treated wall cloth has been evaluated as a control measure against endophilic sand flies in Kenya (Mutinga et al. 1992). In this study, cotton cloth was treated with permethrin and used to cover the interior walls of houses. The number of sand flies (*P. duboscqi* and *P. martini*) collected using sticky paper traps inside of treated houses was significantly lower (more than 75% lower) than control houses.

Insecticide-treated bednets, which are widely used for protection against mosquito vectors of malaria, have been evaluated as a personal protective measure in many different foci of leishmaniasis. In Kabul, which is an endemic area for ACL, significant differences in the incidence of infection with *L. tropica* have been observed between people using permethrin-treated bednets (2.4%) and people receiving no intervention (7.2%): approximately 65% protective efficacy was reported (Reyburn et al. 2000). In Southeastern Anatolia, another ACL endemic area, people using deltamethrin-treated bednets also had a significantly lower incidence of infection than controls; no impact was detected on the overall abundance of sand flies (Alten

et al. 2003). A large-scale study was conducted in over a dozen villages near Aleppo suggested that the use of deltamethrin-treated bednets reduced incidence of ACL (Jalouk et al. 2007). The study also found that when the use of bednets was interrupted, incidence of ACL rebounded to pre-intervention levels within 1 to 2 years, suggesting that the use of insecticide-treated bednets as a personal protective measure must be sustainable in order to be effective.

In a ZCL hyperendemic area near Isfahan, the use of deltamethrin-treated bednets also has been evaluated as a personal protective measure against sand fly bites and protection against infection with Leismania parasites (Yaghoobi-Ershadi et al. 2006). Rates of infection with *L. major* were significantly different after insecticide-treated bednets were used, and the incidence also was significantly lower than the control. There was no reduction in the mean total density of *P. papatasi* in areas that received insecticide-treated bednets, presumably because this sand fly feeds on a number of non-human hosts.

Insecticide-treated bednets also have been used to prevent sand fly bites and infection with the causative agents of visceral leishmaniasis (*L. infantum* and *L. donovani*). In a case control study in Nepal, analysis of several potential risk factors indicated that the ownership and use of a bednet was a significant protective factor against visceral leishmaniasis (Bern et al. 2000). In a VL endemic area of Sudan, lambdacyhalothrin-treated bednets were found to completely protect people sleeping beneath them, as indicated by human landing catches (Elnaiem et al. 1999). In practice, however, it was found that few men were protected against sand fly bites when they used insecticide-treated bednets because the peak biting-time of vector sand flies was earlier than most men went to bed. The authors suggested that children (the group with the highest incidence of VL) could be protected against VL by going to bed earlier.

In a New World VL endemic area in Brazil, deltamethrin-treated bednets have been evaluated as a protective measure against *L. longipalpis* sand flies, the primary vector of *L.*

infantum parasites (Courtenay et al. 2007). Compared with untreated nets, insecticide-treated nets significantly decreased the human-landing rate and increased the 24-h mortality rate of sand flies inside the bednets. However, as indicated by human landing catches, the peak activity period of host seeking *L. longipalpis* sand flies is between 19 – 23 h, and a substantial part of this period is before people have gone to bed. Furthermore, despite the 24-h mortality observed for sand flies that contacted or penetrated insecticide-treated bednets, death was not immediate and would allow time for sand flies to bite and potentially transmit *Leishmania infantum* parasites.

In parts of the Middle East and Southwest Asia, people frequently sleep outdoors during warmer months, and in the absence of any potential protection against sand flies conferred by residual insecticides or insecticide treated materials, expose themselves to sand fly bites (Alten et al. 2003). To address this issue, a control trial of top sheets (chaddars) treated with permethrin was conducted (Reyburn et al. 2000). The incidence of cutaneous leishmaniasis for people provided with treated sheets was 65% lower than controls, and was found to be equally effective as insecticide treated bednets, and more effective than residual spraying. However, insecticide treated top sheets were the least popular intervention (compared to residual spraying or bednets) among people included in the study.

Repellents

The first documented evaluations of a chemical repellent used to protect against sand flies and sand fly-borne diseases were conducted during World War II. United States Army personnel in Egypt (and the Eastern Mediterranean as a whole) experienced a large number of cases of sand fly fever; the sand fly fever case rate at the study site in Egypt was reported to be 25% in 1943 (Philip et al. 1944). A placebo-controlled trial of topical applications of dimethyl phthalate (DMP) as a repellent against the vector species, *P. papatasi*, was conducted, and a higher percentage (43%) of treated participants reported a relief from sand fly bites compared to

patients receiving placebo (12%). The treatments also appeared to protect against infection, and six times as many cases were reported among participants receiving placebo than among participants receiving DMP treatments. Around the same time, Soviet researchers determined that xanthic disulphide ("K preparation") was an effective topical repellent against sand flies (Jukova 1944).

The first laboratory comparison of the efficacy of repellents against sand flies was conducted in 1969 (Schmidt and Schmidt 1969). Nine repellents (diethyltoluamide (DEET); O-ethoxy-N,N-diethylbenzamide; dimethyl carbate; ethyl hexanediol; dimethyl phthalate; O-chloro-N,N-diethylbenzamide; N-butyryl-1,2,3,4-tetrahydroquinoline; indalone, and 2,2,4-tetramethyl-1,3-pentanediol) were applied to the forearms of participants, and the repellent effect against *P. papatasi* sand flies was evaluated. The mean duration of the repellent effect of three of the nine compounds (DEET; O-ethoxy-N,N-diethylbenzamide; and O-chloro-N,N-diethylbenzamide) was found to be greater than four hours when used at 5% concentrations.

The first laboratory evaluations of different repellent compounds against a New World sand fly species were conducted more than a decade later (Buescher et al. 1982). When applied to human skin, Indalone, DEET, and Citronyl were found to be effective repellents against *L. longipalpis* sand flies. Four other experimental compounds were found to be more repellent than DEET when they were applied to rabbits: Rohm & Haas 398; 3-[N-(n-butyl)-N-acetyl]aminoproprionic acid-ethyl ester; N-(u-hexyl)-2-oxazolidine; and methyl N,N-di-(n-hexyl)-ethylenediamine monocarbamate. The sand fly *L. longipalpis* was found to be more sensitive to DEET and other repellents than mosquitoes and other insect pests (Buescher et al. 1982).

Repellents against species of New World sand flies were first evaluated in the field in Panama in the 1980s (Schreck et al. 1982). In addition to DEET, four other compounds (p-

isopropyl-N,N-dimethylbenzamide; 1-(3-cyclohexen-1-ylcarbonyl)piperidine; hexahydro-1-[(2methylcyclohexyl) carbonyl]-1H-azepine; and N,N-dipropylcyclohexanecarboxamide) provided significant protection against bites of *Lutzomyia* spp., including two that are known vectors of *Leishmania* parasites in Panama. However, only one of the compounds (hexahydro-1-[(2-methylcyclohexyl)carbonyl]-1H-azepine) provided complete protection against sand fly bites even in situations of low sand fly biting pressure on control subjects (fewer than 30 bites per hour).

Three repellents (DEET, indalone, and MGK11) have been evaluated against *P. perniciosus*, an important sand fly vector of *L. infantum* in Europe (Fossati and Maroli 1986). Comparing median effective dosages (ED_{50}) of the repellents, the authors found that indalone and MGK11 were similarly effective against *P. perniciosus*, while the ED_{50} of DEET was significantly higher, indicating that it was less repellent.

The repellent and deterrent effects of DEET, picaridin, and SS220 have been evaluated for *P. papatasi* (Klun et al. 2006). As shown previously, when applied to the skin of human participants, the compounds deterred sand flies from feeding. Also, when the compounds were applied to cloth, sand flies were repelled from (and never landed on) the treated surfaces. These three compounds were shown to act primarily through affecting olfactory sensation of sand flies; biting activity of sand flies on treated participants was reduced by approximately one-half compared to control participants (Klun et al. 2006).

Naucke et al. (2006) evaluated the laboratory efficacy of the repellents IR3535 and DEET against the sand flies *P. duboscqi* and *P. mascittii*. Both IR3535 and DEET provided approximately 6 h protection against *P. duboscqi*. The mean protection time against *P. mascittii* was around 9 h with DEET, and more than 10 h with IR3535.

Several compounds also have been evaluated as area repellents for sand flies. In a CL endemic area of Rajasthan, India, neem oil on a heated electric mat was shown to significantly reduce the number of sand flies (*P. papatasi*) collected in rooms compared to control rooms (Dhiman and Sharma 1994). In Turkey, allethrin was found to act as an area repellent and significantly reduced the number of sand flies collected during human landing catches (Alten et al. 2003). In Israel, citronella, linalool, and geraniol candles were evaluated as indoor area repellents for sand flies (Muller et al. 2008). Citronella candles were found to have only a slight repellent effect against sand flies (24.7%), while linalool and geraniol candles significantly reduced the number of sand flies collected during human landing catches (55.2% and 79.7%, respectively).

CHAPTER 2. LABORATORY EVALUATION OF DIFLUBENZURON AS A FEED-THROUGH FOR CONTROL OF IMMATURE SAND FLIES (DIPTERA: PSYCHODIDAE)[*]

2.1 Introduction

[*]Phlebotomine sand flies (Diptera: Psychodidae) 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 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 is the primary vector of *Leishmania major*, which is the causative agent of zoonotic cutaneous leishmaniasis (ZCL).

In arid and semi-arid foci, *P. papatasi* exhibits a close association with the semi-fossorial 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 comprise the burrows. Additionally, even successful treatments are short-lived and would require frequent reapplication (Seyedi-Rashti

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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*), face flies (*Musca autumnalis*), stable flies (*Stomoxys calcitrans*), and horn flies (*Haematobia irritans*) (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 (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, *Mesocricetus auratus*, fed a diet containing diflubenzuron was evaluated.

2.2 Materials and Methods

2.2.1 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. Diflubenzuron [89.7% active ingredient (a.i.), 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 mg/kg. 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 three days. The hamsters were ranked by mean daily food intake, and the twelve 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 mg/kg 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 nine days. Remaining food was removed every 24 h, and food intake was calculated. Daily doses 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 doses 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 nine days, placed in uncovered plastic cups, and dried at room temperature for one week. The samples of hamster feces were stored at -80 °C until used.

2.2.2 Bioassay

A colony of *P. papatasi* was established from larvae obtained from a long-standing colony at the Walter Reed Army Institute of Research (WRAIR, Department of Entomology, 503 Robert Grant Ave., 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, Brentwood, MO) 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 (approximately 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 5 larval groups (3 treated and 2 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 prior to the experiment, and was blotted with filter paper to remove standing water immediately before use.

Ten 2nd instars (13±1-d old) were transferred to each vial using a moistened wooden applicator stick. Each vial was closed with a polyethylene cap that was pierced ten 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% relative humidity (RH), 14:10 (L:D) photoperiod.

Larval mortality was recorded daily; larvae were considered dead if they did not respond within 15 sec 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 percent 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 percent 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).

2.3 Results

2.3.1 Feeding Protocol

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 mg/kg 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 doses 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 mg/kg diflubenzuron, respectively.

2.3.2 Bioassay

Evidence of food ingestion was found for all larvae in each larval diet group. The mean percent survival from 2nd instar to adult was 95±5.5% for the control hamster feces larval group and 96.7±5.2% for the rabbit feces-rabbit chow larval group. Mean percent 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 2.1). 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 successfully pupated in the groups fed feces from hamsters fed diets containing diflubenzuron (Table 2.1; Fig. 2.1). 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 mg/kg diflubenzuron, respectively (Table 2.1). There was no significant difference in the age at death of the sand flies in the three diflubenzuron treatment groups (Table 2.1).

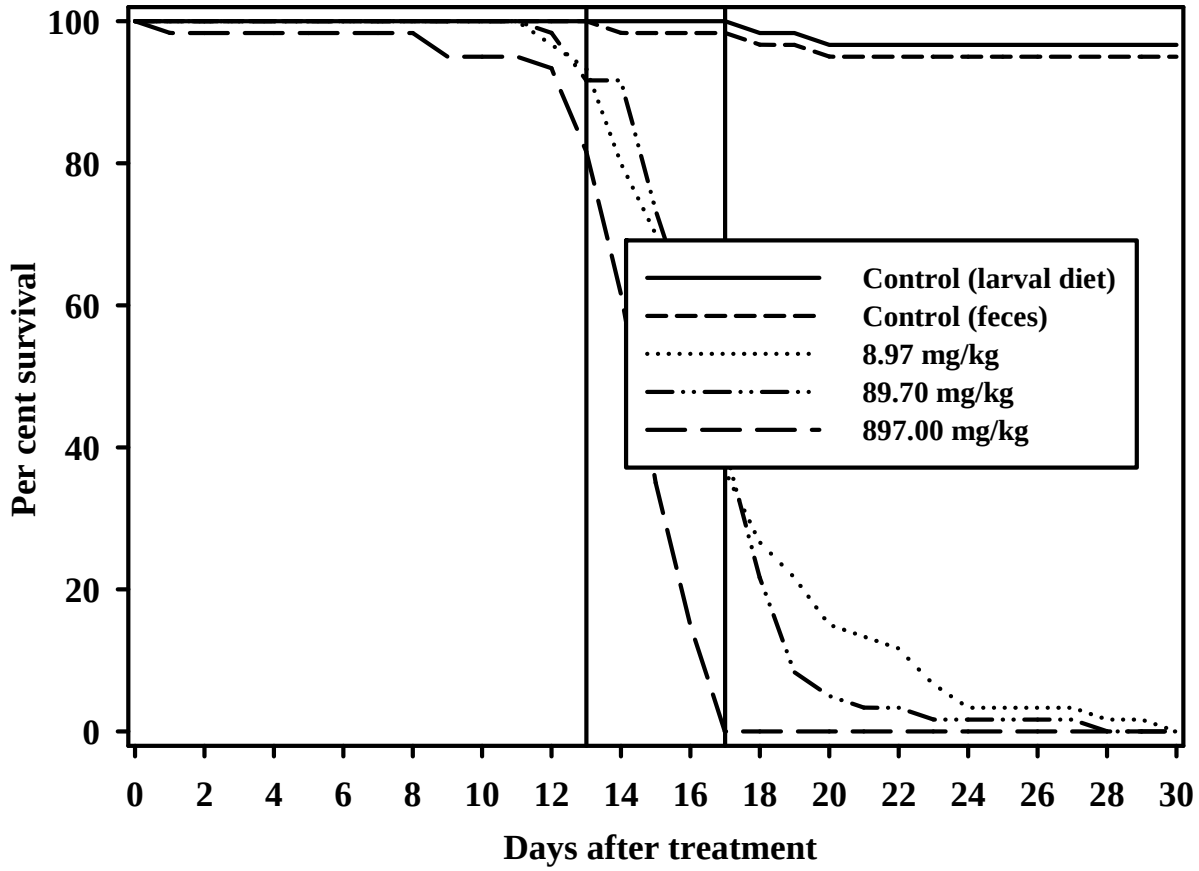

Figure 2.1 Cumulative Per Cent Survival of 2nd Instar (13±1-d old) *P. papatasi* Larvae Fed Feces from Three Treatment Groups of Syrian Hamsters Receiving Diets Containing Different Concentrations of Diflubenzuron, Feces from Untreated Control Syrian Hamsters, or an Untreated Control Laboratory Larval Diet (a 1:1 Rabbit Feces-Rabbit Chow Diet). Vertical Reference Lines Indicate the First Appearance of Pupae (13 d) and Adults (17 d) in Control Vials

Table 2.1 Percent Mortality and Age at Death of 2nd Instar (13±1 Days Old) *P. papatasi* Fed Feces from Three Treatment Groups of Syrian Hamsters Receiving Different Oral Doses 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 % (mean* ± SE)#	Sand fly age at death d (mean* ± SE)#
Diflubenzuron		
0.68 ± 0.09 mg/kg body weight	100.0[a]	30.4 ± 3.6[a]
6.26 ± 0.66 mg/kg body weight	100.0[a]	30.0 ± 2.5[a]
62.28 ± 7.03 mg/kg body weight	100.0[a]	27.6 ± 2.5[a]
Control		
Hamster feces	5.0 ± 5.5[b]	32.0 ± 1.4[a]
Laboratory larval diet	3.3 ± 5.2[b]	30.3 ± 3.1[a]

* Six replicates, ten larvae per replicate
# Values within a column followed by the same letter are not significantly different. P> 0.05

2.4 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 appeared very thin a delicate.

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 2000, Yaghoobi-Ershadi 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 be used effectively as a vehicle to deliver insecticides to the larval habitats of sand flies that 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, Miller 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.

CHAPTER 3. EVALUATION OF NOVALURON AS A FEED-THROUGH INSECTICIDE FOR CONTROL OF IMMATURE SAND FLIES (DIPTERA: PSYACHODIDAE)*

3.1 Introduction

Phlebotomine sand flies (Diptera: Psychodidae) are the vectors of the protozoan parasites that cause leishmaniasis and are notorious pests of humans. Worldwide, there are an estimated 2 million new cases of leishmaniasis annually, and an estimated 12 million people are currently infected (WHO 1991). Throughout Asia and North Africa, the sand fly *Phlebotomus papatasi* Scopoli is the primary vector of *Leishmania major*, which is the causative agent of zoonotic cutaneous leishmaniasis (ZCL).

Semi-fossorial rodents serve as the primary reservoir hosts of ZCL in arid and semi-arid Old World foci. In these ZCL foci, which have high diurnal temperatures and low relative humidity, populations of sand flies aggregate in the burrows of the rodent hosts of *L. major*. Sand fly larvae and adults thrive in the microclimate within the burrows where the abundant organic debris serves as the food source for sand fly larvae. In Old World ZCL foci, the larvae of *P. papatasi* frequently have been recovered from animal burrows (Artemiev *et al*. 1972, Morsy *et al*. 1993).

The only historical successes in suppressing the transmission of *L. major* have involved the destruction of large areas of natural habitat to eliminate reservoirs, and vector breeding and resting places (Faizulin 1980). The use of insecticides to control sand flies in Old World ZCL foci has not been successful because insecticide applications introduced into rodent burrows do not reach the microhabitats of adult and immature sand flies due to the length and complexity of the tunnels that make up the burrows (Seyedi-Rashti and Nadim 1973, Karapet'ian et al. 1983). The development of new, efficacious methods for the control of the vectors of ZCL is needed.

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Phlebotomine sand fly larvae have been observed feeding on the feces of rodents (WHO 1968). Feed-through rodent baits that contain insecticides have been suggested as a novel method for sand fly larval control, and the feasibility of this method has been established using diflubenzuron, a benzoylurea chitin synthesis inhibitor, to control larvae of *P. papatasi* (Mascari et al. 2007b). The objective of this study was to assess novaluron, which also is a benzoylurea chitin synthesis inhibitor, as a rodent feed-through to control sand fly larvae. The development and survival of *P. papatasi* larvae fed feces of Syrian hamsters, *Mesocricetus auratus*, which had been fed a diet containing novaluron, was evaluated.

3.2 Materials and Methods

3.2.1 Feeding Protocol

Twelve Syrian hamsters were housed individually in micro-isolator cages. The maintenance of the hamsters and the experimental procedures of this research followed Animal Care & Use Protocol No. 05-074, which was approved by the Institutional Animal Care and Use Committee at Louisiana State University, Baton Rouge, LA.

Four hamster diets were prepared by adding novaluron (98.8% a.i., Makhteshim Agan Industries Ltd., Tel Aviv, Israel) to a meal form laboratory rodent diet (5001 Rodent Diet, LabDiet®, PMI Nutrition International, Brentwood, MO). Novaluron was added to the meal form hamster diet to achieve four concentrations in the diet (0, 9.88, 98.8 and 988 mg/kg) and was thoroughly mixed.

Three hamsters were randomly assigned to each of the four diet groups (0, 9.88, 98.8 or 988 mg/kg novaluron). The initial body weight of the hamsters was measured on the day before the experiment. The body weight of hamsters in different diet groups was compared using analysis of variance (ANOVA), performed with the general linear model (GLM) procedure of SAS (SAS Institute 2001).

At 12:00 h each day for 9 d, each hamster was provided 25 g of their respective diet. The uneaten portion of the food was collected the following day at 12:00 h, and the daily food intake for each hamster was calculated. The daily doses of novaluron that were ingested by the hamsters were calculated by multiplying the daily food intake by the concentration of novaluron in the hamster's diet. Both the daily food intake and the daily doses of novaluron for individual hamsters were compared within hamster diet groups; daily food intake and the daily dose of novaluron also were compared between hamster diet groups. Each comparison was performed using a repeated measures ANOVA, performed with the GLM procedure of SAS (SAS Institute 2001). The Tukey multiple comparison procedure was used to separate significantly different means.

The feces produced by each hamster were collected daily for 9 d. The feces were placed in uncovered glass vials and dried at room temperature for seven days. Once dry, the feces were stored at -80 °C until used.

3.2.2 Bioassay

A laboratory colony of sand flies was established at Louisiana State University using specimens obtained from a long-standing colony of a Turkish strain of *P. papatasi* at the Walter Reed Army Institute of Research (WRAIR, Department of Entomology, 503 Robert Grant Ave., Silver Spring, MD). The sand flies in the colony were reared using a larval diet composed of a dried and decomposed 1:1 mixture of rabbit feces and rabbit chow (Young et al. 1981). The colony was maintained in environmental chambers at 28 °C, 90% RH, 14:10 (L:D) photoperiod.

Six larval diets were used in sand fly larval bioassays. The feces collected from hamsters on day nine were pooled by treatments and crushed using a glass mortar and pestle. Four groups of larvae were fed feces of hamsters in each hamster diet groups. Two additional groups of sand fly larvae were fed the rabbit feces-rabbit chow larval diet containing either 0 or 988 mg/kg

novaluron. This allowed comparisons between the survival of sand fly larvae fed feces of hamsters that had been fed diets without novaluron and the untreated rabbit feces-rabbit chow larval diet, as well as comparisons between the survival of sand fly larvae fed feces of hamsters that had been fed diets containing novaluron and a larval diet treated directly with novaluron.

The larval bioassays were conducted according to the methods described by Mascari et al. (2007a) A 0.1 g portion of the larval diets was transferred to the plaster surface of each bioassay vial. Ten second instars (13±1-d old) were transferred to each bioassay vial and held in an environmental chamber at 28 °C, 90% RH, 14:10 (L:D) photoperiod. Six bioassay vials were used for each of the six larval diet groups.

The larvae were observed under magnification daily. Larval mortality, defined as the lack of response to prodding with a blunt probe after 15 s, was recorded, and the larvae were observed for abnormal behavioral and morphological characteristics. Evidence of feeding, the presence of frass in the vials and dark material in the guts of larvae, also was monitored.

The percent 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 percent survival of sand flies fed feces of untreated hamsters and the untreated rabbit feces-rabbit chow standard larval diet also was compared using Student's t-test (SAS Institute 2001).

3.3 Results

3.3.1 Feeding Protocol

The mean body weight of the twelve Syrian hamsters was 136.0±20.1 g, and the mean body weights of hamsters in the different hamster diet groups were not significantly different (*F*

= 0.57, df = 3, P = 0.65). The mean daily food intake of the hamsters was 7.6±1.7, 8.2±1.7, 7.7±1.3, and 7.6±0.8 g for hamsters receiving diets containing 0, 9.88, 98.8, and 988 mg/kg novaluron, respectively, and was not significantly different (F = 1.00, df = 3, P = 0.40). The estimated mean daily doses of novaluron for hamsters were 0.6±0.1, 6.2±0.9, and 56.6±7.7 mg/kg body weight for hamsters receiving 9.88, 98.8, and 988 mg/kg novaluron, respectively.

3.3.2 Bioassay

Larvae in each of the larval diet groups were observed feeding, and frass was found in each bioassay vial. The mean percent survival from 2nd instar to adult for the sand flies in the untreated hamster feces larval diet group was 100% and was not significantly different from the 98.3±4.2% survival for sand flies in the rabbit feces-rabbit chow larval diet group (t = -1.00, df = 10, P = 0.34; Table 3.1).

Table 3.1 Percent Mortality and Longevity of 2nd Instar (13 ± 1 Day Old) *P. papatasi* Larvae Fed Feces of Syrian Hamsters That Had Been Fed a Diet Containing 0, 9.88, 98.8, and 988 mg/kg, or an Aged 1:1 Rabbit Feces-Rabbit Chow Larval Diet Containing 0 and 988 mg/kg Novaluron

Larval diet group	Mortality % (mean* ± SE)#	Longevity d (mean* ± SE)#
Hamster feces		
988 mg/kg	100.0[a]	4.7 ± 1.9[a]
98.8 mg/kg	100.0[a]	4.9 ± 2.0[a]
9.88 mg/kg	100.0[a]	4.8 ± 1.7[a]
0 mg/kg	0.0[b]	n/a§
Aged rabbit feces-rabbit chow		
988 mg/kg	100.0[a]	4.4 ± 1.6[a]
0 mg/kg	1.7 ± 4.18[b]	n/a§

* Six replicates, ten larvae per replicate
# Values within a column followed by the same letter are not significantly different from each other, P> 0.05
§ Not applicable

Sand fly larvae that were fed feces of hamsters that had consumed diets containing novaluron and larvae that had been fed the rabbit feces-rabbit chow larval diet containing 988

mg/kg novaluron were ataxic, ceased feeding, and died before pupation (Table 3.1). The mean longevity of sand fly larvae fed feces of hamsters that had been fed 9.88, 98.8, and 988 mg/kg novaluron, or the rabbit feces-rabbit chow larval diet containing 988 mg/kg novaluron was not significantly different (Table 3.1).

3.4 Discussion

The quantity of food eaten by the hamsters in this study was not affected by the incorporation of novaluron in a powdered diet. This suggests that novaluron treated diets are palatable to hamsters, and that novaluron could be incorporated into baits for other rodents. Some important rodent reservoirs of *L. major* in parts of the Middle East and Asia, including *Rhombomys opimus* and *Meriones libycus*, are readily attracted to grain-based baits (Yaghoobi-Ershadi 2000, Yaghoobi-Ershadi 2005). In Sub-Saharan Africa rodent reservoirs of *L. major*, such as *Arvicanthis spp, Mastomys spp,* and *Tatera spp*., are granivorous and also could be targeted with treated baits.

Sand fly larvae fed feces of hamsters that had been fed a diet containing novaluron began to die at a time when the control sand flies were molting from second to third instar. This observation is consistent with second instar spined soldier bugs (*Podisus maculiventris*) that had been exposed to a novaluron-treated substrate, and later exhibited ataxia and died as larvae (Cutler et al. 2006). The mortality of 2nd instar *Culex quinquefasciatus* and *Aedes aegypti* principally occurred during the larval stage when they were exposed to 1 ppb novaluron in water (Mulla et al. 2003; Su et al. 2003).

Previously, diflubenzuron was evaluated as a rodent feed-through for sand fly larvae (Mascari et al. 2007a). Unlike the present findings with novaluron, 2nd instar sand flies that were fed feces of hamsters that had been fed diets containing diflubenzuron died during the larva to pupa molt.

The pharmacokinetics of novaluron in mammalian systems makes it an appropriate choice for use in treated rodent baits. Novaluron is of very low toxicity to mammals by ingestion and other routes of exposure (FAO 2005). Following ingestion the majority of novaluron is eliminated unchanged in the feces (FAO 2005). Novaluron is persistent in the environment. In a rotational crop study where 100 g novaluron per ha was applied to soil, between 32 – 49% of the original compound was still present after 127 to 195 d (FAO 2005). The results of this study suggest that a control strategy using rodent baits containing novaluron to control phlebotomine sand flies and zoonotic cutaneous leishmaniasis may be possible.

CHAPTER 4. IVERMECTIN AS A RODENT FEED-THROUGH INSECTICIDE FOR CONTROL OF IMMATURE SAND FLIES (DIPTERA: PSYCHODIDAE)*

4.1 Introduction

Phlebotomine sand flies (Diptera: Psychodidae) are important both as biting pests of humans and as the vectors of human pathogens. Most importantly, sand flies are the vectors of the protozoan parasites that cause leishmaniasis. Worldwide, 2 million new cases of leishmaniasis are believed to occur annually, and as many as 12 million people currently may be infected (WHO 2006). Throughout North Africa, the Middle East, and Southwest Asia, the sand fly *Phlebotomus papatasi* Scopoli is the vector of *Leishmania major*, which is the causative agent of zoonotic cutaneous leishmaniasis (ZCL).

The reservoir hosts of *L. major* in arid and semi-arid Old World foci are burrowing rodents. Sand flies proliferate inside rodent burrows, where the habitat provides high relative humidity and is protected from extreme temperatures. Adult sand flies live in close proximity to sources of blood (from the rodents living within the burrows) and sugar (from plants that grow near the burrow entrances), whereas the larvae develop within the abundant organic matter inside the burrows. The direct treatment of rodent burrows with insecticides has been largely unsuccessful for controlling sand fly populations. Insecticide applications in and around rodent burrows do not reach the microhabitats of adult or immature sand flies that may be located deep within the burrows (Seyedi-Rashti and Nadim 1973, Karapet'ian et al. 1983). Since leishmaniasis is an uncontrolled and emerging disease that disproportionately affects human populations in developing countries, the development of new, efficacious methods for the control of the vectors of ZCL is needed (Saravia 2004).

In ZCL foci in the Old World, rodent burrows are considered to be the primary habitats for immature *P. papatasi*, and larvae have been observed feeding on the feces of rodents (WHO

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1968). Based on this aspect of sand fly ecology, a rodent feed-through method could be a potential means to control sand fly larvae. Proof of concept for this method has been established using two chitin synthesis inhibitors (diflubenzuron and novaluron) against larvae of *P. papatasi* (Mascari et al. 2007a; Mascari et al. 2007b). Ivermectin is a macrocyclic lactone that acts as a broad-spectrum endectocide against numerous nematodes and arthropods, and has been shown to have broad insecticidal effects in many feed-through systems, particularly in cattle (Miller et al. 1981). The objective of this study was to assess ivermectin as a rodent feed-through to control sand fly larvae. The development and survival of *P. papatasi* larvae fed feces of Syrian hamsters, *Mesocricetus auratus*, that had been fed a diet containing ivermectin were evaluated.

4.2 Materials and Methods

4.2.1 Sand Flies

The sand flies used in these studies were from a laboratory colony of a Turkish strain of *P. papatasi* established at Louisiana State University (Mascari et al. 2007b). Larvae were reared using a lab diet consisting of composted and dried rabbit feces and rabbit chow mixed 1:1 (Young et al. 1981). Adults were provided 20% sucrose solution *ad libitum* and obtained blood meals from Syrian hamsters. The colony was maintained in environmental chambers at 28 °C, 90% RH, and 14:10 (L:D) photoperiod.

4.2.2 Syrian Hamsters

A total of 24 Syrian hamsters were housed individually in micro-isolator cages as described by Mascari et al. (2007a). The maintenance of the hamsters and all experimental procedures followed Animal Care & Use Protocol No. 05-074, which was approved by the Institutional Animal Care and Use Committee at Louisiana State University, Baton Rouge, LA. Research involving the hamsters 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.

4.2.3 Feed-Through

Two separate feed-through experiments were conducted using different concentrations of ivermectin. Hamster diets were prepared by adding technical ivermectin (Merck & Co., Inc., Whitehouse Station, NJ, USA) to a meal form laboratory rodent diet (5001 Rodent Diet, LabDiet®, PMI Nutrition International, Brentwood, MO) and thoroughly mixing the diets.

Ivermectin was added to hamster food to obtain diet concentrations of 2, 6, and 10 mg/kg in the first experiment, and 20, 60, and 100 mg/kg in the second experiment. In each experiment, three hamsters were randomly assigned to each of the three diet groups containing ivermectin and to a control diet group (0 mg/kg ivermectin). At 12:00 h each day for nine days, the hamsters were provided with 15 g of their respective diets. The uneaten portion of the food was collected the following day at 12:00 h, and the daily food intake for each hamster was calculated. The daily doses of ivermectin that were ingested by the hamsters were calculated in mg/kg body weight. The body weight of the hamsters was measured on the day before the experiment. The feces produced by each hamster were collected daily for nine days. The feces were dried at room temperature for seven days, and then were stored at -80 °C until used.

In each experiment, the body weight and daily food intake of hamsters in the four diet groups was compared using repeated measures ANOVA, performed with the GLM procedure of SAS (SAS Institute 2001).

4.2.4 Larval Bioassay

Hamster feces collected during the first and second feed-through experiments were assayed separately. The feces voided by hamsters after nine days of feeding were pooled by hamster diet group, and were crushed using a glass mortar and pestle.

In the first experiment, sand fly larvae were fed the feces of hamsters in each hamster diet group (0, 2, 6, and 10 mg/kg ivermectin). Two control larval diet groups also were included: an untreated control fed standard larval diet to identify potential differences in the survival of larvae fed hamster feces and the standard colony larval diet, and a positive control group fed larval diet containing 100 mg/kg ivermectin. In the second experiment, sand fly larvae were fed feces of hamsters that had been fed a diet containing 0, 20, 60, 100 mg/kg ivermectin for 9d.

Larval bioassays were conducted as described by Mascari et al. (2007a). A 200 mg sample of larval diet (hamster feces or the lab diet) was transferred to the plaster surface of each bioassay vial. Ten 2nd instar (13±1-d old) larvae were transferred to each bioassay vial and held in an environmental chamber at 28 °C, 90% RH, 14:10 (L:D) photoperiod. Six bioassay vials were prepared for each larval diet group.

The larvae were observed under magnification daily. Larval mortality (defined as the lack of response to prodding with a blunt probe after 15 s) was recorded, and the larvae were observed for abnormal behavioral and morphological characteristics. Evidence of feeding (the presence of frass in the vials and dark material in the guts of larvae) also was monitored.

Data collected in the bioassays using hamster feces from the first and second experiments were analyzed separately. The percent survival of immature sand flies to adult emergence after being fed their respective diets was compared with repeated measures ANOVA performed with the GLM procedure (SAS Institute 2001). The Tukey multiple comparison procedure was used to separate significantly different means.

4.3 Results

4.3.1 Feed-Through

In the first feed-through experiment, the mean body weight of the 12 Syrian hamsters fed diets containing 0, 2, 6, and 10 mg/kg ivermectin was 128.2±9.1 g, and the mean body weights

of hamsters in these hamster diet groups were not significantly different (F = 0.87, df = 3, P = 0.49). The mean daily food intake of the hamsters was 9.1±1.2, 9.6±1.5, 9.1±1.3, and 8.9±1.4 g for hamsters receiving diets containing 0, 2.0, 6.0, and 10.0 mg/kg ivermectin, respectively, and was not significantly different (F = 1.27, df = 3, P = 0.29). The estimated mean daily doses of ivermectin for hamsters were 0.2±0.1, 0.4±0.1, and 0.7±0.1 mg/kg body weight for hamsters receiving 2, 6, and 10 mg/kg ivermectin, respectively.

In the second feed-through experiment, the mean body weight of the 12 Syrian hamsters that were fed diets containing 0, 20, 60, and 100 mg/kg ivermectin was 124.2±14.6 g, and the mean body weights of hamsters in the different hamster diet groups were not significantly different (F = 2.78, df = 3, P = 0.11). The mean daily food intake of the hamsters was 7.2±2.6, 7.2±1.5, 5.6±1.1, and 4.6±1.5 g for hamsters receiving diets containing 0, 20, 60, and 100 mg/kg ivermectin, respectively. The means of daily food intake of hamsters fed diets containing 0 and 20 mg/kg ivermectin were significantly different from the mean daily food intake of hamsters fed diets containing 60 and 100 mg/kg ivermectin (F = 10.21, df = 3, P < 0.01). The means of daily food intake of hamsters fed diets containing 60 and 100 mg/kg were 22 and 36% lower, respectively, than the mean daily food intake of the hamsters fed a diet containing 0 mg/kg ivermectin. The estimated mean daily doses of ivermectin for hamsters were 1.2±0.3, 2.8±1.0, and 4.2±1.8 mg/kg body weight for hamsters receiving 20, 60, and 100 mg/kg ivermectin, respectively.

4.3.2 Larval Bioassay

In both the first and second experiments, larvae in each of the larval diet groups were observed feeding, and frass was found in each bioassay vial. In the first experiment, the mean percent survival was not significantly different between larval groups fed either feces from untreated hamsters or the lab diet (t = 0.54, df = 10, P = 0.60; Table 4.1). In the bioassay using

hamster feces collected in the first experiment, the mean percent emergence for the sand fly larvae fed feces from ivermectin treated hamsters was significantly different from larvae fed feces from untreated hamsters (F = 37.27, df = 3, $P < 0.01$; Table 4.1). The mean longevity of larvae after they were fed feces of hamsters that had been fed diets containing 2, 6, and 10 mg/kg ivermectin was 5.3±2.9, 6.2±4.1, and 4.0±3.2 d, respectively. Larvae fed the rabbit feces-rabbit chow diet containing 100 mg/kg ivermectin all died within 3 d. The larvae that were fed feces voided by ivermectin-treated hamsters and lab diet containing ivermectin became rigid and ceased feeding before they died.

Table 4.1 Mortality of Second Instar Sand Flies Fed Feces Voided by Ivermectin-Treated or Untreated Hamsters, and Ivermectin-Treated or Untreated Laboratory Larval Diet (1:1 Rabbit Feces-Rabbit Chow w:v)

Larval diet	Mortality % (mean* ± SE)
Experiment one	
Hamster feces#	
0	5.0 ± 5.5
2	85.0 ± 16.4
6	80.0 ± 22.8
10	93.3 ± 12.1
Laboratory diet§	
0	8.3 ± 7.5
100	100
Experiment two	
Hamster feces#	
0	5.0 ± 8.4
20	100
60	100
100	100

* Six replicates, ten larvae per replicate
# Concentration (mg/kg) of ivermectin in hamster diet
§ Concentration (mg/kg) of ivermectin in laboratory diet

In the larval bioassay using hamster feces collected in the second feed-through experiment, the mean mortality of larvae fed feces from untreated hamsters was 5%. The

mortality of larvae that were fed feces from ivermectin-treated hamsters was 100% (Table 4.1). The mean longevity of larvae after being fed feces of hamsters that had been fed diets containing 20, 60, and 100 mg/kg ivermectin was 4.5±2.3, 3.5±1.9, and 4.3±2.6 d respectively. The larvae fed feces from ivermectin-treated hamsters in this bioassay also became rigid and ceased feeding before death.

4.4 Discussion

The sand fly larvae in this study readily fed on hamster feces, including the feces of hamsters that had been fed diets containing ivermectin. Larvae died soon after being fed feces from ivermectin-treated hamsters, typically within one week. These findings are consistent with the findings of Miller et al. (1981) in which horn fly, face fly, house fly, and stable fly larvae died after being fed feces from ivermectin-treated cattle.

The quantity of food that was consumed by the hamsters tested in this study was not affected by the incorporation of 2, 6, 10, or 20 mg/kg ivermectin in their diet. However, hamsters that were fed diets containing 60 and 100 mg/kg ate significantly less than the control hamsters. The diet concentration of 20 mg/kg ivermectin did not reduce hamster feeding and was more effective than lower diet concentrations as a feed-through against sand fly larvae. The corresponding mean daily dose of ivermectin for hamsters fed a diet containing 20 mg/kg ivermectin (1.16±0.27 mg/kg body weight) was below the LC_{50} observed in orally dosed rats (42.8 to 52.8 mg/kg body weight), as well as the level at which sublethal effects (such as moderate incoordination) have been observed (4 mg/kg body weight; IPCS 1994).

Previously, diflubenzuron and novaluron were evaluated as rodent feed-through insecticides for immature sand flies, and the feces of hamsters treated with these chitin synthesis inhibitors affected the development of sand fly larvae (Mascari et al. 2007a, 2007b). Diflubenzuron interrupted the development of larvae during the molt from larva to pupa, and

novaluron affected sand flies during larval molts. Sand fly larvae may survive for several days after ingesting diets containing chitin synthesis inhibitors because these compounds act at specific developmental stages in sand flies. In contrast, ivermectin induces an acute response in insects by enhancing glutamate-nergic neural and neuromuscular transmission that leads to paralysis and death. As expected, sand fly larvae fed feces from ivermectin-treated hamsters died rapidly, and their death was not linked to an event in their development.

Ivermectin has pharmacokinetic properties that make it an appropriate feed additive for the control of fly larvae that feed on animal feces. Over 90% of orally administered ivermectin is excreted by various mammals (cattle, sheep, pigs, and rodents) unchanged in the feces (Campbell et al. 1983). Ivermectin excreted in animal feces also degrades at a slow rate under field conditions. Sommer and Steffansen (1993) did not observe a reduction in the amount of ivermectin in cow dung that was in a pasture for 45 d, and Madsen et al. (1990) found that dung from ivermectin-treated cattle remained toxic to house fly larvae after two months.

The results of this study suggest that ivermectin-treated diets are effective as feed-through for control of sand fly larvae at concentrations that are palatable to hamsters. In future field trials, several important rodent reservoirs of *L. major* could be targeted with ivermectin-treated baits, particularly, *Rhombomys opimus* and *Meriones libycus* in parts of the Middle-East and Southwest Asia, and *Arvicanthis spp*, *Mastomys spp*, and *Tatera spp*. in Sub-Saharan Africa, all of which can be baited with grains (Yaghoobi-Ershadi et al. 2000, Yaghoobi-Ershadi et al. 2005). If shown to be effective in field trials, rodent baits containing ivermectin may play a role in reducing sand fly populations, the burden of sand flies feeding on people, and the incidence of ZCL.

CHAPTER 5. EVALUATION OF JUVENILE HORMONE ANALOGUES AS RODENT FEED-THROUGH INSECTICIDES FOR CONTROL OF IMMATURE SAND FLIES (DIPTERA: PSYCHODIDAE)

5.1 Introduction

Phlebotomine sand flies (Diptera: Psychodidae) are significant biting pests of humans, and are the vectors of several human pathogens including *Bartonella bacilliformis*, Toscana Virus, Sicilian Virus, and Naples Virus. Most importantly, sand flies are the vectors of the protozoan parasites that cause leishmaniasis. Worldwide, 2 million new cases of leishmaniasis are believed to occur annually, and as many as 12 million people currently may be infected (WHO 2006).

The sand fly species *Phlebotomus papatasi* Scopoli occurs in Mediterranean littoral countries and throughout Southwestern and Central Asia. In arid areas within its distribution, *P. papatasi* is the vector of *Leishmania major*, the causative agent of zoonotic cutaneous leishmaniasis (ZCL). The reservoir hosts of *L. major* are various species of locally abundant burrowing rodents. Sand flies aggregate within rodent burrows, which provide the microclimatic conditions they require for survival (darkness, high relative humidity, and protection from extreme temperatures). Adult sand flies live in close proximity to sources of blood (from the rodents living within the burrows) and sugar (from plants that grow near the burrow entrances), while the sand fly larvae develop within the organic matter inside the burrows.

The close association between sand flies and rodent burrows has been demonstrated in many different sand fly/rodent associations in Old World ZCL foci. However, targeting burrows with insecticides has not been effective at controlling sand fly populations because insecticide applications in and around rodent burrows do not reach deep within the burrows where adult and immature sand flies are located (Seyedi-Rashti & Nadim 1973; Karapet'ian *et al*. 1983). Since leishmaniasis is an emerging disease that disproportionately affects human populations in

developing countries, the development of new and efficacious methods for the control of the vectors of ZCL is needed (Saravia 2004).

The primary habitat for immature *P. papatasi* in ZCL foci is considered to be organic debris in rodent burrows, and sand fly larvae have been observed feeding on the feces of rodents (WHO 1968). Therefore, the use of rodent feed-through insecticides may be a potential method to control sand fly larvae. Proof of concept for rodent feed-through control of larvae of *P. papatasi* has been established in laboratory studies using two benzoylurea chitin synthesis inhibitors (diflubenzuron and novaluron) and a macrocyclic lactone (ivermectin) (Mascari *et al*. 2007a, b; Mascari *et al*. 2008). The objective of this study was to evaluate the juvenile hormone analogs methoprene and pyriproxyfen as rodent feed-through insecticides to control sand fly larvae. The development and survival of *P. papatasi* larvae fed feces of Syrian hamsters, *Mesocricetus auratus*, that had been fed a diet containing methoprene or pyriproxyfen were measured.

5.2 Materials and Methods

5.2.1 Sand Flies

The sand flies used in these studies were from a laboratory colony of a Turkish strain of *P. papatasi* established at Louisiana State University (Mascari *et al.* 2007b). The sand fly larvae in the colony were reared using a larval diet composed of a composted and dried 1:1 mixture of rabbit feces and rabbit chow (Young *et al*. 1981). Adult sand flies were provided 20% sucrose solution *ad libitum*, and obtained blood-meals from Syrian hamsters. The colony was maintained in environmental chambers at 28 °C, 90% RH, and 14:10 (L:D) photoperiod.

5.2.2 Syrian Hamsters

A total of twenty-four Syrian hamsters were housed individually in micro-isolator cages. The maintenance of the hamsters and all experimental procedures followed Animal Care & Use

Protocol No. 05-074, which was approved by the Institutional Animal Care and Use Committee at Louisiana State University, Baton Rouge, LA, USA. Research involving the hamsters 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.

5.2.3 Feed-Through

Hamster diets were prepared by adding pyriproxyfen 98.2% a.i., Valent USA Corporation, Walnut Creek, CA, USA] and methoprene (97.88% a.i., Central Life Sciences, Walnut Creek, CA, USA) to a meal form laboratory rodent diet (5001 Rodent Diet, LabDiet®, PMI Nutrition International, Brentwood, MO, USA). Pyriproxyfen was added directly to hamster food to achieve three concentrations (9.82, 98.2, or 982 mg/kg), and the diets were thoroughly mixed. An untreated control diet also was prepared. Technical methoprene is in liquid form, and it was diluted in pure soybean oil before being added to powdered hamster food. Diluted methoprene was added to hamster food at a rate of 100 g / 900 g powdered hamster food yielding hamster food containing three concentrations of methoprene: 9.788, 97.88, or 978.8 mg/kg. An additional control diet was prepared by adding soybean oil at a rate of 100 g / 900 g hamster food.

Three hamsters were randomly assigned to each of the eight diet groups (three concentrations of pyriproxyfen, three concentrations of methoprene, a soybean oil control diet group, and an untreated control diet group). The hamsters were provided with 25 g of their respective diets each day for 9 d. The uneaten portion of the food was collected the following day, and the daily food intake for each hamster was calculated. The daily doses of pyriproxyfen and methoprene that were ingested by the hamsters were calculated in mg/kg body weight (the body weight of the hamsters was measured on the day before the experiment). The feces voided

by each hamster were collected daily for 9 d. All feces were dried at room temperature for seven days, and then were stored at -80 °C until used.

The daily food intake of hamsters was compared using repeated measures analysis of variance (ANOVA), performed with the GLM procedure of SAS (SAS Institute 2001). The Tukey multiple comparison procedure was used to separate significantly different means. The daily doses of pyriproxyfen or methoprene for individual hamsters were compared within hamster diet groups using the same statistical analysis.

5.2.4 Larval Bioassay

Feces voided by hamsters after nine days of feeding on their respective diets were used as diets for sand fly larvae. The feces were pooled by hamster diet group and were manually crushed using a sterilized glass mortar and pestle.

Larval bioassays were conducted according to the methods described by Mascari et al. (2007a). A 0.4 g portion of feces was transferred to the plaster surface of each bioassay vial. Ten second instars (13±1-d old) were transferred to each bioassay vial and held in an environmental chamber at 28 °C, 90% RH, 14:10 (L:D) photoperiod. Five bioassay vials were used for each of the eight larval diet groups.

The larvae were observed under magnification daily. Mortality, which was defined as the lack of response to prodding with a blunt probe after 15 s, was recorded; and the sand flies were observed for abnormal behavioral and morphological characteristics. Evidence of feeding, which was defined by the presence of frass in the vials and dark material in the guts of larvae, also was monitored.

The percent survival of sand flies and the age of the sand flies at death in each larval diet group were compared with repeated measures ANOVA performed with the GLM procedure

(SAS Institute 2001). The Tukey multiple comparison procedure was used to separate significantly different means

5.3 Results

5.3.1 Feed-Through

The mean body weight of the 24 hamsters in this study was 132.6±6.4 g, and the body weights of hamsters assigned to different diet groups were not significantly different (F = 0.03, df = 7, P > 1.0000). The mean daily food intake of the 24 hamsters in this study was 9.6±1.8 g, and there were significant differences between the mean daily food intake of hamsters fed diets containing soybean oil (0, 9.788, 97.88, and 978.8 mg/kg methoprene) and without soybean oil (0, 9.82, 98.2, and 982 mg/kg pyriproxyfen; F = 17.64, df = 7, P < 0.0001). The mean daily food intake of hamsters fed diets containing soybean oil (10.7±1.6) was 24.4% higher than the mean daily food intake of the hamsters fed a diet without soybean oil (8.6±1.3). The amount of food eaten by hamsters in different diet groups containing soybean oil was not significantly different (F = 2.19, df = 3, P = 0.0941), and the amount of food eaten by hamsters in different diet groups without soybean oil also was not significantly different (F = 0.30, df = 3, P = 0.8242).

The mean daily doses of methoprene for hamsters were 0.8±0.1, 7.8±1.3, and 80.5±12.1 mg/kg body weight for hamsters fed diets containing 9.788, 97.88, or 978.8 mg/kg methoprene, respectively. The mean daily doses of pyriproxyfen for hamsters were 0.6±0.1, 6.5±1.1, and 62.6±11.3 mg/kg body weight for hamsters fed diets containing 0, 9.82, 98.2, and 982 mg/kg pyriproxyfen, respectively.

5.3.2 Larval bioassay

The sand fly larvae in each larval diet group were observed feeding, and frass was found in every vial. The mean percent adult emergence was not significantly different between sand flies fed feces of hamsters fed an untreated diet or hamsters fed a diet containing untreated

soybean oil (F = 1.20, df = 1, P = 0.3052; Table 5.1). Control larvae (larvae that had been fed feces of hamsters fed an untreated diet or a diet containing soybean oil) first pupated when the sand flies were 24 d old. Adult emergence was first observed in both control groups when the sand flies were 30 d old.

Table 5.1 Percent Pupation and Adult Emergence of Immature Sand Flies Fed Feces of Hamsters Fed Diets Containing Methoprene or Pyriproxyfen, or Control Diets

Hamster diet (mg/kg)	Pupation % (mean[*] ± SE)[#]	Adult emergence % (mean[*] ± SE)[#]	Age at death d (mean* ± SE)[#]
Control			
Untreated	94.0 ± 8.9[a]	94.0 ± 8.9[a]	n/a
Soybean oil[§]	90.0 ± 10.0[a]	88.0 ± 8.4[a]	n/a
Pyriproxyfen			
9.82	0[b]	0[b]	34.8 ± 6.0[a]
98.2	0[b]	0[b]	34.2 ± 5.6[a]
982	0[b]	0[b]	30.6 ± 5.3[a]
Methoprene			
9.788	10.0 ± 14.1[b]	4.0 ± 5.5[b]	34.0 ± 7.5[a]
97.88	2.0 ± 4.5[b]	0[b]	36.4 ± 7.2[a]
978.8	8.0 ± 8.4[b]	0[b]	32.7 ± 4.7[a]

[*] Five replicates, ten larvae per replicate
[#] Values within a column followed by the same letter are not significantly different from each other (P > 0.05).
[§] Rodent food + soybean oil (9:1 w/w)

The rates of pupation for larvae fed feces of hamsters fed three concentrations of methoprene (9.788, 97.88, or 978.8 mg/kg) were significantly lower than the pupation rate of larvae in the control groups (F = 89.62, df = 3, P < 0.0001; Table 5.1). None of the larvae that were fed feces of hamsters that had been fed diets containing 97.88 or 978.8 mg/kg methoprene emerged as adults. Only 4.0±5.5% of sand flies fed feces of hamsters that had been fed a diet containing 9.788 mg/kg methoprene emerged as adults, which was significantly lower than the percent adult emergence of sand flies fed feces of control hamsters (F = 352.80, df = 1, P <

0.0001; Table 5.1). The age at death of sand flies fed feces of methoprene-treated hamsters as larvae was over 32 d (Table 5.1).

In the pyriproxyfen larval bioassay, 100% mortality was observed during the larval stage for sand flies fed feces of hamsters fed diets containing 9.82, 98.2 or 982.0 mg/kg pyriproxyfen. The mean age of the larvae at death was over 30 d (Table 5.1).

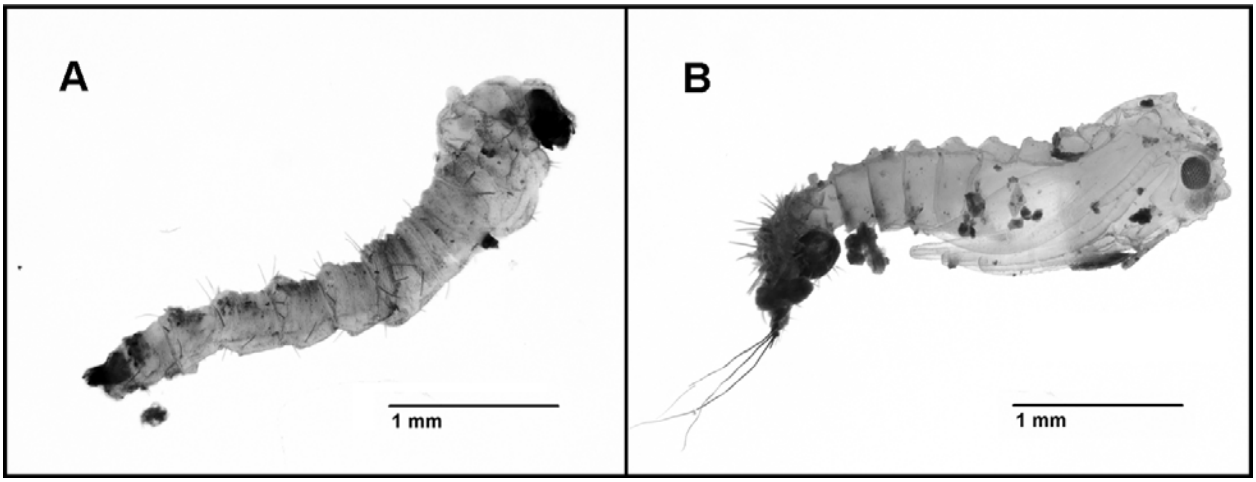

Fig. 5.1 A Pupa-Form Larva That Had Been Fed Feces of Hamsters Fed a Diet Containing 9.82 mg/kg Pyriproxyfen as a Larva (A), and a Normal Pupa That Had Been Fed Feces of Untreated Hamsters as a Larva (B)

The majority of the sand fly larvae that were fed feces of hamsters offered diets containing methoprene or pyriproxyfen died as late 4th instar larvae. The larvae in these groups developed at a normal rate (the same rate as control larvae). Like the control larvae, the larvae in the treatment groups eventually ceased feeding and cleared their guts as late 4th instar larvae. However, rather than progressing to the pupal stage, most of the sand fly larvae in the treatment groups remained as late 4th instar larvae for up to 19 d before eventually dying. Some of the larvae that were fed feces of methoprene- or pyriproxyfen-treated hamsters did transform from 4th instar larvae into pupa-form larvae before dying (Fig. 5.1). These larvae developed normally as 2nd, 3rd, and 4th instar larvae, but became an intermediate form between larva and pupa after

they ceased feeding and cleared their guts. Pupa-form larvae survived for several days, but eventually died without becoming pupae.

5.4 Discussion

The food intake of the hamsters in this study was not affected by the methoprene or pyriproxyfen treatments at any of the concentrations tested. This finding suggests that the treated diets were palatable to hamsters. Furthermore, significantly more food was consumed by hamsters when it contained soybean oil, independent of insecticide treatment. The bait preferences are known for the rodents involved in many of the sand fly/rodent associations found in Old World ZCL foci, and insecticide-treated baits could be developed for use in field trials in these scenarios. In Southwest Asia *Rhombomys opimus* and *Meriones* spp. are readily baited with oats, and in Sub-Saharan Africa five rodent genera known be reservoirs of *L. major* (*Mastomys, Taterillus, Aethomys, Tatera,* and *Arvicanthis*) have been successfully captured in traps baited with corn flour (Githure *et al.* 1986; Yaghoobi-Ershadi *et al*. 2000, 2005).

The results of this study suggest that both methoprene and pyriproxyfen remained pharmacologically active after passing through the guts of hamsters, and that the compounds were present at sufficiently high concentrations to affect the development and survival of immature sand flies. As juvenile hormone analogues, both methoprene and pyriproxyfen were expected to have the same effect on the development of immature sand flies. The development of immature sand flies fed feces of hamsters fed diets containing methoprene or pyriproxyfen was identical to that of control sand flies until the 4^{th} larval instar. At this point, nearly all of the surviving control larvae subsequently pupated. Larvae that had been fed feces of pyriproxyfen-treated hamsters remained as 4^{th} instar larvae or became pupa-form larvae, and all of these sand flies eventually died before pupation. The development of sand fly larvae fed feces of methoprene-treated hamsters was similar to that of larvae fed feces of pyriproxyfen-treated

hamsters. However, pupation of larvae fed feces of hamsters fed diets containing methoprene was observed at all concentrations, and adult emergence was seen at the lowest concentration. The finding that pyriproxyfen treatments fully prevented pupation and adult emergence at all concentrations tested while similar concentrations of methoprene resulted in some pupation and adult emergence is consistent with other studies that compared the effectiveness of methoprene and pyriproxyfen against other insects. The LC_{50} for methoprene was more than 20 x higher than pyriproxyfen in an evaluation of the relative toxicity of methoprene and pyriproxyfen in topsoil against immature *Ctenocephalides felis* (Rajapakse *et al*. 2002). Similarly, pyriproxyfen was found to be 21.5 x more toxic than methoprene to larvae of *Aedes albopictus* (Ali *et al*. 1995). Against larvae of *Culex quinquefasciatus* and *A. albopictus*, methoprene provided significant but incomplete inhibition of adult emergence, even at the highest concentrations tested (Nayar *et al*. 2002).

The results of this study add the juvenile hormone analogues methoprene and pyriproxyfen to the list of insecticides that potentially can be used as rodent feed-throughs for the control of phlebotomine sand flies in certain sand fly/rodent associations. The identification of multiple insecticides that have been found to be effective as rodent feed-throughs against sand fly larvae in the laboratory increases the likelihood that a suitable compound will be found for use in field trials. However, future studies on the relative residual activity and environmental persistence of the compounds will be required before field trials can be conducted.

CHAPTER 6. EVALUATION OF NOVALURON AS A RODENT FEED-THROUGH UNDER SIMULATED FIELD CONDITIONS FOR CONTROL OF SAND FLY LARVAE (DIPTERA: PSYCHODIDAE)

6.1 Introduction

Phlebotomine sand flies are major biting pests of man and are the vectors of the protozoan parasites that cause leishmaniasis. Worldwide, there are an estimated 2 million new cases of leishmaniasis annually, and 12 million people are currently believed to be infected (WHO 2006). Throughout North Africa, the Middle East and SW Asia, *Phlebotomus papatasi* is the primary vector of *Leishmania major*, the causative agent of zoonotic cutaneous leishmaniasis (ZCL).

In spite of their importance, there are no effective control or preventive measures currently available for sand flies in ZCL foci. In arid and semi-arid foci, *P. papatasi* exhibits a close association with several burrowing rodent that serve as reservoirs of *L. major*. In ZCL foci in the Old World, rodent burrows are considered to be the primary immature habitats for *P. papatasi*, and sand fly larvae have been observed feeding on the feces of rodents. Therefore, rodent feed-through insecticides are a potential means of controlling sand fly larvae.

Proof of concept for rodent feed-through control of larvae of *P. papatasi* has been established in laboratory studies using the benzoylurea chitin synthesis inhibitor novaluron (Mascari *et al*. 2007b). All diet concentrations of novaluron tested in a preliminary rodent feed-through study (9.88, 98.8 and 988 mg/mg) were 100% effective in killing sand fly larvae that fed on the feces of novaluron-treated rodents (Mascari et al. 2007b). However, additional laboratory studies are required to determine if novaluron would be appropriate for field use. There were three objectives of this study: 1) to determine the minimum concentration of novaluron mixed with hamster feces that would prevent development of sand fly larvae and the minimum dose of novaluron for hamsters that would be effective as a feed-through against sand fly larvae 2) to

determine the persistence of the larvicidal effect of novaluron in hamster feces held under simulated field conditions 3) to determine the effectiveness of novaluron as a feed-through in preventing the development of sand fly larvae when novaluron-treated food makes up only a portion of a hamster's daily diet.

6.2 Materials and Methods

6.2.1 Sand Flies

The sand flies used in these studies were from a laboratory colony of a Turkish strain of *P. papatasi* established at Louisiana State University (Mascari et al. 2007b). The sand fly larvae in the colony were reared using a larval diet composed of a composted and dried 1:1 mixture of rabbit feces and rabbit chow (Young et al. 1981). Adult sand flies were provided 20% sucrose solution *ad libitum*, and obtained blood meals from Syrian hamsters. The colony was maintained in environmental chambers at 28 °C, 90% RH.

6.2.2 Hamsters

Syrian hamsters were housed individually in micro-isolator cages. The maintenance of the hamsters and all experimental procedures followed Animal Care & Use Protocol No. 05-074, which was approved by the Institutional Animal Care and Use Committee at Louisiana State University, Baton Rouge, LA. Research involving the hamsters 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.

6.2.3 Experiment 1: Direct Treatment of Hamster Feces with Novaluron

A series of ten-fold dilutions of technical novaluron (98.8% a.i., Makhteshim Agan Industries Ltd., Tel Aviv, Israel) was prepared in acetone. The feces of untreated hamsters were collected and dried at room temperature for 7 d. The feces were crushed using a sterilized glass

mortar and pestle and treated with novaluron solutions (at a rate of 1 mL/1 g feces) yielding eight concentrations ranging from $9.88x10^{-5}$ to 988 mg/kg novaluron. A solvent-control diet (made by treating feces with acetone) also was prepared. The acetone was allowed to evaporate for 12 h, and the feces were stored at -80 °C until used. Larval bioassays were conducted as described by Mascari et al. (2007). A 200 mg portion of feces was placed on the plaster surface of each bioassay vial. Ten 2nd instar (13±1-d old) larvae then were transferred to each bioassay vial and held in an environmental chamber at 28 °C, 90% RH. Four bioassay vials were prepared for each concentration of novaluron and for the solvent-control group.

Larvae were observed under magnification daily, and larval mortality (defined as the lack of response to prodding with a blunt probe after 15 s) and the percentage of sand flies that successfully emerged as adults were recorded. Larvae were observed for abnormal behavioral and morphological characteristics. Evidence of feeding (the presence of frass in the vials and dark material in the guts of larvae) also was monitored. The percent survival of sand flies and the age of sand flies at death were compared with repeated measures ANOVA performed with the GLM procedure (SAS Institute 2001). The Tukey multiple comparison procedure was used to separate significantly different means.

6.2.4 Experiment 2: Minimum Effective Dose of Novaluron as a Feed-Through

Hamster diets were prepared by adding technical novaluron to a meal-form laboratory rodent diet (5001 Rodent Diet, LabDiet®, PMI Nutrition International, Brentwood, MO). Novaluron and the diet were thoroughly mixed to achieve six concentrations ($9.88x10^{-5}$, $9.88x10^{-4}$, $9.88x10^{-3}$, $9.88x10^{-2}$, $9.88x10^{-1}$, and 9.88 mg/kg). A control diet (untreated laboratory rodent diet) also was prepared. Three hamsters were randomly assigned to each of the seven hamster diet groups (six novaluron treatment groups and one control group).

At 12:00 h each day for nine days, the hamsters were provided with 15 g of their respective diet. The uneaten portion of the food was collected the following day at 12:00 h, and the daily food intake and daily doses of novaluron that were ingested by the hamsters were calculated. The daily doses of novaluron for individual hamsters were compared within hamster diet groups using repeated measures ANOVA, performed with the GLM procedure of SAS (SAS Institute 2001). The Tukey multiple comparison procedure was used to separate significantly different means. The feces voided by each hamster were collected daily for nine days. The feces of each hamster were placed in uncovered containers, dried at room temperature for seven days, and then stored at -80 °C until used.

Feces voided by the three hamsters in each diet group were pooled. Larval bioassays were conducted as described above using hamster feces collected after 9 d of feeding as larval diets. Six bioassay vials were prepared for each of the six larval diets (feces of hamsters fed a diet containing five concentrations of novaluron or a control diet). The development and survival of sand fly larvae were monitored and statistically analyzed as described above for Experiment 1.

6.2.5 Experiment 3: Aging Feces under Simulated Field Conditions

A meal-form hamster diet containing 988 mg/kg technical novaluron and an untreated control hamster diet were prepared. Twelve hamsters were weighed and randomly assigned to each of the two hamster diet groups (total of 24 hamsters). Hamsters were fed their respective diets for 9 d as described above. The daily food intake and the daily doses of novaluron which were ingested by the hamsters were calculated, and were statistically analyzed as described above for Experiment 2.

A total of 12 g of feces voided by control or novaluron-treated hamsters after 9 d of feeding on their respective diets (1 g of feces voided by each hamster) was placed in a 120 ml specimen cup with a 2 cm thick basal layer of plaster of Paris. The specimen cups containing

feces were stored in an environmental chamber at 28 °C, 90% RH (conditions which simulated the temperature and humidity within a rodent burrow; Kay and Whitford 1978). The specimen cups were placed on filter papers that were kept saturated with distilled water in glass dishes. Samples of the aged feces (2 g) were taken from the specimen cups at 30 d intervals for 150 d, and the aged feces were stored at -80 °C until used in sand fly larval bioassays.

Aged hamster feces were fed to second instar sand flies as described above in Experiment 1. Six bioassay vials were used for each of the 12 larval diet groups (feces of control or novaluron-treated hamsters aged for six time periods: 0, 30, 60, 90, 120, or 150 d). The development and survival of sand fly larvae were monitored and statistically analyzed as described above for Experiment 1.

6.2.6 Experiment 4: Partial Consumption of Novaluron-Treated Food by Hamsters

Meal-form hamster diets containing 0 or 988 mg/kg novaluron were prepared. Three hamsters were randomly assigned to each of the following three groups: hamsters fed exclusively a diet containing 988 mg/kg novaluron for nine days, hamsters fed exclusively an untreated diet, or hamsters fed 1 g of diet containing 988 mg/kg novaluron for 3 h each day and then afterwards provided with untreated diet. The hamsters were fed using these protocols for 9 d. The daily food intake and daily doses of novaluron for each hamster were calculated as described above. The feces voided by each hamster were collected daily for 9 d and were processed as described above.

The feces collected from hamsters after feeding for 9 d were pooled by treatments, crushed using a mortar and pestle, and used in sand fly larval bioassays. The larval bioassays were conducted as described above. Six bioassay vials were used for each of the three larval diet groups (feces of hamsters exclusively fed novaluron-treated or control diets, or feces of hamsters

fed novaluron-treated food as a portion of their diet). The development and survival of sand fly larvae were monitored and statistically analyzed as described above.

6.3 Results

6.3.1 Experiment 1: Direct Treatment of Hamster Feces with Novaluron

Larvae in each of the larval diet groups were observed feeding, and frass was found in each bioassay vial. The percent survival from 2nd instar to adult was 100% for larvae fed acetone treated hamsters feces. At the concentrations of novaluron tested, the mean percent survival of sand fly larvae fed novaluron-treated hamster feces ranged from 0 to 100% (Table 6.1). Mortality of larvae fed feces containing as little as $9.88x10^{-1}$ mg/kg novaluron was significantly different from mortality of control larvae (F = 199.47, df = 8 $P < 0.0001$); mortality was 100% at 9.88 mg/kg and above (Table 6.1). The mean longevity of sand fly larvae that died after being fed novaluron-treated feces ranged from 3.0±0.2 to 4.1± 0.3 d (Table 6.1).

Table 6.1 Mortality and Longevity of 2nd Instar Sand Flies Fed Hamster Feces Directly Treated with Novaluron Solutions

Concentration (mg/kg) of novaluron in larval diet	Mortality % (mean* ± SE)#	Longevity d (mean* ± SE)
0	0.0±0.0[a]	n/a
$9.88x10^{-5}$	5.0±5.8[a]	n/a
$9.88x10^{-4}$	2.5±5.0[a]	n/a
$9.88x10^{-3}$	0.0±0.0[a]	n/a
$9.88x10^{-2}$	0.0±0.0[a]	n/a
$9.88x10^{-1}$	35.0±19.1[b]	4.0±0.4
9.88	100.0±0.0[c]	4.1±0.3
98.8	100.0±0.0[c]	3.0±0.3
988	100.0±0.0[c]	3.0±0.3

* Six replicates, ten larvae per replicate
# Values within a column followed by the same letter are not significantly different from each other, P> 0.05
n/a not applicable

6.3.2 Experiment 2: Minimum Effective Dosage of Novaluron as a Feed-Through

The mean body weight of the 12 hamsters in this study was 143.3±3.0 g, and the body weights of hamsters assigned to different diet groups were not significantly different (F = 0.24, df = 5, P = 0.9358). The mean daily food intake of the 12 hamsters in this study was 7.2±1.4 g, and the amount of food eaten by hamsters in different diet groups was not significantly different (F = 0.28, df = 5, P = 0.9256). The mean daily doses of novaluron for hamsters ranged from $4.8x10^{-5}$ to $5.1x10^{-1}$ (Table 6.2).

Table 6.2 Means (±SE) of Body Weight, Food Intake, and Daily Dosages of Novaluron for Syrian Hamsters

Concentration (mg/kg) of novaluron in diet	Body weight g (mean ± SE) [#]	Food intake g/d (mean[*] ± SE) [#]	Daily dose mg/kg (mean[*] ± SE)[#]
0	142.3±3.2[a]	7.3±1.5[a]	n/a
$9.88x10^{-4}$	144.4±3.5[a]	6.9±1.6[a]	$4.8x10^{-5}$±$1.1x10^{-5}$
$9.88x10^{-3}$	144.0±1.0[a]	7.2±1.4[a]	$5.0x10^{-4}$±$9.4±10^{-5}$
$9.88x10^{-2}$	143.0±4.6[a]	7.2±1.1[a]	$5.0x10^{-3}$±$7.8x10^{-4}$
$9.88x10^{-1}$	144.0±3.6[a]	7.1±1.4[a]	$4.9x10^{-2}$±$9.7x10^{-3}$
9.88	142.0±3.6[a]	7.3±1.1[a]	$5.1x10^{-1}$±$8.4x10^{-2}$

* Nine replicates, three hamsters per replicate
[#] Values in a column followed by the same letter are not significantly different from each other, P>0.05
n/a not applicable

Evidence of feeding was observed for larvae in each of the larval diet groups. The percent survival from 2nd instar to adult was 90.0±8.2% for larvae fed feces of untreated hamsters (Table 6.3). At the tested concentrations of novaluron fed to hamsters, the mean percent survival of sand fly larvae fed feces of novaluron-treated hamsters ranged from 0.0 to 95.0% (Table 6.3). Mortality of larvae fed feces of hamsters fed a diet containing as little as $9.88x10^{-1}$ mg/kg novaluron was significantly different from mortality of larvae fed feces of hamsters fed an

untreated diet (F = 188.61, df = 5, P < 0.0001; Table 6.3). The mean longevity of sand fly larvae that died after being fed feces of novaluron-treated hamsters ranged from 4.2 to 5.6 (Table 6.3).

Table 6.3 Mortality and Longevity of 2nd Instar Sand Flies Fed Feces of Hamsters Fed Diets Containing Novaluron

Hamster diet novaluron concentration (mg/kg)	Mortality % (mean* ± SE)#	Longevity d (mean* ± SE)
0	10.0±8.2[a]	n/a
$9.88x10^{-4}$	7.5±9.6[a]	n/a
$9.88x10^{-3}$	12.5±9.6[a]	n/a
$9.88x10^{-2}$	5.0±5.0[a]	n/a
$9.88x10^{-1}$	100.0±0.0[b]	5.6±0.6
9.88	100.0±0.0[b]	4.2±0.6

* Six replicates, ten larvae per replicate
# Values within a column followed by the same letter are not significantly different from each other, P> 0.05
n/a not applicable

6.3.3 Experiment 3: Aging Feces under Simulated Field Conditions

The mean body weight of the 36 hamsters was 150.8±10.2 g, and the mean body weights of hamsters in the two hamster diet groups were not significantly different (F = 0.16, df = 1, P = 0.85; Table 6.4). The mean daily food intake for hamsters fed diets containing 988 mg/kg novaluron or acetone alone were not significantly different (F = 1.65, df = 1, P = 0.19; Table 6.4). The estimated mean daily dosage of novaluron was 63.1±10.0 mg/kg body weight.

Table 6.4 Means (±SE) of Body Weight, Food Intake, and Daily Dosages of Novaluron for Syrian Hamsters

Hamster diet group	Body weight g (mean* ± SE) #	Food intake g/d (mean* ± SE) #	Daily dose mg/kg (mean* ± SE)
Control (0 mg/kg)	151.0±11.7[a]	9.5±1.7[a]	n/a
Novaluron (988 mg/kg)	151.8±8.6[a]	9.7±1.6[a]	63.1±10.0

* Nine replicates, three hamsters per replicate
# Values in a column with the same letter are not significantly different from each other, P>0.05
n/a not applicable

Larvae in each of the larval diet groups were observed feeding, and frass was found in each bioassay vial. The mean percent adult emergence for sand flies fed feces of untreated hamsters that had been aged for any of the time periods (0 to 150 d) was over 90%. There were no significant differences between percent adult emergence at different aging periods (F = 230.34, df = 11, $P < 0.0001$; Fig. 6.1).

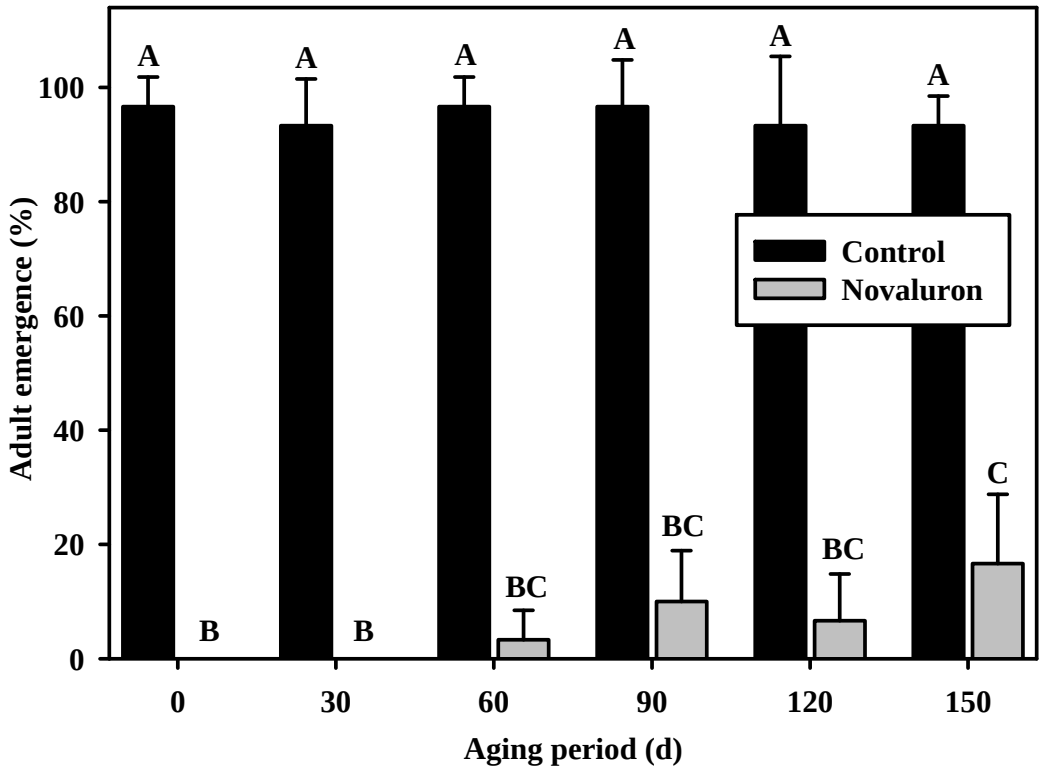

Figure 6.1 Percent Adult Emergence of Sand Flies Fed Feces of Control or Novaluron-Treated Hamsters; Feces Were Aged under Simulated Field Conditions (28 °C, 90% RH) for up to 150 d

The mean percent adult emergence for sand flies fed feces of novaluron-treated hamsters was significantly different from sand flies fed feces of control hamsters when the feces were aged for any of the time periods (Fig. 6.1). All larvae that had been fed feces of novaluron-treated hamsters aged for 0 or 30 d died before adult emergence. The mean percentage of larvae that emerged as adults after being fed feces of novaluron-treated hamsters that had been aged for

60 (3.3±5.2%), 90 (10.0±8.9%), or 120 d (6.7±8.2%) was significantly lower than for larvae fed feces of control hamsters (Fig. 6.1). The mean percent adult emergence for larvae fed feces of novaluron-treated hamsters aged for 150 d was 16.7±12.1%, which was significantly different from both larvae fed feces of control hamsters and larvae fed feces of novaluron-treated hamsters aged for 0 or 30 d (Fig. 6.1).

6.3.3 Experiment 4: Partial Consumption of Novaluron-Treated Food by Hamsters

The mean daily food intake of the hamsters was 7.3±0.8 g. The mean daily food intake of hamsters fed novaluron-treated food as a portion of their daily diet was significantly different from the food intake of hamsters fed exclusively untreated or novaluron-treated diets (F = 6.30, df = 2, P = 0.0029; Table 6.5). The mean body weight of hamsters in this study was 136.0±13.0 g, and the mean body weights of hamsters in the three hamster diet groups were not significantly different (F = 0.40, df = 2, P = 0.6846; Table 6.5).

Hamster diet containing 988 mg/kg novaluron constituted 14.8±1.9% of the total daily food intake of hamsters in the partial feeding group (Table 6.5). The mean daily dose of novaluron for hamsters in the different diet groups are reported in Table 6.5.

Table 6.5 Means (±SE) of Body Weight, Food Intake, and Daily Doses of Novaluron for Syrian Hamsters Offered Food Containing Novaluron as All, Part, or None of Their Diet

Concentration (mg/kg)	Body weight g (mean* ± SE)#	Food intake g/d (mean* ± SE)#	Portion of diet§ % (mean* ± SE)	Daily dose mg/kg (mean* ± SE)
0	141.3±20.5[a]	7.4±0.8[a]	0	0
988 (all of diet)	131.0±8.9[a]	7.6±0.7[a]	100	57.4±6.0
988 (part of diet)	135.7±9.9[a]	6.9±0.9[b]	14.8±1.9	7.3±0.4

* Nine replicates, three hamsters per replicate

Values in a column followed by the same letter are not significantly different from each other, P>0.05

§ Portion of diet that was novaluron-treated food

Larvae in each of the larval diet groups were observed feeding, and frass was found in each bioassay vial. The mean percent survival from 2nd instar to adult for the sand flies in the untreated hamster feces larval diet group was 96.7±3.3%. Sand fly larvae that were fed feces from hamsters that had consumed diets containing novaluron died before pupation (Table 6.6). Larvae that had been fed either feces from hamsters that exclusively had been fed a diet containing novaluron or both a diet containing 988 mg/kg novaluron and untreated hamster food were ataxic and ceased feeding, and none pupated. The mean longevity of sand fly larvae fed feces from hamsters that exclusively had been fed a diet containing 988 mg/kg novaluron and feces from hamsters were fed both diets containing novaluron and untreated diets was not significantly different (F = 0.95, df = 1, P = 0.3317; Table 6.6).

Table 6.6 Mortality and Longevity of 2nd Instar Sand Flies Fed Feces of Hamsters Fed Diets Containing Novaluron. Hamsters Were Fed Novaluron-Treated Food as All, Part, or None of Their Daily Diet

Hamster diet group	Mortality % (mean* ± SE)#	Longevity d (mean* ± SE)#
Control	3.3 ± 5.2[a]	n/a
Novaluron-treated diet	100.0[b]	3.6 ± 1.2[a]
Partial consumption	100.0[b]	3.8 ± 1.6[a]

* Six replicates, ten larvae per replicate
# Values within a column followed by the same letter are not significantly different from each other, P> 0.05
n/a not applicable

6.4 Discussion

In experiments 1 and 2, complete control of sand fly larvae was observed when the larvae were fed either hamster feces containing 9.88 mg/kg novaluron or feces of hamsters fed a diet containing $9.88x10^{-1}$ mg/kg novaluron. The concentrations that were 100% effective against sand fly larvae in experiments 1 and 2 were different by an order of magnitude, but diet and fecal concentrations of novaluron cannot be equated. Laboratory studies on the metabolism and

excretion of novaluron in rats have shown that 95.4% of novaluron is eliminated in feces for up to 168 h (FAO 2005). If hamsters excrete novaluron at a rate similar to rats, an increase in the concentration of novaluron in the feces of hamsters over the 9-d period of this study would be expected. In experiment 2, the mean amount of food consumed by a hamster each day (7.2±1.4 g) was higher than the amount of feces voided by a hamster (approximately 2 g). Since the majority of novaluron is eliminated in feces, the concentration of novaluron in the feces of a hamster should be higher than the concentration of novaluron in a hamster's diet. The purpose of these experiments was to measure the effect of the treatments on the survival of sand flies fed feces of novaluron-treated hamsters, but the rate at which novaluron is eliminated from hamsters could be an important variable to measure in future studies.

When feces of novaluron-treated hamsters were held under simulated field conditions (28 °C, 90% RH) for up to 30 d, all larvae that consumed these feces died before pupation. A significant reduction in treated larval survival relative to control was observed when the feces were aged for up to 150 d. Based on the findings of experiment 1, we can conclude that feces of hamsters fed a diet containing 988 mg/kg that had been aged for 30 d contained at least 9.88 mg/kg novaluron (a concentration that caused complete larval mortality).

Studies on the fate of novaluron in soil have shown that between 32 and 49% of the compound can be present in soil after 127-195 d (FAO 2005). Therefore, baits containing 316 mg/kg novaluron (32% of 988 mg/kg novaluron) could be expected to be 100% effective as a rodent feed-through for larval sand fly control. However, novaluron may have been degraded at a faster rate in hamster feces than in soil due to the rapid proliferation of fungi. While we approximated the temperature and humidity of a rodent burrow in experiment 3, in an actual field setting novaluron in feces could degrade at an even faster rate than we observed in this study.

Therefore, the rate at which novaluron in feces is degraded under different simulated field conditions could be another important topic for future studies.

Novaluron-treated diet made up approximately 15% of the food consumed daily by hamsters in experiment 4, and the feces of these hamsters was equally as effective against sand fly larvae as feces of hamsters exclusively fed a novaluron-treated diet. The results of experiment 4 suggest that when novaluron is eliminated by orally dosed hamsters, it is uniformly distributed in the feces. This is an important observation because artificial baits for wildlife do not fully supplant naturally available food sources. The results of experiment 4 indicate that novaluron would be effective under circumstances where baits make up only a small portion of the diet of the target rodents in a field setting.

While target rodents in field trials of feed-through control measures for sand fly larvae cannot be expected to exclusively consume novaluron-treated baits, a key component of potential field work would be to identify baits that are readily consumed by target rodents in different foci. The amount of food consumed by study hamsters was not affected by novaluron treatments at any of the concentrations tested in experiments 2, and 3 (significant differences were only observed in experiment 4 when hamsters were fed novaluron-treated food as a portion of their diet, and this observation may have been a result of the frequent changing of food which disrupted the hamsters' feeding). This observation is consistent with findings of Mascari et al (2007b), in which the authors reported that the quantity of food consumed by hamsters was not affected by concentrations of novaluron as high as 988 mg/kg. It is not known whether the food intake of the different rodents that could be targeted in field trails would be similarly unaffected by novaluron treatments. However, the grain-based baits that would be prepared for field trials could contain a palatability agent, such as a vegetable oil. Palatability agents are commonly used

in conjunction with rodenticides in order to increase bait uptake by target animals and may mask any potential odor or taste of novaluron.

The important characteristics of an insecticide used in a rodent bait for control of sand fly larvae would be A) that it is excreted in feces of bait-fed rodents rather than metabolized, B) that it persists in the environment, and C) that it is effective in preventing the development and survival of sand fly larvae when the bait makes up only a portion of a target rodent's diet. In this study, significant control of sand fly larvae was observed when they were fed feces of novaluron-treated hamsters that had been aged for up to 150 d or feces of novaluron-treated hamsters when only 15% of their daily diet was novaluron-treated food. Therefore, novaluron is a good candidate for further evaluation as a rodent feed-through insecticide against sand fly larvae. Since the results of this study suggest that novaluron could be effective as a rodent feed-through insecticide in a field setting, the next step would be to evaluate the effects of novaluron-treated baits on sand fly populations in different rodent/sand fly associations.

CHAPTER 7. EFFECT OF ORAL IVERMECTIN TREATMENT OF RODENTS ON SURVIVAL OF SAND FLY (DIPTERA: PSYCHODIDAE) LARVAE FED ON THE RODENT FECES AND FEMALE SAND FLIES FED ON THE RODENTS

7.1 Introduction

Sand flies are hematophagous Diptera of the subfamily Phlebotominae, and, with the exception of New Zealand and some Pacific islands, are found in most parts of both the New World and Old World between 50 °N and 40 °S. Sand flies are often significant biting pests of man; their bites can cause acute dermatitis and delayed-type hypersensitivity reactions. Sand flies also are vectors of medically important viruses, bacteria, and protozoa. The protozoa of at least 20 *Leishmania* spp. are transmitted by sand flies and are the causative agents of human leishmaniasis (WHO 2008). Leishmaniasis is a zoonotic disease with a broad clinical spectrum that is estimated to affect as many as 12 million people, and 2 million new cases of leishmaniasis are believed to occur annually (WHO 2006).

In the Old World, *Leishmania major* is the causative agent of zoonotic cutaneous leishmaniasis (ZCL), which has an enzootic transmission cycle among populations of locally abundant, burrow-dwelling rodents. Man becomes infected with *L. major* by the bite of infectious sand flies (*Phlebotomus papatasi* in Southwestern Asia, the Middle East, and North Africa; *Phlebotomus duboscqi* in Sub-Saharan Africa) in settlements located near areas of intense transmission or as a result of movement into enzootic areas, such as during military operations or during suburban expansion (Faulde et al. 2008**;** Traore et al. 2001).

No effective preventive or control measures are currently available for Old World ZCL. Personal protective measures (including the use of repellents, bednets, and insecticide treated materials) provide inconsistent and incomplete protection against sand fly bites and infection with *L. major* (Jumaian 1998). Despite the close association of rodent burrows and vector

species, direct treatment of burrows with insecticides has not been shown to have a significant effect on sand fly populations (Karapati'an et al. 1983).

Proof of concept for ivermectin as a rodent feed-through insecticide for control of larval sand flies has been established; the feces of hamsters fed a a diet containing 20 mg/kg ivermectin were shown to be 100% effective against sand fly larvae (Mascari et al. 2008). While the majority of the drug is rapidly eliminated in the feces, ivermectin reaches detectable levels in the blood of orally dosed mammals (Pound et al. 2004; Campbell et al. 1983). Several field studies have demonstrated significant reductions in survival of mosquitoes that obtained bloodmeals from ivermectin-treated hosts (Tesh 1990; Cartel 1991; Foley 2000, Bockarie 1999). The toxicity of ivermectin has been demonstrated for phlebotomine sand flies fed ivermectin-treated blood through an artificial membrane (Kassem et al. 2001). Therefore, targeting rodents with an ivermectin feed-through also could affect post-bloodmeal survival of sand flies.

The primary objective of this research was to determine whether the post-bloodmeal survival of adult sand flies would be affected by feeding their rodent hosts a diet containing ivermectin. In this study the insecticidal effect of ivermectin treatments against bloodfed sand flies was monitored for 14 d after rodents were withdrawn from their ivermectin-treated diets, and sand fly larval bioassays were conducted using feces voided by ivermectin-treated rodents over this same time period.

7.2 Materials and Methods

7.2.1 Sand Flies

The sand flies used in these experiments were from a laboratory colony of a Turkish strain of *P. papatasi* established at Louisiana State University (Mascari et al. 2007b). The sand fly larvae in the colony were reared using a larval diet made of a composted and dried 1:1 mixture of rabbit feces and rabbit chow (Young et al. 1981). Adult sand flies were provided with

20% sucrose solution *ad libitum*, and they obtained bloodmeals from Syrian hamsters. The colony was maintained in environmental chambers at 28 °C, 90% RH.

7.2.2 Hamsters

Syrian hamsters were housed individually in micro-isolator cages. The maintenance of the hamsters and all experimental procedures followed Animal Care & Use Protocol No. 05-074, which was approved by the Institutional Animal Care and Use Committee at Louisiana State University, Baton Rouge, LA. Research involving the hamsters 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.

7.2.3 Hamster Treatments

Ivermectin (Merck & Co., Inc., Whitehouse Station, NJ) was added to a meal-form laboratory rodent diet (5001 Rodent Diet, LabDiet®, PMI Nutrition International, Brentwood, MO). A diet containing 20 mg/kg ivermectin was prepared, and a control diet (untreated meal-form rodent diet) also was prepared.

Three hamsters were assigned randomly to each of the two diet groups (ivermectin-treated diet or control diet). At 18:00 h each day for nine days, the hamsters were provided with 25 g of their respective diets. The uneaten portion of the food was collected the following day at 18:00 h, and the daily food intake for each hamster was calculated. The daily doses of ivermectin that were ingested by the hamsters also were calculated. Feces voided by each hamster were collected daily for 9 d during feeding. Feces were air-dried at room temperature for 7 d, and then stored at -80 °C until used in larval bioassays. After being fed their respective diets for 9 d, all hamsters were withdrawn from their meal-form diets and provided with an untreated pellet diet

(5001 Rodent Diet, LabDiet®, PMI Nutrition International, Brentwood, MO). Feces then were collected after 3, 7, and 14 d.

7.2.4 Adult Bloodfeeding Assays

Adult sand flies were allowed to feed on hamsters 0, 3, 7, and 14 d after they were withdrawn from untreated or ivermectin-treated meal-form diet. Bloodfeeding bioassays were conducted in clear polycarbonate boxes with a cloth sleeve to allow hamsters and sand flies to be introduced into the container. Thirty 2 to 4-d old nulliparous female sand flies were transferred using a mouth aspirator into each bioassay container.

Hamsters were chemically immobilized with an anesthetic mixture of ketamine HCl (100 mg/kg body weight) and xylazine HCl (10 mg/kg body weight) administered via intra-peritoneal (IP) injection. The depth of anesthesia was considered sufficient when hamsters were no longer ambulatory, and had lost their righting reflex (defined as the ability of hamsters to regain sternal recumbency) and superficial pain response (determined by pinching the skin over the anterior surface of the feet). Once immobilized, ophthalmic ointment was placed in the hamsters' eyes to protect them against potential sand fly bites.

A single immobilized hamster was placed in each bioassay container. The sand flies were allowed to feed for 50 min before the hamsters were removed from the containers (Fig. 7.1). When the hamsters were recovered from anesthesia, they were returned to their micro-isolator cages. The number of engorged sand flies was recorded immediately, and they were provided with a piece of filter paper saturated with 20% sucrose solution. The sand flies were kept in the bioassay container for 24 h to allow them to form a peritrophic membrane around the bloodmeal; past experience has shown that moving sand flies by mouth aspirator before the peritrophic membrane is formed may increase mortality rates. After 24 h the mortality was recorded, and live sand flies were transferred using a mouth aspirator to a 150 mL glass jar with a plaster of

Paris base and a fine mesh lid. The sand flies were provided with 20% sucrose solution on a cotton pad. Mortality (defined as lack of movement during 30 s observation) was recorded again after 24 h (48 h post-bloodmeal).

Figure 7.1 Sand Flies Taking a Bloodmeal from a Chemically Immobilized Ivermectin-Treated Syrian Hamster

Differences in mean survivorship of sand flies that had taken bloodmeals from ivermectin-treated or control hamsters that had been withdrawn from their meal-form diets for different time periods were compared after 24 and 48 h using repeated measures ANOVA (SAS Institute 2001). Treatment means were separated by Tukey's honestly significant difference (HSD) multiple comparison test.

The viability (successful hatching) of eggs deposited by bloodfed sand flies on the plaster surface of the jars also was recorded. Sand flies were allowed to lay eggs until 12 d post-bloodmeal (sand flies from this colony typically lay eggs 5 d post-bloodmeal, and eggs begin to

hatch 7 d after being deposited). Eggs were observed for signs of hatching up to 14 d after the first eggs were deposited. After the eggs hatched, larvae were counted and removed from the jars to allow an accurate count of newly hatched eggs each day. The mean number of eggs per jar and the mean percent of eggs that hatched were calculated and compared using Student's t-test (SAS Institute 2001).

7.2.5 Larval Feed-Through Bioassays

Feces voided by hamsters 0, 3, 7, and 14 d after being withdrawn from an untreated or ivermectin-treated meal-form diet were collected. Feces of the three hamsters in each diet group were pooled and then crushed using a sterilized glass mortar and pestle.

Larval bioassays were conducted as described by Mascari et al. (2007a). A 200 mg sample of crushed hamster feces was transferred to the plaster surface of each bioassay vial. Ten 2^{nd} instar (13±1-d old) larvae were transferred to each bioassay vial (six vials per treatment) and held in an environmental chamber at 28 °C, 90% RH, 14:10 (L:D) photoperiod. Larvae were fed feces of control or ivermectin-treated hamsters collected at each of the time periods (0, 3, 7, and 14 d after being returned to untreated, pellet diet).

The larvae were observed under magnification daily. Larval mortality (defined as the lack of response to prodding with a blunt probe after 15 s) was recorded, and the larvae were observed for abnormal behavioral and morphological characteristics. Evidence of feeding (the presence of frass in the vials and dark material in the guts of larvae) also was monitored.

The percent survival of immature sand flies to adult emergence after being fed their respective diets was compared using repeated measures ANOVA performed with the GLM procedure (SAS Institute 2001). The Tukey multiple comparison procedure was used to separate significantly different means.

7.3 Results

7.3.1 Hamster Treatments

The mean body weight of the six hamsters in this study was 137.3±18.6 g, and the body weights of hamsters assigned to different diet groups were not significantly different (F = 0.2, df = 1, P = 0.6816). The mean daily food intake of the hamsters was 7.4±1.4 g. The amount of food consumed by ivermectin-treated hamsters was significantly greater than for control hamsters (F = 5.31, df = 1, P = 0.0253; Table 7.1).

Table 7.1 Means (±SE) of Body Weight, Food Intake, and Daily Dosages of Ivermectin for Syrian Hamsters

Hamster diet group	Body weight g (mean ± SE) [#]	Food intake g/d (mean[*] ± SE) [#]	Daily dosage mg/kg (mean[*] ± SE)
Control	141.0±21.7[a]	7.0±1.4[a]	0
Ivermectin (20 mg/kg)	133.7±18.9[a]	7.9±1.2[b]	1.0±0.3

* Nine replicates, three hamsters per replicate

[#] Values in a column followed by the same letter are not significantly different from each other, P>0.05

7.3.2 Adult Bloodfeeding Assays

All sand flies that were exposed to a hamster for 50 min successfully took a bloodmeal. All sand flies survived for at least 48 h after taking bloodmeals from hamsters that had been withdrawn from an untreated meal-form diet for 0, 3, 7, or 14 d (Table 7.2; Table 7.3). The 24 h post-bloodmeal mortality of sand flies that had taken bloodmeals from hamsters withdrawn from an ivermectin-treated diet for 0, 3, or 7 d was greater than 50% (Table 7.2). The 48 h survival was 0% for sand flies that took a bloodmeal from hamsters withdrawn from an ivermectin-treated diet for 0, 3, or 7 d (Table 7.3). The 24 and 48 h post-bloodmeal survival was 100% for sand flies fed on hamsters withdrawn from an ivermectin-treated diet for 14 d.

Table 7.2 Post-Bloodmeal (24 h) Survival of Sand Flies Fed on Ivermectin-Treated Hamsters

Hamster diet	Survival (at 24 h) % (mean* ± SE) [#]			
	0 DAT[§]	3 DAT[§]	7 DAT[§]	14 DAT[§]
Control				
0 ppm	100.0±0.0[a]	100.0±0.0[a]	100.0±0.0[a]	100.0±0.0[a]
Ivermectin				
20 ppm	42.2±8.3[b]	13.3±14.5[b]	46.7± 18.6[b]	100.0±0.0[a]

* 3 hamsters, 30 sand flies per hamster

[#] Values in a column followed by the same letter are not significantly different from each other, P>0.05

§ Days after hamsters were withdrawn from an untreated or ivermectin-treated meal-form diet

Table 7.3 Post-Bloodmeal (48 h) Survival of Sand Flies Fed on Ivermectin-Treated Hamsters

Hamster diet	Survival (at 48 h) % (mean* ± SE) [#]			
	0 DAT[§]	3 DAT[§]	7 DAT[§]	14 DAT[§]
Control				
0 ppm	100.0±0.0[a]	100.0±0.0[a]	100.0±0.0[a]	100.0±0.0[a]
Ivermectin				
20 ppm	0.0±0.0[b]	0.0±0.0[b]	0.0±0.0[b]	100.0±0.0[a]

* 3 hamsters, 30 sand flies per hamster

[#] Values in a column followed by the same letter are not significantly different from each other, P>0.05

§ Days after hamsters were withdrawn from an untreated or ivermectin-treated meal-form diet

The mean number of eggs deposited by sand flies that took bloodmeals from untreated or ivermectin-treated hamsters 14 d after withdrawal from their respective meal-form diets was not significantly different (t = 0.17, df = 4, P = 0.8725; Table 7.4). Additionally, the mean percentage of eggs that hatched after being deposited by sand flies that took bloodmeals from untreated or ivermectin-treated hamsters 14 d after withdrawal from their respective meal-form diets also was not significantly different (t = 0.28, df = 4, P = 0.7913; Table 7.4).

Table 7.4 Mean Number and Viability of Eggs Deposited by Sand Flies That Had Taken Bloodmeals from Hamsters 14 d after Being Withdrawn from an Untreated or Ivermectin-Treated Diets

Hamster diet	No. eggs		Percent hatch	
	mean* ± SE[#]	Range	mean* ± SE[#]	range
Control				
0 mg/kg	831.3±142.0[a]	689-974	71.9±6.1[a]	64.9-76.0
Ivermectin				
20 mg/kg	808.3±184.6[a]	690-1021	74.4±13.4[a]	59.5-85.6

* 3 repetitions, 30 bloodfed sand flies per repetition

[#] Values in a column followed by the same letter are not significantly different from each other, P>0.05

7.3.3 Larval Feed-Through Bioassays

Feces voided by hamsters on the same day they were withdrawn from an ivermectin-treated diet (0 d) were fed to sand fly larvae and yielded 100% larval mortality. The percent survival

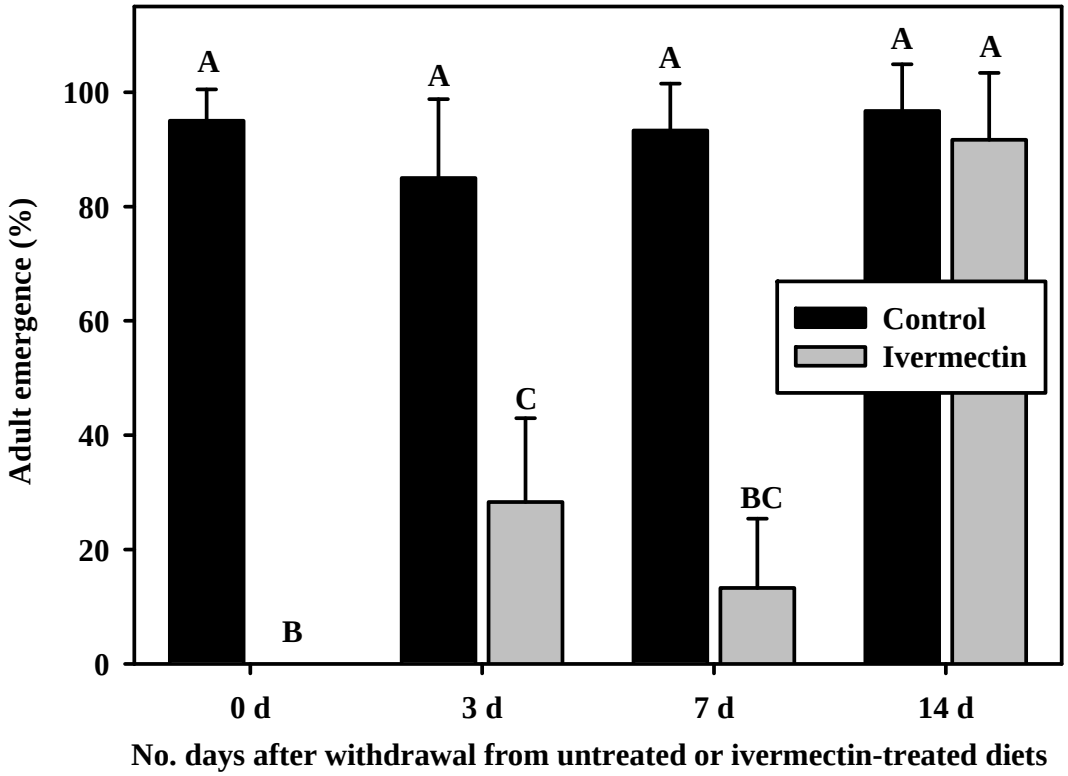

Figure 7.2 Percent Adult Emergence (Mean ± SE) of Sand Flies Fed as 2nd Instars the Feces of Untreated or Ivermectin-Treated Hamsters; Feces Used in This Bioassay Were Voided by Hamsters 0, 3, 7, or 14 d after the Hamsters Were Withdrawn from Their Respective Diets

of sand fly larvae fed feces of hamsters withdrawn from an ivermectin-treated diet for 3 and 7 d (28.3±14.7% and 13.3±12.1%, respectively) was significantly different from the survival of larvae fed feces of untreated hamsters collected at the same time periods (85.0±13.8% and 93.3±8.2%, respectively; $F = 96.60$, df = 7, $P < 0.0001$). The mean percent survival was not significantly different between larvae fed feces voided by hamsters 14 d after they were withdrawn from an ivermectin-treated or untreated diet (91.7±11.7% and 96.7±8.2%, respectively).

7.4 Discussion

Ivermectin treatment of hamsters was 100% effective against bloodfeeding sand flies for up to 7 d after hamsters were withdrawn from ivermectin-treated diets. This is consistent with other studies showing the effects of ivermectin against a broad range of nematodes and arthropods. In humans, ivermectin is commonly used in mass drug administrations (a single dose of 0.2 mg/kg body weight) to clear microfilaria of *Wuchereria bancrofti* and *Onchocerca volvulus*. During these mass drug administrations, ivermectin also has been shown to remain active against mosquitoes feeding on treated humans for long periods of time after treatment. Post-bloodmeal survival of *Anopheles farauti* mosquitoes fed on ivermectin-treated humans was significantly lower than control for up to 44 d post-treatment (Foley et al. 2000). Remarkably, a significant reduction in post-bloodmeal survival of *Aedes polynesiensis* mosquitoes fed on ivermectin-treated humans was reported for up to 6 months post-treatment (Cartel et al. 2001). The survival of ticks also has been shown to be affected by treating hosts with ivermectin. Significant mortality was observed for the ticks *Ornithodoros moubata* and *Boophilus microplus* that had fed on ivermectin-treated cattle, and the effect persisted for up to 21 d post-treatment (Centurier and Barth 1980, Nolan et al. 1981). However, the post-treatment duration of the insecticidal effects of ivermectin on bloodfeeding sand flies was less than 14 d. The hamsters in

this study received a mean daily dose of 1.0±0.3 mg/kg ivermectin for 9 consecutive days, which is substantially higher than the 0.2 mg/kg administered once orally or by injection to humans and other animals. The shorter duration of effect against sand flies suggests that ivermectin may be rapidly eliminated by hamsters or that sand flies are less susceptible to the serum concentrations of ivermectin that may be present in hosts more than 1 wk after treatment (Chiu and Lu 1989).

The results of this study also can be used to direct the frequency with which ivermectin-treated baits should be delivered in a field setting. Since the effects of oral treatment of rodents on sand fly adults and larvae persisted for at least 1 wk, treatment of target rodents with ivermectin (or access of target rodents to ivermectin-treated baits) does not need to be continuous, but should not be withdrawn for more than 1 wk.

Fecundity was not significantly different for sand flies that took a bloodmeal from an untreated or ivermectin-treated hamster 14 d post-treatment. A previous study reported that sand flies that had ingested a sub-lethal dose of ivermectin in a bloodmeal produced around 2/3 the number of eggs as control sand flies (Kassem et al. 2001). It is possible that the results of this study did not detect a similar effect of ivermectin on sand fly fecundity because a sub-lethal serum concentration of ivermectin may have occurred in hamsters between 7 and 14 d post-treatment. The percent hatch of eggs of sand flies that ingested sub-lethal doses of ivermectin was not lower than control sand flies in the study conducted by Kassem et al. (2001), nor was it lower for sand flies that took a bloodmeal from an ivermectin-treated hamster in this study.

Feces of ivermectin-treated hamsters were 100% effective against sand fly larvae when collected immediately after hamsters were withdrawn from ivermectin-treated diets (0 d), which is consistent with the results of Mascari et al. (2008). The results of this study also are consistent with the elimination profile of ivermectin in other rodents. In rats, only about 2% of ivermectin is eliminated via urine of orally dosed rats; the remainder is eliminated in feces (Chiu and Lu

1989). The rate at which ivermectin is eliminated in feces also has been described using a rat model; 57.4 to 58.4% of the administered drug is eliminated 1 d after administration. These figures increase to between 83.0% and 91.7% five days after administration (WHO Food Additives Series 27: 696. Ivermectin). Despite the rapid elimination of ivermectin in feces, the high dose of ivermectin administered to the hamsters in this study led to the significant effects against sand fly larvae that were observed up to 7 d after hamsters were withdrawn from an ivermectin-treated diet.

This study confirms that ivermectin-treated rodent baits developed as a feed-through to control sand fly larvae also could have a collateral effect on bloodfeeding adult sand flies, thus affecting a larger portion of the sand fly population than would be estimated due to larvicide activity alone. While ivermectin treatment of rodent hosts of *L. major* may reduce the overall population of sand flies, this approach would have its greatest impact on the most epidemiologically important subset of the sand fly population: females that have taken a bloodmeal from potentially infected rodents. In this study sand flies that have taken a bloodmeal from an ivermectin-treated hamster died within 2 d. The extrinsic incubation period of *L. major* in *P. papatasi* is approximately 6 d, and female *P. papatasi* sand flies take bloodmeals every 5 to 7 d. Therefore, targeting rodents in ZCL foci with ivermectin-treated baits could, in effect, turn *L. major*-infected hosts into dead-end hosts in the transmission cycle by killing sand flies before the extrinsic incubation period for *L. major* can be completed. Furthermore, since female sand flies that take bloodmeals from ivermectin-treated rodents would not feed again, the ivermectin treatments would eliminate any infectious sand flies, serving as a dead-end in transmission of *L. major* parasites for 5 to 7 d.

Sand fly species that feed on the feces of rodents as larvae could be controlled using feed-through insecticides, and for those species that do not feed on rodent feces, treating rodent hosts

with a systemic insecticide could be an effective control measure. Whereas domesticated animals that serve as reservoirs for *Leishmania infantum*, such as dogs, can be topically treated with insecticides or fitted with insecticide treated collars to prevent sand fly bites and transmission to humans, this approach is not practical for wild mammals (Halbig et al. 2000, Mencke et al. 2003). In fact, with the exception of foci of visceral leishmaniasis in which dogs serve as the primary reservoir, there are no effective control measures for sand flies. In these situations, reservoirs of *Leishmania* spp. could be targeted with baits containing a systemic insecticide to control sand flies and potentially reduce the incidence of human infection.

Additional studies (such as on the persistence of ivermectin in feces over time under simulated field conditions and the effectiveness of ivermectin-treated baits when they make up only a portion of a target rodent's daily diet) will be required before conducting a field evaluation of ivermectin-treated rodent baits to control sand flies. Results of these studies would determine whether ivermectin would be effective as a stand-alone feed-through and systemic insecticide, or whether it would be more suitable as a systemic insecticide used in conjunction with a potentially more effective feed-through insecticide. Additional insecticides also should be evaluated for potential use as systemic or dual systemic/feed-through agents against phlebotomine sand flies.

CHAPTER 8. EVALUATION OF RHODAMINE B AS AN ORALLY DELIVERED BIOMARKER FOR RODENTS AND A FEED-THROUGH TRANS-STADIAL BIOMARKER FOR PHLEBOTOMINE SAND FLIES (DIPTERA: PSYCHODIDAE)

8.1 Introduction

Phlebotomine sand flies are major biting pests of man and are the vectors of the protozoan parasites that cause leishmaniasis. Worldwide, there are an estimated 2 million new cases of leishmaniasis annually, and 12 million people are currently believed to be infected (WHO 2006). Throughout North Africa, the Middle East and SW Asia, *Phlebotomus papatasi* is the primary vector of *Leishmania major*, the causative agent of zoonotic cutaneous leishmaniasis (ZCL).

In arid and semi-arid foci, *P. papatasi* exhibits a close association with several burrowing rodent reservoirs of *L. major*. Sand fly larvae are found in habitats that provide darkness, a moist substratum, organic matter for food, and protection from unfavorable weather conditions (for example temperature and precipitation). In ZCL foci in the Old World, rodent burrows are considered to be the primary immature habitats for *P. papatasi*.

While larvicides are commonly used to control mosquitoes and many other flies of medical and veterinary importance, there is no current use of larvicides for phlebotomine sand fly control. There are hundreds of species of phlebotomine sand fly found in a diverse range of habitats around the world, and the larval habitats remain entirely unknown for many species. Identifying and locating the larval habitats of sand flies is challenging due to the difficulty of sampling for sand fly larvae, which can involve the processing of large quantities of soil with little or no result (Feliciangeli 2004). For the few species for which the larval habitats are known to be within rodent burrows, delivering insecticides to the precise larval habitats can be difficult to achieve, and attempts have largely been unsuccessful (Karapati'an et al. 1983). Nevertheless,

some insecticides (*Bacillus thuringiensis israelensis* and *Bacillus sphaericus*) have been shown to be effective against immature sand flies in the laboratory (Wahba 2000, Wahba et al. 1999).

Larvae of several species of phlebotomine sand flies have been recovered from soil taken from rodent burrows including *P. papatasi*, *P. langeroni*, *P. martini*, *P. duboscqi*, and *P. chinensis* (Mutinga et al. 1986, Doha et al. 1990, Artemiev et al. 1972, Morsy et al. 1993, Nicolescu and Bilbie 1980). In Old World ZCL foci, sand fly larvae also have been observed feeding on the feces of rodents (WHO 1968). Because of this fact, rodent feed-through insecticides are a potential means of controlling sand fly larvae, and this approach has recently been evaluated in laboratory studies using chitin synthesis inhibitors (diflubenzuron and novaluron), juvenile hormone analogs (methoprene and pyriproxyfen), and ivermectin (Mascari et al. 2007a, Mascari et al. 2007b, Mascari et al. 2008). The results of these studies constituted proof of concept for feed-through rodent baits for the control of sand fly larvae.

Prior to or simultaneous with field evaluations of feed-through control of sand flies in the different sand fly/rodent associations that exist, establishing whether the larvae of different species of sand fly feed on the feces of rodents must be demonstrated. While sand fly larvae have been recovered from rodent burrows and have been observed feeding on the feces of rodents, larval sampling is an impractical method to demonstrate the larval diet of sand flies. However, there are currently no alternative methods available to directly demonstrate if the larval diet of phlebotomine sand flies is largely or exclusively rodent feces.

The primary objective of this study was to develop a method to identify adult sand flies that had fed as larvae on the feces of bait-fed rodents. Numerous techniques exist for marking insects including tagging, painting, mutilation, dusting with fluorescent powder, dyeing, marking with radio-isotopes or trace elements. Of these, the use of dyes, radio-isotopes, and trace-elements are most appropriate for use in mark-capture studies (as opposed to mark-release-

recapture studies) because the insects can be self-marked in nature by contact with or ingestion of the markers. The use of a dye was selected for this study because of cost, safety, and the ability to detect small quantities of certain dyes at low concentrations using a fluorescence microscope or a spectrofluorometer. Rhodamine B (a xanthene dye with fluorescent properties) was chosen as a potential feed-through dye because of its reported low mammalian toxicity, because it is eliminated in the feces of orally dosed mammals, and because rhodamine B is an efficient fluorophore (has a high quantum yield) allowing its detection at low concentrations. Currently, rhodamine B is used as a biomarker to determine the diet of the nematodes *Trichostrongylus colubriformis* and *Nippostrongylus braziliensis* that parasitize the intestines of mammals (Bansemir and Sukhdeo 2001, Bottjer and Bone 1984). Rhodamine B also was shown to function as a trans-stadial marker for sawflies; larvae were fed a diet containing rhodamine B and the dye was detected in larvae, pupae, and adults (Heron 1968). The primary objective of this research was to develop a fluorescent tracer technique using rhodamine B as a rodent feed-through to identify adult sand flies that had fed on the feces of rhodamine-B treated hamsters. We also made observations on rhodamine B marking of bait-fed rodents and the female flies that fed upon them.

8.2 Materials and Methods

8.2.1 Sand Flies

A laboratory colony of a Turkish strain of *P. papatasi* was established at Louisiana State University (Mascari et al. 2007). The sand flies in the colony were reared using a larval diet consisting of a dried and decomposed 1:1 mixture of rabbit feces and rabbit chow (Young et al. 1981). The colony was maintained in environmental chambers at 28 °C, 90% RH, 14:10 (L:D) photoperiod.

8.2.2 Syrian Hamsters

Twelve Syrian hamsters were housed individually in micro-isolator cages. The maintenance of the hamsters and the experimental procedures of this research followed Animal Care & Use Protocol No. 05-074 which was approved by the Institutional Animal Care and Use Committee at Louisiana State University, Baton Rouge, LA. Research involving the hamsters 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.

8.2.3 Feed-Through

Rhodamine B (Sigma-Aldrich, St. Louis, MO) was added to a meal-form laboratory rodent diet (5001 Rodent Diet, LabDiet®, PMI Nutrition International, Brentwood, MO) containing soybean oil (100 g/kg) as a palatability and sticking agent. Three concentrations of rhodamine B in rodent diet were prepared (50, 500, and 5,000 mg/kg); a control rodent diet also was prepared (a powdered rodent diet containing 100 g/kg soybean oil).

Three hamsters were assigned randomly to each of the four diet groups (three diets containing rhodamine B, and one control diet). At 12:00 h each day for nine days, the hamsters were provided with 25 g of their respective diets. The uneaten portion of the food was collected the following day at 12:00 h, and the daily food intake for each hamster was calculated. The daily doses of rhodamine B also were calculated for each hamster (in mg/kg body weight). Feces voided by each hamster were collected daily for 9 d during feeding. Feces were air-dried in darkness at room temperature for 7 d, and then stored at -80 °C until being examined for the presence of rhodamine B or used in larval bioassays.

After being fed their respective diets for 9 d, the hamsters were returned to an untreated pellet diet (5001 Rodent Diet, LabDiet®, PMI Nutrition International, Brentwood, MO). The

hamsters were examined weekly for 8 wk under white light using the overhead lighting of the vivarium, and the presence and location of markings (pink color) on the hamsters was recorded.

8.2.4 Bioassay

Feces voided after nine days of feeding in the rhodamine B feed-through experiment were collected, pooled by hamster diet group, and crushed using a glass mortar and pestle. Four larval diets were used in sand fly larval bioassays: feces of hamsters fed 0, 50, 500, or 5,000 mg/kg rhodamine B. The larval bioassays were conducted according to the methods described by Mascari et al. (2007a). Portions of the larval diets (0.2 g) were transferred to the plaster surfaces of each bioassay vial. Ten 2nd instar larvae (13±1-d old) were transferred to each bioassay vial and were held in darkness in an environmental chamber at 28 °C, 90% RH. Six bioassay vials were used for each of the four larval diet groups.

The larvae were observed under magnification daily in subdued lighting, and larval mortality (defined as the lack of response to prodding with a blunt probe after 15 s) was recorded. Evidence of feeding, the presence of frass in the vials, dark material in the guts of larvae, and the visual presence of rhodamine B in larvae also was monitored. Sand flies were killed by freezing within 1 d of emergence and were stored in darkness at -80 °C. The percent adult emergence of sand flies in each larval diet group was compared using repeated measures ANOVA performed with the GLM procedure (SAS Institute 2001). The Tukey multiple comparison procedure was used to separate significantly different means.

8.2.5 Blood-Feeding

Hamsters were fed diets containing 0 and 5,000 mg/kg rhodamine B for 9 d. On the ninth day, the hamsters were chemically immobilized with an anesthetic mixture of ketamine HCl (100 mg/kg body weight) plus xylazine HCl (10 mg/kg body weight) administered via IP injection. When the hamsters were immobilized, they were placed individually in clear, polycarbonate

cages (30.5 x 30.5 x 30.5 cm) containing 15 adult female sand flies (2 to 4-d old). The sand flies were allowed to feed on the hamsters for 30 min before the hamsters were removed.

Immediately after feeding, five sand flies from each group were transferred to a 150 mL jar lined with plaster of Paris and were provided with 20% sucrose solution *ad libitum*; the remaining blood-fed sand flies were killed by freezing, transferred to 2 mL cryogenic vials, and stored at -80 °C. After 5 d (after the sand flies had fully digested their bloodmeals), the sand flies were transferred to 120 mL jars with a moistened plaster of Paris base, and the sand flies were allowed to lay eggs. The oviposition jars were stored at 28 °C, 90% RH. After 3 d (8 d post-bloodmeal), the adult sand flies were killed by freezing, transferred to 2 mL cryogenic vials, and stored at -80 °C. Eggs were monitored daily, and upon hatching the larvae were fed a larval diet *ad libitum* (rabbit feces-rabbit chow). As adult sand flies emerged, they were killed by freezing, transferred to 2 mL cryogenic vials, and stored at -80 °C.

8.2.6 Fluorescence Microscopy

All adult sand flies that had been killed by freezing in the larval and bloodfeeding bioassays were examined using fluorescence microscopy; three randomly selected fecal pellets voided by each hamster also were examined using fluorescence microscopy. Individual specimens (adult sand flies or hamster feces) were placed in the well of a glass concavity slide and covered with a glass cover-slip to prevent air currents in the lab from moving specimens during observation. The slides were placed on the stage of a fluorescence stereomicroscope (Zeiss SteREO Lumar.V12, Zeiss, Göttingen, Germany) and observed using incandescent illumination. Digital images were captured using Zeiss AxioVision (version 4.6) using a 200 ms exposure time. The specimens then were observed using fluorescence microscopy using a rhodamine filter cube (excitation wavelength 540 nm, emission wavelength 625 nm). Three exposure times (1, 4, and 15 s) were used for capturing images during fluorescence microscopy

to identify the optimal exposure time for distinguishing fluorescence due to rhodamine B in specimens experimentally exposed to the dye from potential auto-fluorescence in control specimens. Specimens were considered positive for the presence of rhodamine B using fluorescence microscopy if they appeared red and could be distinguished readily from the black background.

8.3 Results

8.3.1 Feed-Through

The mean body weight of the hamsters was 133.8±5.3 g, and the mean body weights of hamsters in the different hamster diet groups were not significantly different (F = 0.83, df = 3, P = 0.4795; Table 8.1). The mean daily food intake of the hamsters was 10.8±1.2 and there was no significant difference between hamsters in the different diet groups (F = 0.95, df = 3, P = 0.4600; Table 8.1). The mean daily dose of rhodamine B for treated hamsters ranged from 4.0 to 401.8 mg/kg (Table 8.1).

Table 8.1 Means (±SE) of Body Weight, Food Intake, and Daily Dosages of Rhodamine B for Syrian Hamsters Fed Rhodamine B-Treated or Untreated Diets for 9 d

Concentration (mg/kg) of Rhodamine B in diet	Body weight g (mean* ± SE)#	Food intake g/d (mean* ± SE)#	Daily dose mg/kg (mean* ± SE)#
0	132.0±4.0[a]	10.7±1.4[a]	0
50	131.3±5.0[a]	10.6±1.2[a]	4.0±0.5
500	134.0±6.0[a]	10.8±1.0[a]	40.4±3.9
5,000	138.0±6.0[a]	11.1±1.0[a]	401.8±38.3

* Nine replicates, three hamsters per replicate

Values in a column followed by the same letter are not significantly different from each other, P>0.05

Hamsters fed diets containing 50, 500, or 5,000 mg/kg rhodamine B were marked by the dye after feeding on their diets for 1 d. Bright pink coloration was present on the skin of the

hamsters' footpads, muzzle, tail, and urogenital and anal areas, and also on the pelage (Table 8.2; Fig 8.1). The markings on hamsters fed diets containing 50 or 500 mg/kg rhodamine B faded within 2 wk of being withdrawn from a rhodamine B-treated diet. The markings on hamsters fed diets containing 5,000 mg/kg rhodamine B had not diminished noticeably after observation over 8 wk.

Table 8.2 Duration and Location of Markings after Hamsters Were Withdrawn from Untreated Diets or Diets Containing Rhodamine B

Diet concentration mg/kg	Marked																			
	1 wk					2 wk					3 wk					8 wk				
	D	O	P	U	T	D	O	P	U	T	D	O	P	U	T	D	O	P	U	T
0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
50	+	+	+	+	+	-	+	-	+	+	-	-	-	-	-	-	-	-	-	-
500	+	+	+	+	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-
5,000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

D = dorsal pelage, O = oral mucosa, P = Paws, U = urogenital area, T = tail

Fig. 8.1 A Hamster That Had Been Fed a Diet Containing Rhodamine B. Pink Coloration Was Visible on the Dorsal Pelage (A), Oral Mucosa (B), Paws (C), and Urogenital Area and Tail (D)

Feces of hamsters fed a diet containing rhodamine B were pink, and the feces could be distinguished from feces of control hamsters when they were viewed under incandescent illumination (Fig. 8.2). An increasing intensity of the pink coloration in feces appeared to be associated with increasing doses of rhodamine B. When examined using fluorescence microscopy with a 1 s exposure time, feces of hamsters fed diets containing all three concentrations of rhodamine B (50, 500, 5,000 mg/kg) appeared red, while feces of control hamsters appeared as a black field (Fig. 8.2).

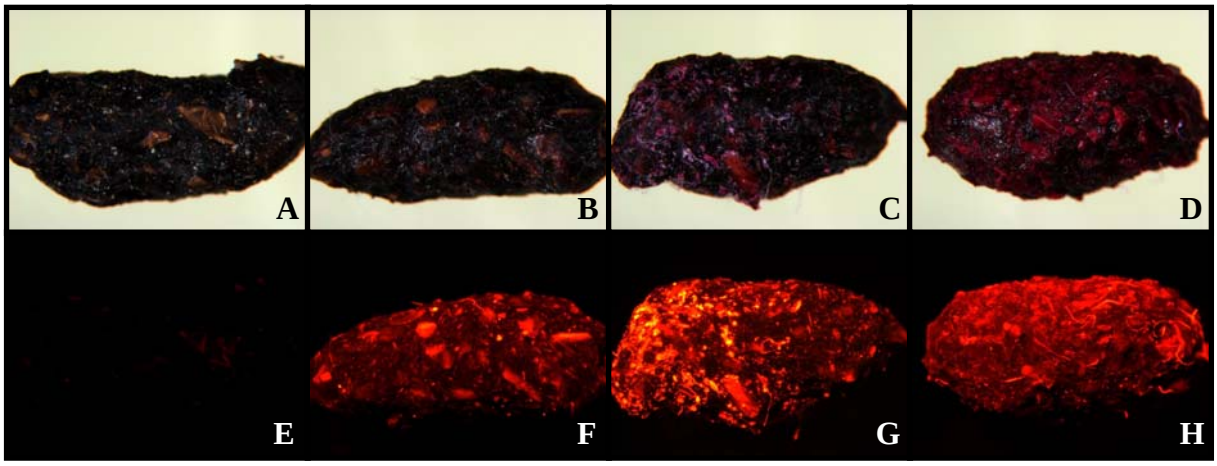

Fig. 8.2 Images of Feces of Four Hamsters Taken under Incandescent Lighting (A, B, C, and D), and Using Fluorescence Microscopy with a 1 sec Exposure Time (E, F, G, and H). The Feces Pictured Are from a Hamster Fed a Control Diet (A and E), or a Diet Containing 50 (B and F), 500 (C and G) or 5,000 mg/kg Rhodamine B (D and H)

8.3.2 Bioassay

The mean percent adult emergence in the larval bioassay was 97.5±5.3 d, and there was no significant difference in percent emergence of sand flies in different larval diet groups (F = 0.18, df = 3, P = 0.9118; Table 8.3). The mean age of sand flies at adult emergence was 30.0±1.3 d, and there was no significant difference in age of emergence of sand flies in different larval diet groups (F = 1.35, df = 3, P = 0.2592; Table 8.3).

Table 8.3 Results of the Rhodamine B Feed-Through Larval Bioassay (Percent Adult Emergence, Age at Adult Emergence, and Fluorescence of Adult Sand Flies That Were Fluorescent when Observed Using Fluorescence Microscopy). Second Instar Sand Flies Were Fed Feces of Hamsters That Had Been Fed a Diet Containing 0, 50, 500, or 5,000 mg/kg Rhodamine B

Larval diet mg/kg	Adult emergence % (mean* ± SE)[#]	Age at adult emergence d (mean* ± SE)[#]	Marked
0	98.3 ± 4.1[a]	30.2 ± 1.4[a]	not marked
50	96.7 ± 5.2[a]	30.0 ± 1.3[a]	inconsistent marking
500	98.3 ± 4.1[a]	29.7 ± 1.3[a]	all marked
5,000	96.7 ± 8.2[a]	30.1 ± 1.2[a]	all marked

* Six replicates, ten larvae per replicate
# Values within a column with the same letter are not significantly different, P> 0.05

Adults that as larvae were fed feces of rhodamine B-treated hamsters were positive for the presence of rhodamine B (appeared red) when examined using fluorescence microscopy. An exposure time of 15 s was used to detect fluorescence due to rhodamine B in sand flies. At this exposure time, adult sand flies fed feces of untreated hamsters as larvae appeared as a black field. All sand flies fed feces of hamsters that had been fed a diet containing 5,000 mg/kg rhodamine B as larvae were fluorescent (n = 58); sand flies fed feces of hamsters fed a diet containing 500 mg/kg rhodamine B exhibited a fainter fluorescence, but were all still discernable from control flies (n = 59). Adult sand flies that had been fed as larvae the feces of hamsters fed a diet containing 50 mg/kg rhodamine B were difficult or impossible to distinguish from control sand flies. As indicated by fluorescence, rhodamine B was principally located in the thoraces and femurs, but often also was present in the abdomen (Fig. 8.3; Fig. 8.4). Rhodamine B appeared to be absent from the head, wings, and the legs below the femur.

8.3.3 Blood-Feeding

All of the sand flies exposed to anesthetized hamsters successfully obtained bloodmeals (Fig. 8.5). The sand flies that were killed immediately after feeding on rhodamine B-treated

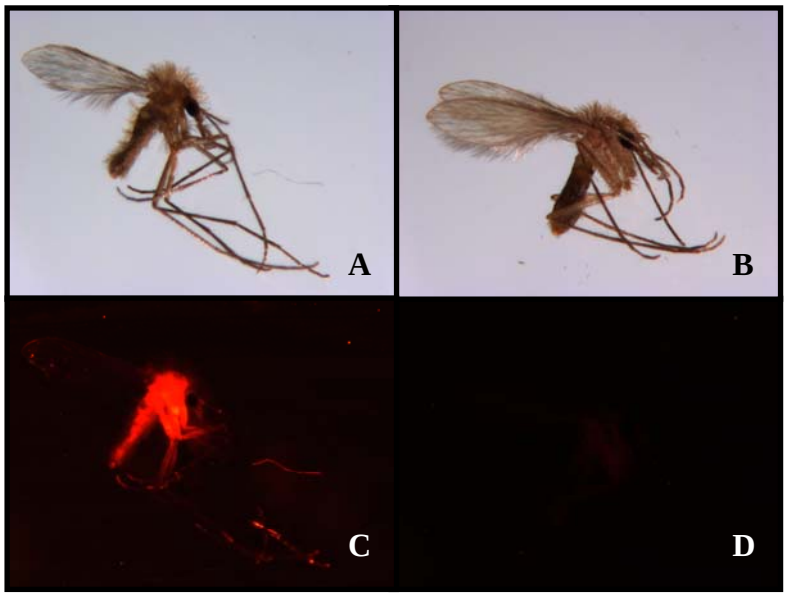

Fig. 8.3 Images of Two Female Sand Flies Taken under Incandescent Lighting (A and B), and Using Fluorescence Microscopy (C and D). The Sand Fly Pictured in the First Column (A and C) Had Been Fed as a Larva the Feces of a Hamster That Had Been Fed a Diet Containing 5,000 mg/kg Rhodamine B, and the Sand Fly Pictured in the Second Column (B and D) Had Been Fed as a Larva the Feces of a Hamster That Had Been Fed a Control Diet

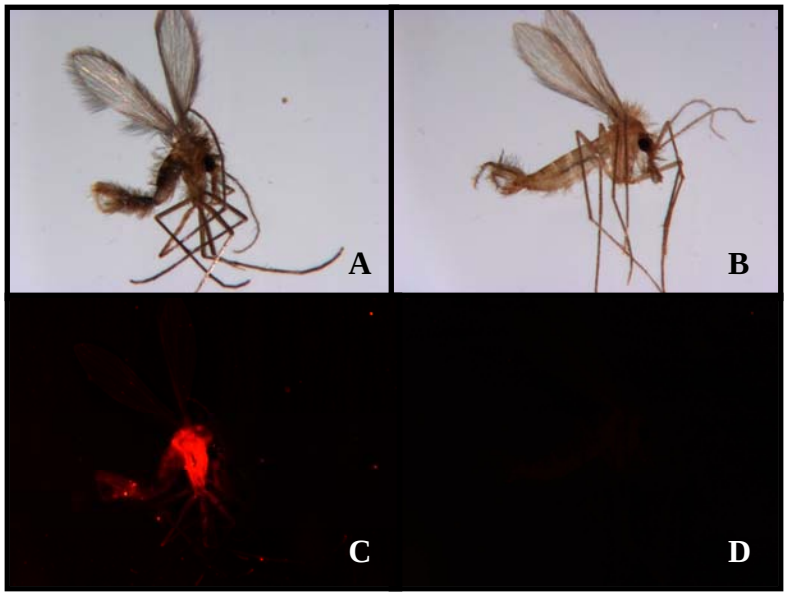

Fig. 8.4 Images of Two Male Sand Flies Taken under Incandescent Lighting (A and B), and Using Fluorescence Microscopy (C and D). The Sand Fly Pictured in the First Column (A and C) Had Been Fed as a Larva the Feces of a Hamster That Had Been Fed a Diet Containing 5,000 mg/kg Rhodamine B, and the Sand Fly Pictured in the Second Column (B and D) Had Been Fed as a Larva the Feces of a Hamster That Had Been Fed a Control Diet

hamsters were positive for the presence of rhodamine B (appeared red) when examined using fluorescence microscopy with an exposure time of 15 s. As indicated by fluorescence, rhodamine B was located is in the thorax, femur, posterior tip of the abdomen, and the malpighian tubules. Rhodamine B appeared to be absent from the head, the legs below the femur, and the bloodmeal itself (Fig. 8.6). Sand flies that took bloodmeals from untreated hamsters appeared as a black field using fluorescence microscopy with an exposure time of up to 15 s exposure time.

Fig. 8.5 A Female Sand Fly Taking a Bloodmeal from the Hind Foot of an Anesthetized, Rhodamine B-Treated Hamster

Sand flies that were killed 8 d after feeding on control or rhodamine B-treated hamsters were not marked (were not considered positive for the presence of rhodamine B) when examined with fluorescent microscopy using an exposure time up to 15 s. Similarly, the eggs, larvae, and adult progeny of sand flies that took a bloodmeal from a control or rhodamine B-treated hamster

also were not marked when examined with fluorescence microscopy using a 15 sec exposure time.

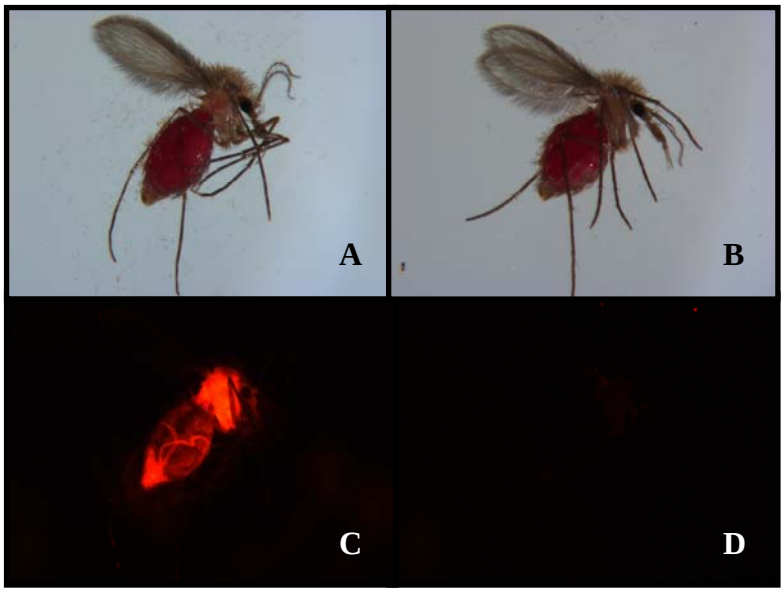

Fig. 8.6 Images of Two Bloodfed Female Sand Flies Taken under Incandescent Lighting (A and B), and Using Fluorescence Microscopy (C and D). The Sand Fly Pictured in the First Column (A and C) Had Taken a Bloodmeal from a Hamster That Had Been Fed a Diet Containing Rhodamine B, and the Sand Fly Pictured in the Second Column (B and D) Had Taken a Bloodmeal from a Hamster Fed a Control Diet

8.4 Discussion

To be an effective biomarker, the marker must be non-toxic and readily consumed by target animals. In this study, there was no significant difference in the amount of food consumed by hamsters at any of the diet concentrations of rhodamine B tested. Furthermore, rhodamine B is a dye with low mammalian toxicity (LD_{50} 887 mg/kg mouse oral), and has been used safely in a wide variety of mammals (Fisher 1999). The finding that the food intake of hamsters was not affected by rhodamine B treatments is consistent with results for other small mammals. For example, there was no difference in the bait preference of ground squirrels targeted with untreated baits or baits containing 2500 mg/kg rhodamine B (Sullens and Verts 1978). Similarly, red-backed voles and field voles did not discriminate between untreated food or food containing

rhodamine B (Buckner 1968). The results of this study support the idea that rhodamine B does not affect the palatability of baits for rodents, and suggest that effective baits containing rhodamine B could be prepared for the various rodents present in different ZCL foci.

All hamsters that consumed diets containing rhodamine B in this study were visibly marked. Hamsters fed a diet containing 50 or 500 mg/kg rhodamine B were marked for a period of up to 2 wk, while hamsters fed a diet containing 5,000 mg/kg rhodamine B were marked for more than 8 wk. Persistence of markings due to rhodamine B have been shown to vary between different mammalian species. For example, coypu fed a diet containing 5,000 mg/kg rhodamine B remained marked for up to 225 d, whereas mountain beavers fed a diet containing 1000 to 3400 mg/kg rhodamine B remained marked for only 2 wk (Fichet-Calvet 1999; Lindsey 1983). This is the first report of rhodamine B being used as a biomarker for hamsters and adds support for the use of rhodamine B as an orally delivered marker for mammals. The findings in this study also demonstrate that the persistence of rhodamine B as a biomarker in hamsters is associated with the dose of rhodamine B.

Numerous biomarkers have been used to monitor the ingestion of baits by mammals including fat-soluble markers such as Dupont oil blue A, antimicrobial markers such as tetracycline hydrochloride and sulfadimethoxine, and the cholecyctographic agent iophenoxic acid (Southey et al. 2002; Creekmore et al. 2002). Unlike rhodamine B, none of these oral biomarkers for mammals have been detected in the feces of orally dosed mammals. In rats, only 3-5% of orally dosed rhodamine B is excreted unchanged in feces (Webb and Hansen 1961). Nevertheless, in this study feces of hamsters that consumed rhodamine B-treated diets were fluorescent. This finding is consistent with studies using rhodamine B to mark other small mammals. For example, feces of black-tailed jackrabbits and cottontail rabbits orally dosed with rhodamine B also were reported to be visibly marked and fluorescent under UV illumination

(Evan and Griffith 1973). The results of this study suggest that examining feces for rhodamine B could provide an alternative measure of bait uptake by target rodents that would be less invasive and time consuming than examining the rodents themselves.

Sand fly larvae fed feces of hamsters that had been fed a diet containing 500 or 5,000 mg/kg rhodamine B were fluorescent as adults when examined using fluorescence microscopy. The results of this study constitute the first report of a feed-through biomarker for larvae and the subsequent adult insects for a medically important insect. In this study, fluorescence due to rhodamine B was detected primarily in the thorax of sand flies. This finding indicates that a marking system using rhodamine B would be compatible with the processing required to identify sand flies. Sand flies are cleared before identification, and key taxonomic characteristics are located in the head and abdomen (the cibarium of a sand fly is used to distinguish medically important *Phlebotomus* spp. from *Sergentomyia* spp., and the spermatheca of female sand flies or the external genitalia of male sand flies are necessary to differentiate species within *Phlebotomus* spp.).

The results of this study suggest that fluorescence due to rhodamine B is transient in sand flies that have taken a bloodmeal from rhodamine B-treated hamsters. Using fluorescence microscopy, rhodamine B was observed in the malpighian tubules of recently engorged sand flies. In insects, the malpighian tubules are the primary system responsible for excretion, suggesting that rhodamine B is rapidly eliminated as the bloodmeal is processed. This hypothesis is further supported by the absence of fluorescence due to rhodamine B in the bloodmeal itself.

A biomarker incorporated into a rodent bait to demonstrate the suitability of rodent feed-through control of sand fly larvae in a rodent/sand fly association must: A) be palatable to rodents, B) mark the rodents and their feces, C) be excreted unchanged in the feces of bait-fed rodents (rather than metabolized), and D) be detectable in adult sand flies that have consumed

feces of bait-fed rodents as larvae. In this study hamsters readily consumed food containing high concentrations of rhodamine B (up to 5,000 mg/kg), and markings due to rhodamine B were detected on the hamsters and their feces. Adult male and female sand flies that had been fed feces of rhodamine B-treated hamsters as larvae could be distinguished from control sand flies using fluorescence microscopy. Therefore, rhodamine B is a good candidate for further evaluation as a biomarker for rodents and sand flies in feed-through studies. Since the results of this study suggest that rhodamine B could be an effective biomarker, the next step would be to evaluate potential interactions between rhodamine B and feed-through insecticides, to evaluate rhodamine B in additional rodent and sand fly models, and to develop a high-throughput and quantitative technique, such as spectrofluorometry, for detecting rhodamine B in specimens. Additional fluorescent dyes with non-overlapping excitation an emission wavelengths also could be evaluated for potential use in conjunction with rhodamine B and feed-through or systemic insecticides.

Using rhodamine B incorporated rodent baits in the field could allow the identification of specific foci with sand fly/rodent associations that would be susceptible to control using feed-through or systemic insecticides, through the detection of adult male and female sand flies that had fed on the feces of baited rodents as larvae, and adult female sand flies that have taken a bloodmeal from bait-fed rodents. The development of rhodamine B as a feed-through biomarker also could have an enormous impact on future studies on sand flies, and could be the breakthrough for several unanswered questions: What percentage of sand flies in an area are associated with rodents? Which of the sand fly species feed on rodent feces as larvae? What percentage of human-biting flies in an area could potentially be eliminated using rodent baits containing insecticides? How far do sand flies disperse from their immature habitat? What

distance around human settlements must be targeted with insecticide-treated rodent baits to create an appropriate barrier?

Using rhodamine B as a feed-through biomarker also could provide detection of changes in sand fly populations due to larval control measures. Currently, monitoring the effects of larval control of phlebotomine sand flies during field trials is a problematic. The ideal method of demonstrating successful larval control would be to directly measure reductions in the larval population through larval sampling, which is impractical. Alternatively, adult sampling techniques (such as sticky paper traps and light traps) could be used, but there are inherent problems with adult sampling to demonstrate larval control, including potential false negative interpretations of results (such as not detecting control when it happens, which could happen through immigration of adult sand flies into the study area) or false positive interpretations of results (which would be attributing a reduction in adult populations to successful larval control, when it actually is a natural seasonal population decline or differential changes in microclimates of test sites).

In conclusion, this study is the first report of a fluorescent dye fed to larvae and then detected in adults of a medically important insect. The value of this method is that it can mark rodents and their feces to establish the consumption of treated-baits, mark adult female sand flies that feed rodents for the duration of persistence of the dye in rodents, and mark adult male and female sand flies that had fed on feces of bait-fed rodents as larvae. In specific rodent sand fly associations, the differential marking of both male and female sand flies or only female sand flies can be used to direct field control studies: a rodent bait containing a systemic insecticide would be used at sites where only female sand flies that fed on rodents are marked, while if male and female sand flies are marked, feed-through insecticides also could be used.

SUMMARY AND CONCLUSIONS

In North Africa, the Middle East, and Southwest Asia, the sand fly *P. papatasi* is the vector of *L. major*, the causative agent of ZCL. All life stages of *P. papatasi* exhibit a close association with the burrowing rodent reservoirs of *L. major*. Despite this close association, currently there are no effective control measures available for the sand fly *P. papatasi*. However, the primary larval habitats of *P. papatasi* in ZCL foci are rodent burrows, and sand fly larvae have been observed feeding on the feces of rodents. Because of this fact, novel control methods targeting sand fly larvae that feed on rodent feces (using rodent feed-through insecticides) and adult sand flies that take bloodmeals from rodents (using a systemic insecticide) were evaluated in this study. Furthermore, a novel biomarker system was evaluated that marked rodents and their feces, adult female sand flies that took bloodmeals from treated rodents, and adult male and female sand flies that had fed on feces of bait-fed rodents as larvae. This biomarker system could be a valuable tool for monitoring host-targeted control trials for sand flies in the field.

The studies presented in Chapters 2 to 5 of this dissertation evaluated the development and survival of *P. papatasi* larvae fed feces from Syrian hamsters that had been fed a diet containing the chitin synthesis inhibitors diflubenzuron and novaluron, the juvenile hormone analogs pyriproxyfen and methoprene, or the macrocyclic lactone ivermectin. In the studies on diflubenzuron, novaluron, methoprene, and pyriproxyfen, the mean daily food intake of the insecticide-treated hamsters was not significantly different from the food intake of control hamsters at any of the concentrations tested (up to approximately 1000 mg/kg a.i.), but the mean daily food intake of hamsters was significantly lower than control for hamsters fed a diet containing more than 20 mg/kg ivermectin. Sand fly larvae (2^{nd} instars) that had been fed feces of hamsters fed diets containing approximately 10, 100, or 1000 mg/kg diflubenzuron, novaluron, or pyriproxyfen, or 20 mg/kg ivermectin all died before adult emergence. Feces of

hamsters fed a diet containing up to 978.8 mg/kg methoprene caused significant but less than 100% mortality of immature sand flies. The results of the studies in Chapters 2 to 5 constitute proof of concept for feed-through control of sand fly larvae using diflubenzuron, novaluron, pyriproxyfen, and ivermectin.

The studies presented in Chapter 6 of this dissertation determined the minimum effective dose of novaluron as a feed-through for control of sand fly larvae, and evaluated novaluron feed-through under simulated field conditions. Complete control of sand fly larvae was observed when they were fed hamster feces containing 9.88 mg/kg novaluron, or when they were fed the feces of hamsters fed a diet containing $9.88x10^{-1}$ mg/kg novaluron. Feces of hamsters fed a diet containing 988 mg/mg novaluron were aged for up to 150 d at 28 °C and 90% RH. All larvae that had been fed feces of novaluron-treated hamsters aged for 0 or 30 d died before adult emergence, and the mortality of sand fly larvae fed feces aged up to 150 d was significantly different from control. The last experiment presented in Chapter 6 evaluated the effectiveness of novaluron as a feed-through in preventing the development of sand fly larvae when novaluron-treated food made up only a portion of a hamster's daily diet. All larvae that had been fed either feces from hamsters that exclusively had been fed a diet containing novaluron or hamsters that consumed novaluron-treated food as approximately 15% of their daily diet died before pupation.

In this study, significant control of sand fly larvae was observed when they were fed feces of novaluron-treated hamsters that had been aged for up to 150 d or feces of novaluron-treated hamsters when only a portion of their daily diet was novaluron-treated food. Therefore, novaluron is a good candidate for further evaluation as a rodent feed-through insecticide against sand fly larvae. Since the results of this study suggest that novaluron could be effective as a rodent feed-through insecticide in a field setting, the next step would be to evaluate the effects of novaluron-treated baits on sand fly populations in different rodent/sand fly associations.

The studies presented in Chapter 7 of this dissertation evaluated whether the post-bloodmeal survival of adult female sand flies would be affected by feeding their rodent hosts a diet containing ivermectin. The 48 h survival was 0% for sand flies that took a bloodmeal from hamsters withdrawn from an ivermectin-treated diet for 0, 3, or 7 d, but was 100% for sand flies fed on hamsters withdrawn from an ivermectin-treated diet for 14 d. Feces voided by ivermectin-treated hamsters also were collected 0 to 14 d after the hamsters were withdrawn from their ivermectin-treated diets, and the feces were fed to sand fly larvae. Mortality was 100% for larvae fed feces voided 0 d after withdrawal, and was significantly higher than control larvae for up to 7 d after withdrawal. The mean percent survival of sand fly larvae fed feces of ivermectin-treated hamsters 14 d after they were withdrawn from an ivermectin-treated diet was not significantly different from control.

This study confirms that ivermectin-treated rodent baits developed as a feed-through to control sand fly larvae also could have a collateral effect on bloodfeeding adult sand flies, thus affecting a larger portion of the sand fly population than would be estimated due to larvicide activity alone. While ivermectin treatment of rodent hosts of *L. major* may reduce the overall population of sand flies, this approach would have its greatest impact on the most epidemiologically important subset of the sand fly population: females that have taken a bloodmeal from potentially infected rodents.

In the studies presented in Chapter 8 of this dissertation, hamsters were fed diets containing rhodamine B, and the mean daily food intake of the rhodamine B-treated hamsters was not significantly different from the food intake of control hamsters at any of the concentrations tested (50, 500, or 5000 mg/kg rhodamine B). Hamsters fed diets containing rhodamine B were marked by the dye after feeding on their diets for up to 8 weeks. Feces of rhodamine B-treated hamsters also were marked when examined using fluorescence microscopy.

The development and survival of sand fly larvae fed feces of rhodamine B-treated hamsters was not significantly different from control sand flies. Adult male and female sand flies that had were fed as larvae the feces of rhodamine B-treated hamsters were fluorescent when examined using fluorescent microscopy, and could be distinguished from control sand flies. Adult female sand flies that took bloodmeals from rhodamine B-treated hamsters were fluorescent, but not when they were examined 8 d after bloodfeeding.

Using rhodamine B incorporated rodent baits in the field could allow the identification of specific foci with sand fly/rodent associations that would be susceptible to control using feed-through or systemic insecticides, through the detection of adult male and female sand flies that had fed on the feces of baited rodents as larvae, and adult female sand flies that have taken a bloodmeal from bait-fed rodents. The development of rhodamine B as a feed-through biomarker also could have an enormous impact on future studies on sand flies, and could be the breakthrough for several unanswered questions about sand fly ecology.

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APPENDIX A. COPYRIGHT PERMISSION LETTERS

August 26, 2008

Thomas M Mascari, MSc DLSHTM
402 Life Sciences Building
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Baton Rouge, LA 70803

Dear Mr. Mascari,

The Entomological Society of America grants you permission to use the articles cited below as part of your doctoral dissertation at Louisiana State University, that is titled "Novel Methods for Control of Phlebotomine Sand Flies (Diptera:Psychodidae)."

Mascari, T.M., Mitchell, M.A., Rowton, E.D., and Foil, L.D. 2007a. Laboratory evaluation of diflubenzuron as a feed-through for the control of immature sand flies (Diptera: Psychodidae). Journal of Medical Entomology. 44:171-174.

Mascari, T.M., Mitchell, M.A., Rowton, E.D., and Foil, L.D. 2007b. Evaluation of novaluron as a feed-through insecticide for control of immature sand flies (Diptera: Psychodidae). Journal of Medical Entomology. 44: 714-717.

Please provide proper acknowledgement.

Sincerely,

Alan Kahan
Director of Communications
Entomological Society of America
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Dear Thomas Mascari,

The American Mosquito Control Association (AMCA) kindly grants you permission to use excerpts of the following article which was published in the Journal of the American Mosquito Control Association:

Mascari, T.M., Mitchell, M.A., Rowton, E.D. and Foil, L.D. 2008. Ivermectin as a rodent feed-through insecticide for control of immature sand flies (Diptera: Psychodidae). Journal of the American Mosquito Control Association 24: 323-326

Please note that this material is copyrighted by the American Mosquito Control Association and all content is required to be properly cited. The Journal of the American Mosquito Control Association must be listed as the source of any information.

Please do not hesitate to contact me with any questions or concerns.

Thank you,

Sarah Gazi
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Mount Laurel, NJ 08054
Ph: 856-439-9222
Fax: 856-439-0525

VITA

Thomas Michael Mascari was born to Walter and Kate Mascari in New Orleans, Louisiana, in May 1979. Thomas completed a Bachelor of Arts at Louisiana State University, majoring in Anthropology, in 2001. In 2002, he completed a Master of Science at the London School of Hygiene and Tropical Medicine, under the late Professor Christopher Curtis. The title of his masters project was "Baseline data collection on *Wuchereria bancrofti* infection prevalence and intensity, the evaluation of LF diagnostic tools, and the measurement of lymphoedema patient leg volumes in Newala District, Tanzania." In 2003, he returned to Louisiana State University and began work on a Doctor of Philosophy under Dr. Michael Perich. Following Dr. Perich's death later in 2003, Thomas continued his studies under Dr. Lane Foil.