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UNDERSTANDING THE MECHANISMS OF INSECTICIDE RESISTANCE IN
PHLEBOTOMUS PAPATASI AND *LUZOMYIA LONGIPALPIS* SAND FLIES
(DIPTERA: PSYCHODIDAE: PHLEBOTOMINAE)

by

David S. Denlinger

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Biology

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Logan, Utah

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ABSTRACT

Understanding the Mechanisms of Insecticide Resistance in *Phlebotomus papatasi* and *Lutzomyia longipalpis* Sand Flies (Diptera: Psychodidae: Phlebotominae)

by

David S. Denlinger, Doctor of Philosophy

Utah State University, 2017

Major Professor: Dr. Scott A. Bernhardt
Department: Biology

The prevalence of insecticide resistance in vector species around the world is a continuous threat for any success at mitigating the spread of vector-borne diseases. With a limited arsenal of new insecticides, it is crucial for public health programs to understand the geographic range and the genetic mechanisms of resistance to best approach controlling insect vectors. Insecticide resistance is being increasingly observed in phlebotomine sand fly (Diptera: Psychodidae) populations in both the Old World and New World. Sand flies transmit the protozoans that cause leishmaniasis, a disfiguring disease that kills tens of thousands of people each year. The goal of this dissertation was to have both an applied and basic research focus towards understanding resistance in phlebotomines. I began by comparing *in vivo* and *in vitro* methods for blood-feeding two species of sand flies, *Phlebotomus papatasi* and *Lutzomyia longipalpis*, in the laboratory, both of which are important leishmaniasis vectors. I investigated the susceptibility of both species to ten different insecticides by calculating lethal concentrations that caused varying levels of mortality. Based on these results, I determined diagnostic doses and diagnostic times for both species to the same ten insecticides using an accepted, but novel, assay for sand flies. Finally, I tested for known mechanisms of insecticide resistance in four artificially

resistant-selected colonies of sand flies, as well as tested for novel resistance mechanisms.

Through applied research, I developed methods for efficient sand fly rearing and for determination of population resistance to insecticides, tools that have worldwide applicability.

Through basic research, I determined that laboratory populations of sand flies have sufficient standing genetic variation needed to survive sublethal doses of insecticides; however, I was unable to develop artificially-selected colonies resistant to these insecticides. My research has generated information to provide new insights into the evolution of insecticide resistance in natural sand fly populations. My results support that resistance development may be possible, but evolutionary challenging, an encouraging finding that may be exploited by vector biologists and public health officials to prevent or slow the development of resistance in sand flies to insecticides

(257 pages)

PUBLIC ABSTRACT

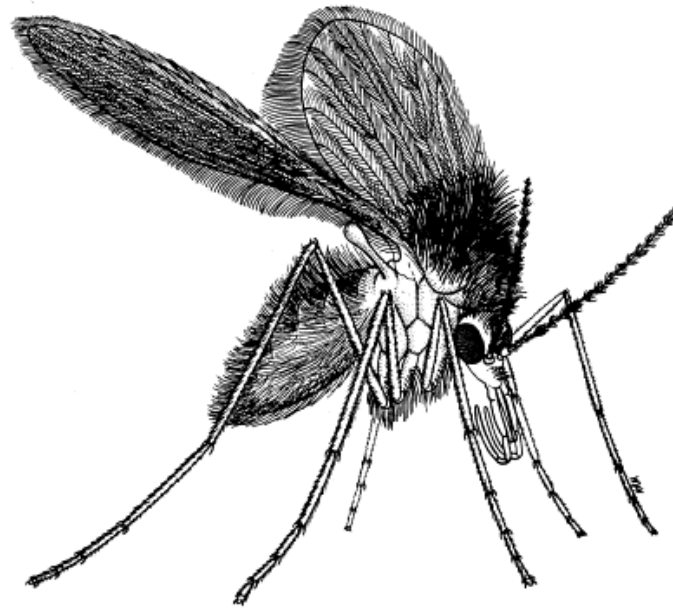
Understanding the Mechanisms of Insecticide Resistance in *Phlebotomus papatasi* and *Lutzomyia longipalpis* Sand Flies (Diptera: Psychodidae: Phlebotominae)

David S. Denlinger

Sand flies, like mosquitoes, ticks, fleas, and lice, transmit pathogens that cause disease in humans. Leishmaniasis, caused by pathogens transmitted by sand flies, kills tens of thousands of people every year. Insecticides have been used to control sand flies, but there is evidence of insecticide resistance in populations of sand flies around the world. The goal of this dissertation was to develop tools to maintain sand flies in the laboratory, develop the ability to identify insecticide-resistant populations of sand flies, and to investigate the genetic mechanisms of how sand flies become resistant to insecticides. I began by comparing live animal and artificial techniques for blood-feeding two species of sand flies, *Phlebotomus papatasi* and *Lutzomyia longipalpis*, in the laboratory, both of which are important leishmaniasis vectors. Next, I investigated how susceptible laboratory colonies of both species are to ten insecticides that are used worldwide to control sand flies. Based on my results, I determined diagnostic concentrations and diagnostic exposure times for the laboratory colonies to the ten insecticides using a known assay that has been used very little for sand flies, which allows researchers to determine if a population of sand flies is resistant to an insecticide. Finally, I tested for known mechanisms of insecticide resistance in four artificially resistant-selected colonies of sand flies, and I also looked for novel mechanisms. This dissertation is useful in that it provides researchers practical approaches to maintain sand flies to be used for further research and to determine resistance in the field. It also demonstrates that sand fly populations are homogenous, and it implies that it is challenging for a population to become resistant to insecticides. This aspect can

be exploited by sand fly researchers and public health officials in effectively controlling sand fly populations, which is also beneficial for slowing the transmission of leishmaniasis.

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This dissertation is dedicated to my grandfathers, Herb Denlinger and Ralph Mease, two military men who served honorably in Korea and World War II. Fair winds and following seas...

“It seemed as though all the insect life of the entire region had congregated here in anticipation of a glorious picnic...Ants built catacombs beneath our couches, land crabs burrowed up through the fungus-grown floor to inspect our resting places, woodticks climbed the tent-walls, whence they could select the most favorable lodging place, flies covered our food as with sackcloth and endeavored to rob us even of its scantiness, mosquitoes of unrivaled force and ferocity plied their lancets with merciless vigor, and, when their appetite were appeased, rested on the ridgepole and mockingly barked at their victim until he went to sleep, great hairy spiders built nests in the peak, strange things whizzed and buzzed and boomed through the darkness, cannon dropping on our faces with a sharp thud as if shot or alighting with sticky feet reluctant of dislodgement. All night

long there was a rustling and a crackling of well-nigh every type of winged and creeping abomination that earth produces.” (Armed Forces Pest Management Board, *Evolution of Military Medical Entomology*, 2008)

-To all the 72Bs, 43MXs, and 230X-1850s who, unknown to most and often unceremoniously, have, are, and will continue to honorably fight six-legged enemies in order to protect those who fight against our two-legged enemies-

I think that there is no fundamentally more collaborative effort in an individual’s academic career than the pursuit of a graduate degree. I certainly would not have reached the end of my degree without the guidance, support, patience, and camaraderie of so many here at Utah State.

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To my Alpine family who have been great friends over the past three years and with who I've built amazing relationships: growing together; living together; playing Banjo Kazooie and watching movies late into the night; having spur of the moment dinners at Toro, Sonic, or Dennys; working on my pickup truck; making coffee with; Fusion; playing golf; Euchre; Banff; and worshipping God together.

To the rest of the Bernhardt lab team who, over the years, helped me to make oviposition pots, helped me take care of the sand flies, did PCRs for me, helped me to get a Handel on the law, took me to lunch and dinner, who I could help mentor and publish with, and who were a joy to do life together down here in the dungeon of BNR.

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CHAPTER 1

INTRODUCTION

The Evolution of Blood-Feeding in Insects. Insects have been successful over the course of their approximate 500-million-year existence because of their taxonomic and ecological diversity (Misof et al. 2014). Insects have evolved to fill an immense number of ecological niches (Gullan and Cranston 2010). One niche, though, has arguably impacted human existence more than any other and has been a significant detriment to our development and our society: hematophagy.

Hematophagy has independently evolved many times within the insect orders Diptera, Hemiptera, Lepidoptera, Phthiraptera, and Siphonaptera (Black IV and Kondratieff 2005). Two hypotheses explain the independent origin of hematophagy in insects. First, hematophagy arose in nidicolous insect lineages that had prolonged associations with vertebrates and that had not yet acquired specializations for blood-feeding. These insects initially fed on organic matter including sloughed skin, hair, or feathers. There was physiological and behavior selection for individuals that had efficient chewing mouthparts and higher propensity for feeding directly on the host. Blood is more nutritious than skin, and therefore, there was selection for mouthparts that allowed insects to gradually transition to blood-feeding (Lehane 2005). Second, insects already had morphological pre-adaptations for piercing, for feeding on other insects and for piercing plants, that evolved into blood-feeding. Their proteases evolved to digest hemoproteins as they began to pierce vertebrate tissues (Lehane 2005).

Blood-Feeding Flies. The ability for insects to imbibe human blood would be a mere annoyance if not for the viral, bacterial, and protozoal pathogens that have exploited this insect-human relationship. In no group of insect vectors has this dynamic evolved more times than in Diptera. Diptera, the true flies, contains the greatest number of families that are hematophagous

and competent of disease transmission. Feeding on blood and/or hemolymph has evolved in more than twenty families of extinct and extant flies: Athericidae, Blephariceridae, Calliphoridae, Carnidae, Ceratopogonidae, Chironomidae, Chloropidae, Corethrellidae, Culicidae, Glossinidae, Hippoboscidae, Muscidae, Nycteribiidae, Oestridae, Piophilidae, Psychodidae, Rhagionidae, Sarcophagidae, Simuliidae, Streblidae, and Tabanidae. Between the extant species, several thousand are of medical and veterinary interest. Diseases vectored by flies afflict greater than 500 million people, and 3.5 billion people are at-risk (Hall and Gerhardt 2009, Wiegmann et al. 2011).

Mosquitoes (Culicidae) vector the agents that cause many viral encephalitis diseases, chikungunya, Zika, dengue, yellow fever, malaria, and filariasis. Biting midges (Ceratopogonidae) vector the viruses that cause bluetongue, epizootic hemorrhagic fever, and African horsesickness in animals, whereas in humans, they vector the agents that cause Oropuche fever and mansonellosis. Black flies (Simuliidae) are best known for vectoring *Onchocerca* nematodes that cause onchocerciasis, or river blindness. Tsetse flies' (Glossinidae) ability to vector the trypanosomes that cause sleeping sickness in humans and nagana in animals has thwarted the development of Africa. Horse and deer flies (Tabanidae) are adamantly known as nuisance pests because of their formidable bites; nevertheless, *Chrysops* tabanids vector *Loa loa*, the African eyeworm, which causes loiasis, and at least in the United States, tabanids mechanically vector *Francisella tularensis* (tularemia). Like tabanids, flies of Muscidae are less known for vectoring diseases, but the bazaar fly, *Musca sorbens*, mechanically transmits *Chlamydia* bacteria that cause trachoma. Even *Liohippelates* flies (Chloropidae) can mechanically transmit the spirochete *Treponema pertenu*e that causes the syphilis-like, disfiguring disease yaws (Adler and McCreadie 2009, Foster and Walker 2009, Hall and Gerhardt 2009, Krinsky 2009, Moon 2009, Mullen 2009, Mullens 2009, Petersen et al. 2009).

Psychodidae. Taxonomically, the family Psychodidae is sister to the family Tanyderidae (primitive crane flies), and together this clade is sister to the family Blephariceridae (net-winged midges). These three families are grouped together into the Psychodomorpha infraorder of Diptera (Wiegmann et al. 2011). Of its six subfamilies, Psychodidae contains two subfamilies with blood-feeding individuals: Sycoracinae, blood-feeders of anurans, and Phlebotominae, blood-feeders of vertebrates (Bravo and Salazar-Valenzuela 2009, Petrulevičius et al. 2011).

The subfamily Phlebotominae (sand flies) contains the only anthroponotic hematophagous insects of the family Psychodidae, which are capable of vectoring viral, bacterial, and protozoan disease agents (Rutledge and Gupta 2009). The term “sand fly” can be a misnomer. Many people think that sand flies are only found at beaches, and even more confusing, colloquially around the world the term “sand fly” is used to describe *Culicoides* midges, simuliid black flies, or mosquitoes (Killick-Kendrick 1999, Maroli et al. 2013).

The nearly one thousand species of the subfamily Phlebotominae has made developing a consistent, reliable taxonomy contentious; the number of genera has fluctuated from six to thirty-one (Curler and Moulton 2012). Sand fly taxonomy has historically been based on phenetics of morphology, but the subfamily’s systematics are improving because of genetic and genomic advances (Akhoundi et al. 2016). These advances are rapidly expanding Phlebotominae’s taxonomy, now to include many tribe and subtribe levels with numerous genera (Akhoundi et al. 2016). Phlebotominae’s expanding taxonomy is complicated by epidemiological issues and may be impractical for medical parasitologists and physicians who have relied on older taxonomies (Ready 2011). However, advances in phlebotomine taxonomy is needed to stimulate research in sand fly vectorial and ecological life histories (Ready 2011). Despite these recent advances, six genera are conservatively agreed upon by many sand fly taxonomists: *Phlebotomus*, *Sergentomyia*, and *Chinius* in the Old World, and *Lutzomyia*, *Brumptomyia*, and *Warileya* in the New World (Akhoundi et al. 2016). The Grace-Lema et al. (2015) recent phylogeny

hypothesizes a tropical Old World origin of Phlebotominae with subsequent diversifications into Asia and Europe, and then into the New World.

Phlebotomine sand flies are distributed in the warm zones of Asia, Africa, Australia, southern Europe, and the Americas. Their northern distribution extends near latitude 50°N in southwest Canada, northern France, and Mongolia. Their southern distribution extends to near 40°S (Lewis 1982, Young and Perkins 1984). Sand flies are not found on New Zealand or on the Pacific Islands (Maroli et al. 2013). The altitudinal distribution maximums extend from below sea level (near the Dead Sea) to 3,300 meters above sea level in Afghanistan (Killick-Kendrick 1999).

Sand flies are holometabolous insects (complete metamorphosis with egg, larva (four instars), pupa, and adult stages) that are terrestrial in all life stages (Rutledge and Gupta 2009). Eggs are 0.3-0.5 mm in length and are oviposited in habitats with rich organic substrate including animal feces or soil, which provide larvae with shelter, nutrition, and moisture. Larvae resemble caterpillars and are best recognized by their prominent caudal setae (Fig. 1.1). Fourth instar larvae will evacuate their gut contents as they search for drier substrate in which to pupate. Finding sand fly larvae and pupae in natural environments has proven unproductive and tedious (Felicangeli 2004). Sand fly pupae resemble chrysalises; the exuvia of the fourth larval instar anchors the pupa to a substrate (Maroli et al. 2013). The life-cycle of a sand fly, from oviposition to adult, lasts approximately five weeks, including laboratory conditions (Volf and Volfova 2011).

Adult sand flies are less than 5 mm in length, delicate with long legs, densely hairy, and usually grey, black, brown, or sandy in color (Fig. 1.1.). They are most active during crepuscular and nocturnal hours when they feed, and during the day they tend to rest in cool, humid, dark microhabitats such as tree buttresses, stables, caves, rock fissures, bird's nests, termitaria, leaf litter, caves, animal burrows, latrines, and in homes (Rutledge and Gupta 2009). Adults are poor



Fig. 1.1. Sample photographs of a larval sand fly (left) and adult sand fly (right) (from Maroli et al. 2013 and Alten et al. 2015)

fliers and are known for their hopping behavior, which has substantiated the belief that sand flies are not very vagile (Killick-Kendrick 1999). Their dispersal seldom exceeds one kilometer from their breeding sites (Quate 1964, Alexander and Young 1992). The reason for their poor dispersal ability may be their inability to fly well in windy conditions.

Both adult male and female sand flies require sugar for growth and development. Sugar sources include plants and honeydew of hemipterans (Schlein and Warburg 1986, Killick-Kendrick 1999). Only adult female phlebotomines blood-feed, which they do on mammals, and the blood provides nutrition for egg development. However, some species are autogenous and are able to complete one gonotrophic cycle without a blood meal (El Kammah 1973, Montoya-Lerma 1992). Females are guided to hosts via CO₂, temperature, and humidity cues (Killick-Kendrick 1999). Mating can happen before, during, and after blood-feeding and usually occurs near the host. Adult females are telmophagic and use their stylet-like mandibles, maxillae, and labrum to lacerate the skin and capillaries from which pooled blood can be imbibed (Black IV and Kondratieff 2005). Because females blood-feed on humans, they are competent to transmit disease agents of medical importance. Sand flies are associated with vectoring the agents that cause sand fly fever, bartonellosis, and leishmaniasis in humans.

Leishmaniasis. Leishmaniasis is regarded as a neglected tropical disease by the World Health Organization (WHO) and is caused by *Leishmania* protozoans (Trypanosomatida: Trypanosomatidae) (WHO 2013a). *Leishmania* are dixenous parasites; they are capable of surviving in two hosts: mammals, including humans, and phlebotomine sand flies (Dostálová and Volf 2012, Maslov et al. 2013). With very rare exceptions, female phlebotomine sand flies are the only way for humans to acquire an infection with *Leishmania* parasites. These exceptions can include human venereal transmission, human congenital transmission, needle transmission, and blood transfusions (Killick-Kendrick 1999, Maroli et al. 2013). In addition, it has been suggested that arthropods other than sand flies are capable of vectoring *Leishmania*: ticks (Coutinho et al. 2005, Dantas-Torres et al. 2010, Paz et al. 2010, Dantas-Torres 2011, Solano-Gallego et al. 2012) and *Forcipomyia* and *Culicoides* midges (Ceratopogonidae) (Dougall et al. 2011, Seblova et al. 2012, Slama et al. 2014).

The first reports that leishmaniasis is caused by a parasite came from Major D.D. Cunningham of Britain and Army physician Peter Borovsky of Russia in the late nineteenth century. The disease leishmaniasis is named after British Colonel W.B. Leishman who described the protozoan agent in 1903 in Dum Dum, India. That same year, British Colonel C. Donovan, a military physician, linked the disease to parasites recovered from a spleen of a living patient. For his work, the amastigote form of *Leishmania* is also referred to as Leishman-Donovan bodies (Crum et al. 2005).

Approximately ten percent of sand flies are competent to vector *Leishmania* to humans, and these species putatively belong to the genera *Phlebotomus* and *Lutzomyia* (Rutledge and Gupta 2009, Maroli et al. 2013, Akhoundi et al. 2016). Several, but not all, requirements needed to incriminate *Sergentomyia* species as vectors have been met (Maia and Depaquit 2016). Competent *Phlebotomus* and *Lutzomyia* species together vector approximately twenty species of *Leishmania* that are pathogenic to humans (Bañuls et al. 2007, Antinori et al. 2012, Maroli et al.

2013). The Phlebotominae ancestor likely evolved in the Triassic period, which preceded the origin of *Leishmania* (Jurassic) and mammals (Paleocene). This means that *Leishmania* likely evolved from a trypanosomatid monoxenous insect parasite that eventually became dioxenous mammal parasites, approximately 90 million years ago (Akhoundi et al. 2016). Three hypotheses exist of the origin of *Leishmania*: Palaearctic (Kerr 2000, Kerr et al. 2000), Neotropical (Noyes et al. 2000), and Afrotropical (Momen and Cupolillo 2000). Murid rodents were likely the first hosts for *Leishmania* and were responsible for dispersing *Leishmania* around the world (Schenk et al. 2013, Akhoundi et al. 2016). Today, each species of *Leishmania* that is pathogenic to humans is maintained in areas where female sand flies, humans, and potentially other mammals overlap. These foci can be divided into two epidemiological groups: zoonotic and anthroponotic leishmaniases (Maroli et al. 2013). Zoonotic leishmaniases include a mammal reservoir host (e.g. opossums, monkeys, sloths, rodents, canines, hyraxes, anteaters) in the transmission cycle with humans (Gramiccia and Gradoni 2005). In anthroponotic leishmaniases, humans are the only source of infection for phlebotomines (Desjeux 1996).

Today, leishmaniasis is endemic in at least ninety-eight countries spanning five continents and is found in situations where there is poor housing, inadequate sanitation, and poverty (Fig. 1.2.) (Alvar et al. 2006, Alvar et al. 2012, WHO 2013a). The disease occurs in semiarid, arid, urban, sylvatic, and rural regions (Desjeux 1996, Rutledge and Gupta 2009). Approximately 310 million individuals are at risk world-wide with an annual incidence of 1.3 million cases, of which 20,000-40,000 deaths are attributed to leishmaniasis (WHO 2013a). Clinically in humans, the disease is manifested in two forms: visceral and cutaneous. Of the 1.3 million new cases of leishmaniasis each year, 300,000 are visceral and 1.0 million are cutaneous (WHO 2013a).

Visceral leishmaniasis, known as kala-azar, is the most severe presentation of the disease. Ninety percent of all visceral leishmaniasis cases occur in India (predominantly in the state of

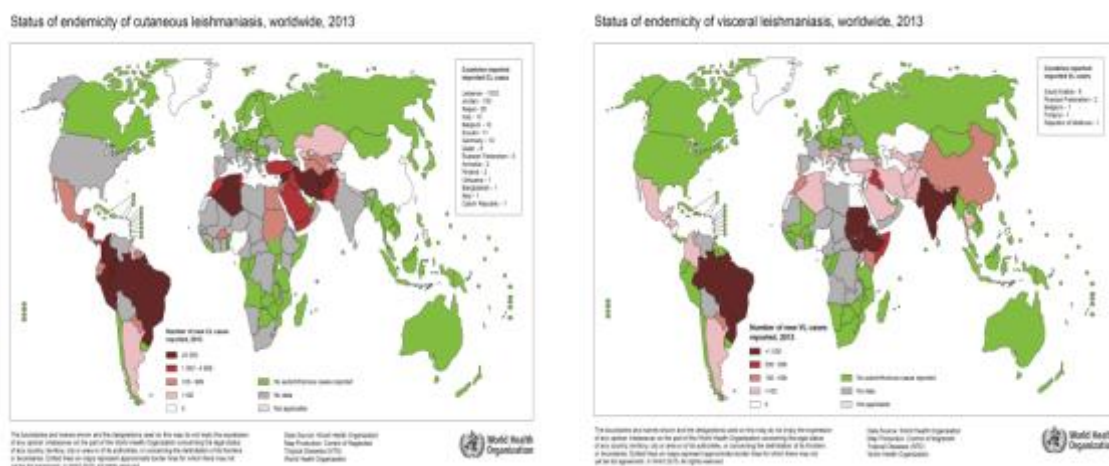


Fig. 1.2. Global distribution of cutaneous (left) and visceral (right) leishmaniasis (from WHO 2013a). The countries highlighted in gray have no data available. The countries highlighted in green represent countries with no autochthonous cases. The countries highlighted in light pink (<math>< 100</math> new cases) to dark purple (>1,000 new cases) represent an increasing annual incidence of leishmaniasis.

Bihar), Bangladesh, Nepal, Brazil, and Sudan (Hailu et al. 2005, WHO 2013a). Symptoms of visceral leishmaniasis appear over a period of weeks and months, and people become increasingly anemic, lethargic, cachectic, and susceptible to secondary infections. Clinical features of the disease include splenomegaly, anemia, pancytopenia, weight loss, weakness, and the disease is almost always fatal if untreated (Hailu et al. 2005) (Fig. 1.3).

Cutaneous leishmaniasis, including the destructive mucocutaneous variant, is more geographically widespread than visceral leishmaniasis. Ninety percent of cases are focused in the Maghreb region, the Middle East, Brazil, and Peru. Recently, surveillance studies have discovered that the incidence of cutaneous leishmaniasis cases is increasing in countries surrounding these regions (Reithinger et al. 2007, Ready 2010). Clinically, there is an initial erythema where the host was bitten by the sand fly. Over the course of a few weeks to several months, the erythema becomes an ulcerating papule and eventually a lesion (Reithinger et al. 2007) (Fig. 1.3). Fortunately, spontaneous healing usually results in lifelong protection from the specific *Leishmania* species, and it may offer cross-protection to other species. A cutaneous scar

remains for the rest of the individual's life. In cases of mucocutaneous leishmaniasis, infection manifests as a non-self-healing, disfiguring, and potentially life-threatening destruction of the mucous membranes as the parasite metastasizes via the lymphatic system (Fig. 1.3.). Often, the lips, cheeks, soft palate, pharynx, and larynx can be destroyed (Reithinger et al. 2007). Besides the obvious physical challenges associated with cutaneous leishmaniasis, there is a potentially more impactful detriment. The social, cultural, familial, and economic stigmas associated with this disease are a severe burden for people (Hotez 2008, Kassi et al. 2008, Hotez 2016).

Cutaneous leishmaniasis has resurged recently in northern Africa and in the Middle East because of the political instability, political terror, and human diaspora (Hotez et al. 2012, Alasaad 2013, Salam et al. 2014, Fawcett and Hay 2015, Hotez 2015, Al-Salem et al. 2016, Berry and Berrang-Ford 2016, Du et al. 2016, Mondragon-Shem and Acosta-Serrano 2016).

***Leishmania* Development in Sand Flies.** In the female sand fly vector, *Leishmania* parasites are contained only within the digestive tract following the blood-meal and undergo cyclodevelopmental horizontal transmission (Sacks 2001, Ramalho-Ortigão et al. 2007,



Fig. 1.3. Left: visceral leishmaniasis with outlined enlargements of spleens and livers. Center: cutaneous leishmaniasis with noticeable pathology on the face. Right: mucocutaneous leishmaniasis with destruction of the face and nasal cavity (Murray et al. 2005, Reithinger et al. 2007, Ekiz et al. 2017).

Dostálová and Volf 2012). Parasites of the subgenus *Leishmania* (suprapylarian leishmaniasis) are restricted only to the sand fly midgut prior to anterior migration while parasites of the subgenus *Viannia* (peripylarian leishmaniasis) enter the sand fly hindgut prior to anterior migration (Kamhawi 2006, Dostálová and Volf 2012). Development of *Leishmania* in sand flies begins with the procyclic promastigote stage and concludes with the mammal-infecting metacyclic stage (Fig. 1.4.).

Early Development and the Peritrophic Matrix. *Leishmania* development in the female sand flies begins when she ingests blood from a vertebrate host containing amastigote-infected macrophages (Rogers et al. 2008, Dostálová and Volf 2012). The intracellular amastigote stage of *Leishmania* is only found within vertebrate host macrophages (Handman and Bullen 2002). Since sand flies are telmophagic, the laceration of vertebrate capillaries triggers macrophages to assist with wound damage and allows for the macrophages to be imbibed into the gut of the sand fly (Bates 2007).

One of the first physiological processes to occur in the sand fly midgut is the formation of

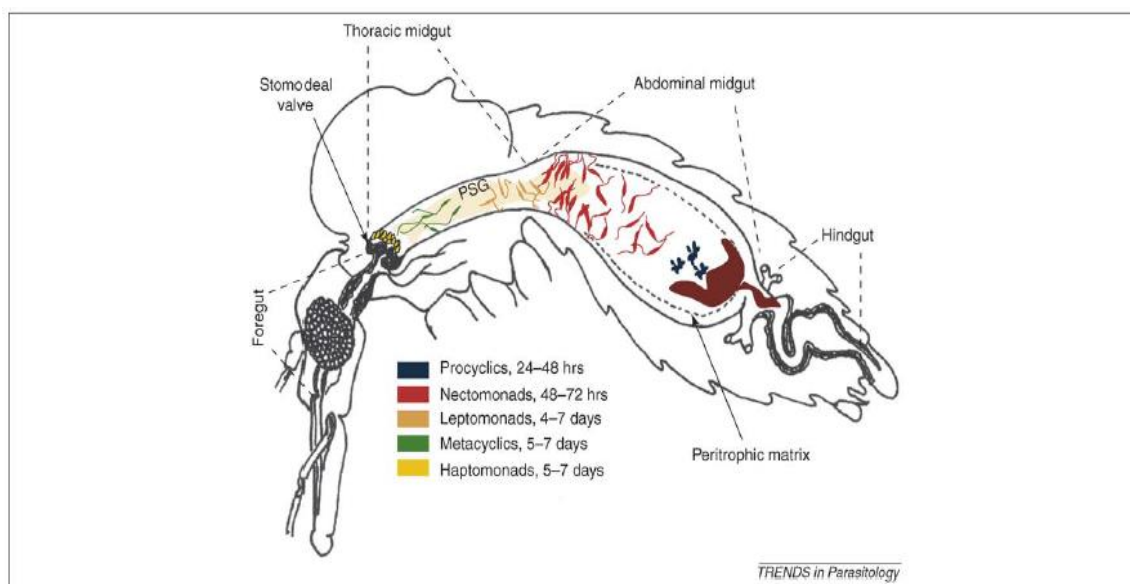


Fig. 1.4. Overview of the developmental life stages of *Leishmania* parasites within a female sand fly and the days that each stage exists after a blood-meal. From Kamhawi (2006).

a Type I peritrophic matrix, which is secreted by the sand fly midgut minutes after the ingestion of the blood meal as midgut cells are stretched and flattened as the volume of blood distends the midgut (Walters et al. 1993, Pimenta et al. 1997, Devenport and Jacobs-Lorena 2005, Sádlová and Volf 2009). The peritrophic matrix is a semipermeable, extracellular layer that separates the luminal side of the midgut epithelial cells from the food bolus (Devenport and Jacobs-Lorena 2005). Because the midgut is not protected with chitinous cuticle, the peritrophic membrane serves many purposes: it prevents clogging of the epithelial cell microvilli from improper food digestion and absorption, it compartmentalizes digestion by acting as a permeability barrier for digestive enzymes, and it protects the midgut from pathogenic microbes by acting as a barrier to their development (Pimenta et al. 1997). This matrix, because it separates the food bolus from the midgut epithelial cells, must be permeable enough to allow digestive enzymes to cross it and reach the food bolus and for the digested products to diffuse in the opposite direction to be absorbed by the midgut (Devenport and Jacobs-Lorena 2005). This feature of the peritrophic matrix is both beneficial and detrimental to the *Leishmania* parasite. The peritrophic matrix is the first physical barrier that *Leishmania* must overcome to complete their lifecycle in the sand fly (Rogers et al. 2008). The peritrophic matrix is now known to serve a dual purpose for the *Leishmania* parasite.

First, the peritrophic matrix serves as a partial physical barrier to the chitinases secreted in the sand fly midgut and protects the *Leishmania* as they transform from amastigotes to procyclic promastigotes in the first 24-48 hours after ingestion (Pimenta et al. 1997). Procyclic promastigotes are weak, motile forms. Changes in biochemical conditions moving from the mammal host to the sand fly gut trigger this morphological transformation (Bates 2007, Dostálová and Volf 2012). The transition from amastigote to procyclic promastigote is when *Leishmania* is most vulnerable to proteolysis because during the same time interval, the ingestion of the blood meal by the female sand fly has induced a secretion of large numbers of enzymes.

These enzymes participate in various physiological processes that include, among others, bloodmeal digestion, and the peritrophic matrix limits the exposure of the parasites to the digestive enzymes (Pimenta et al. 1997, Ramalho-Ortigão et al. 2007, Jochim et al. 2008, Dostálová and Volf 2012, Pruzinova et al. 2015).

Second, the peritrophic matrix serves as a barrier to the *Leishmania*. After surviving the onslaught of proteolytic enzymes, the remaining procyclic promastigotes develop into nectomonads, which are large, slender, motile forms whose function is to escape the peritrophic matrix. The nectomonads must escape the peritrophic matrix and establish infection or they are excreted when the blood meal is defecated 48-72 hours post-blood-meal (Sacks 2001, Kamhawi 2006). In this sense, the peritrophic matrix can be a detriment to *Leishmania* survival and development. Escape from the peritrophic matrix is facilitated with both sand fly- and *Leishmania*- derived chitinases (Pimenta et al. 1997, Ramalho-Ortigão et al. 2001, Ramalho-Ortigão and Traub-Csekö 2003, Joshi et al. 2005, Ramalho-Ortigão et al. 2005).

The temporal relationship between *Leishmania*-derived chitinases and the sand fly-induced chitinases is critical. As Pimenta et al. (1997) describes, overexpression of chitinases by the *Leishmania* is detrimental to itself by degrading the peritrophic matrix too quickly, which makes it more porous for the sand fly-induced proteolytic enzymes to destroy the transforming amastigotes. However, as Rogers et al. (2008) discovered, chitinase-overexpressing *Leishmania* that survive the enzyme attack are better able to escape the peritrophic matrix before it is defecated by using a combination of their own chitinases and the sand fly-induced chitinases.

Overcoming Defecation with Lipophosphoglycan. Next, the *Leishmania* nectomonads must overcome midgut defecation of the blood-meal. This barrier causes most *Leishmania* loss (Sacks 2001, Wilson et al. 2010). It is imperative for *Leishmania* to persist in the midgut through the defecation of the blood-meal. Fluorescent micrographs determined that *Leishmania* survive

blood-meal defecation by anchoring their flagella between the female sand fly's microvilli of the midgut epithelial cells (Warburg et al. 1989).

Leishmania anchor to the midgut epithelial microvilli with their outer surface lipophosphoglycan (LPG) molecules on their flagella (Pimenta et al. 1992, Pimenta et al. 1994, Sacks et al. 2000). Pimenta et al. (1994) demonstrated that differences in the LPG molecules between different species of *Leishmania* contribute to varying sand fly vector competencies. LPG is festooned on the entire outer surface of the parasite and is structurally a dense glycocalyx (Sacks 2001). Each LPG is a tripartite molecule comprised of a proximal glycoposphatidylinositol anchor, a hexasaccharide glycan core, and a distal phosphoglycan (PG) domain. In all *Leishmania* species, the PG moieties all share a common backbone but are distinguished from other species by varying sidechains that come off the PG domain (Turco et al. 1987, Ilg et al. 1992, McConville et al. 1992, McConville et al. 1995, Mahoney et al. 1999, Soares et al. 2002).

Detachment from the Midgut. Having survived the bloodmeal digestion and defecation, the nectomonads must detach from the midgut epithelial microvilli to continue their lifecycle and effective transmission. Metacyclogenesis initiates following microvilli detachment and has been initiated *in vitro* in conditions similar to an evacuated female sand fly midgut (e.g. low pH and nutrient depletion) (Bates 1994, Bates 2008). The process of metacyclogenesis begins with conformational changes in the nectomonads. It involves elongation of the LPG molecules by increasing the number of PG units and a regulating the number of side-chain substitutions (Pimenta et al. 1992, Sacks et al. 1995). During metacyclogenesis, LPG can be shed, PG chains can be gained or elongated and the terminal sugars on the PG chains can become cryptic (Turco et al. 1987, Sacks et al. 1990, McConville et al. 1992, Pimenta et al. 1992, Sacks et al. 1995, Mahoney et al. 1999, Soares et al. 2002, Soares et al. 2005). These changes promote the parasites' ability to be transmitted.

Transmission to the Vertebrate Host. Following detachment from the midgut, the nectomonads migrate anteriorly towards the stomodeal valve. The stomodeal valve separates the foregut and midgut and ensures the unidirectional flow of food by preventing regurgitation (Volf et al. 2004). It consists of a ring of cylindrical epithelial cells and is lined with cuticle. Some nectomonads that reach the stomodeal valve transform into leptomonads, which are short forms that begin to replicate (Rogers et al. 2002, Bates 2007). A percentage of leptomonads further differentiate into metacyclics, which are the vertebrate-infective form (Bates 2007). Other nectomonads anchor into the cuticle-lined surface of the stomodeal valve and become haptomonads (Killick-Kendrick et al. 1974, Volf et al. 2004, Bates 2007, Rogers et al. 2008).

Both the haptomonads and leptomonads play a vital, altruistic role in the transmission of the metacyclics. *Leishmania* haptomonads help to degrade the sand fly's stomodeal valve by separating the stomodeal valve cuticle from its epithelial cells, forcing the valve open, and destroying the valve via their own chitinases (Schlein et al. 1992, Volf et al. 2004, Rogers et al. 2008). Concurrently, the *Leishmania* leptomonads secrete a mucin-like filamentous matrix known as promastigote secretory gel (PSG) (Rogers et al. 2002). PSG was first observed and recognized as a *Leishmania*-secreted substance by Stierhof et al. (1994, 1999). The main component of PSG is proteophosphoglycan (PPG), which is unique to *Leishmania* and makes PSG resistant to proteinases (Ilg et al. 1996, Rogers et al. 2004). The PSG plug is found to extend anteriorly into the stomodeal valve and oesophagus and posteriorly into the midgut. This plug ultimately exerts strong mechanical pressure on the fore- and midgut walls (Rogers et al. 2002, Volf et al. 2004, Rogers et al. 2008). Metacyclics are found at the poles of the plug, and this feature is thought to assist in their transmission (Bates 2007).

Together, the haptomonad-damaged stomodeal valve and leptomonad-secreted PSG gel plug inoculated with metacyclics support the "blocked fly hypothesis" of *Leishmania* transmission (Bates 2007). *Leishmania* physically obstruct the female sand fly's alimentary canal

and must be regurgitated to fully blood-feed. In *Leishmania*-infected female sand flies, the stomodeal valve remains open (because it is damaged and because of the obstructing PSG plug) when blood is imbibed such that the midgut contents and new blood from the host mix together, facilitating parasite transmission (Schlein et al. 1992). This backflow of the midgut contents, including the PSG plug carrying *Leishmania* metacyclics, are deposited on the host skin as the female sand fly probes and feeds along with saliva (Rogers et al. 2004, Volf et al. 2004). Rogers et al. (2004) found that the glycan moieties of the regurgitated PPG, and sand fly saliva, are virulence factors in the mammalian infection with leishmaniasis (Theodos et al. 1991).

Rogers and Bates (2007) examined the potential manipulation that *Leishmania* have on the female sand fly. They found that *Leishmania* behaviorally manipulate their sand fly host, such that the timing of *Leishmania* development is linked to the sand fly feeding persistence and enhancement of *Leishmania* transmission. Specifically, they found that *Leishmania* link their infectivity (transformation of metacyclics) with the formation of PSG, reduce the lifespan of the female sand fly by exerting fitness costs (stomodeal valve destruction, gut distension, and resource diversion), do not alter sand fly fecundity, manipulate the female sand fly to persist in blood-feeding, and manipulate the female sand fly to feed on multiple hosts. These behavioral manipulations are adaptive because they increase the virulence of mammalian infection by increasing the number of parasites per infected host (Rogers and Bates 2007). These manipulations allow *Leishmania* to be evolutionary successful by helping to ensure their transmission to the human host around the world.

Integrated Vector Management for Sand Flies. Effective integrated vector management (IVM) relies on using multiple control methods to reduce populations of vector insects in an effort to delay resistance and mitigate disease transmission (Denholm and Rowland 1992). IVM is effective when there is vertical collaboration of all levels of society, from local communities to businesses to local and state government to world-wide health agencies (WHO

2004). Proper sand fly IVM in a geographic focus requires characterizing sand fly biology and ecology, sand fly population structure, reservoirs, and human population. These factors should guide the implementation of control measures that will have the greatest success (Kishore et al. 2006, Luckhart et al. 2010, Warburg and Faiman 2011). While insecticides remain the primary resource for controlling sand flies, they are not the only resource. IVM incorporates programs with aspects of environmental control, biological control, community control, that all have varying successes in controlling sand flies to diminish the spread of leishmaniasis.

The goal of environmental control is to alter the physical environment to disrupt a sand fly population's ecology, including breeding, resting sites, and oviposition, to disrupt leishmaniasis transmission (Kishore et al. 2006, Amóra et al. 2009). Larval source reduction is a principle approach in environmental control (Beier et al. 2008), but this has been infeasible for sand flies because there is poor knowledge of breeding sites and larval environments (Feliciangeli 2004). Therefore, larval source reduction is not currently considered practical for controlling sand flies in field situations (Warburg and Faiman 2011). Other types of environmental control have been considered for both larval and adult sand flies: treating soil cracks and habitats, plastering cracks and crevices of walls, pruning trees to increase sunshine, increased sewage treatment, disposal of garbage, vegetation removal, and reservoir control (Lane 1991, Coleman et al. 2006, Jassim et al. 2006, Sharma and Singh 2008, Amóra et al. 2009, Faulde et al. 2009, Warburg and Faiman 2011). Which of these approaches will be most effective in vector control programs depends heavily on a strong understanding of the ecology of the sand flies in a particular leishmaniasis focus (Amóra et al. 2009).

Many types of biological control have been considered for controlling sand flies, but there is a paucity of data. Biolarvicides are not considered practical because of the diverse breeding habitats of sand flies (Kishore et al. 2006). Phytochemicals have been demonstrated to be toxic against larvae and adults by interfering with their development and reproduction (Amóra

et al. 2009). Entomopathogens, such as *Bacillus thuringiensis* and *B. sphaericus* have shown some success in the laboratory, although application in the field remains challenging (Amóra et al. 2009). The entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* were effective at causing mortality in the sand flies *P. papatasi*, *P. duboscqi*, and *L. longipalpis* (Warburg 1991, Ngumbi et al. 2011). Attractive toxic sugar baits applied as sprays and mixed with fruit juice in small field studies have shown success (Mascari and Foil 2010, Qualls et al. 2015). Interestingly, phlebotomines are reservoirs for the parasitic *Psychodiella gregarines* (Eugregarinorida: Lecudinidae), which can destroy laboratory colonies of sand flies, of which nearly 100% of flies are infected; in the field, the prevalence of sand fly infection is approximately 25%, which makes using gregarines for biological control unlikely (Votýpka et al. 2009, Lantová and Volf 2014). An intestinal nematode was discovered in *L. longipalpis* from Brazil, which under stressful laboratory conditions increases host colonization. Its infectious and lethal effect on *L. longipalpis* could be exploited for biological control (Secundino et al. 2002). Synthetic pheromones have been shown to be effective, especially when used in conjunction with other methods (Bray et al. 2010, Bray et al. 2014). While these biological control approaches have potential, their applicability to control sand flies in the field at large scales requires further exploration.

Community control focuses on health education for the public where basic knowledge of leishmaniasis is critical and needs to be disseminated (Kishore et al. 2006). There has been much research into the success of community control in Brazil, which has both endemic cutaneous and endemic visceral leishmaniasis. There is a lack of knowledge among the public and health professionals. Public health agencies lack an organized direction, and vector control specialists are employed transiently. The leishmaniasis-affected citizens reside in poor areas and are unable to implement control measures. Despite the collective knowledge of leishmaniasis, there remains

an impetus for continued, multi-disciplinary, focused, efforts to reach individuals who need serious assistance in combating leishmaniasis (Amóra et al. 2009).

One success of an integrated approach for controlling sand flies comes from the U.S. military at Tallil Air Base (TAB) in southern Iraq during the initial stages of Operation Iraqi Freedom and Enduring Freedom. In 2003, over one thousand cases of leishmaniasis were diagnosed in military servicemen (Lay 2004, Aronson et al. 2006, Aronson 2007, Schleier III et al. 2009). Personal protective measures (PPM) including insecticide-treated bed nets and uniforms were poorly implemented to thwart sand fly bites because they were unavailable; because of noncompliance due to the extreme temperatures, blowing sand, and unfamiliarity; because many personnel were unfamiliar with the products; and because many products were ineffective (Coleman et al. 2006). Accordingly, TAB implemented the “Leishmaniasis Control Plan” (LCP). Its four objectives were: 1. sand fly surveillance and testing for *Leishmania* to assess risk. 2. ensuring personnel had access to and used PPM. 3. establishing a control program targeting sand flies and reservoirs such as canines and rodents through the application of residual insecticides to tents or buildings; hand-held, truck-mounted, and aerial insecticide sprays; thermal fogging applications of insecticides; and habitat destruction and 4. educating personnel about the risk of leishmaniasis and the PPM required to protect themselves (Coleman et al. 2006). Over the course of several months, the LCP was successful in drastically lowering the number of leishmaniasis cases in U.S. military personnel (Aronson 2007).

The collective IVM effort from environmental control, biological control, community control, and other approaches including mosquito nets, repellents, and sand fly trapping may still be insufficient for adequate leishmaniasis control (Lane 1991, Casanova 2001, Kishore et al. 2006, Hoel et al. 2007, Gillespie et al. 2016). Novel approaches are required. Remote sensing and geographic information systems have allowed epidemiologists to map information about the geographic landscapes and to determine abiotic factors that are important to sand fly ecology and

leishmaniasis transmission (Kesari et al. 2011). Rodent and cattle feed-through systems show promise in control of both larval and adult sand flies (Mascari et al. 2007, Mascari et al. 2008, Mascari et al. 2011, Poché et al. 2013). Paratransgenic *P. argentipes* have been developed in the laboratory using a bacterial vehicle, but more work is needed to develop the system in the field to make *P. argentipes* refractory to *Le. donovani* (Hurwitz et al. 2011).

Insecticide Control of Sand Flies. The unproven success of certain aspects of integrated vector management has forced public health programs that target sand flies to rely on chemical insecticides (Alexander and Maroli 2003). The first attempt to control adult phlebotomines occurred in 1944 with DDT in the Rimac Valley of Peru, against *Lu. verrucarum*, and in the same year in Naples, Italy, against *P. papatasi*, both of which were considered successful. Since then, synthetic insecticides have been used in many applications to control sand flies. As part of control programs, sand flies have been exposed to four major classes of synthetic insecticides- 1) organochlorines 2) pyrethroids 3) organophosphates, and 4) carbamates. These exposures have been either intentional in directed vector control efforts or have been inadvertent as part of malaria control efforts against anophelines (Alexander and Maroli 2003, Surendran et al. 2005, Alexander et al. 2009, Dinesh et al. 2010, Faraj et al. 2012, Hassan et al. 2012, Saeidi et al. 2012).

Insecticides theoretically should be very effective against sand flies. Sand flies move by hopping in short flights and must land on many surfaces as they approach, allowing for significant contact time (Killick-Kendrick 1999). Insecticides have been applied via residual spraying of homes, tents, and animal shelters; barrier sprays in sylvatic environments on tree trunks and vegetation; in insecticide-treated bed nets; in sprays around termite mounds and animal burrows where sand flies breed and oviposit; in impregnated dog collars because dogs are reservoirs for zoonotic cutaneous leishmaniasis; in fumigants, ultra-low volume sprays, diffusers, foggers, and coils; and in spray clothes and mesh barriers (Alexander et al. 1995, Robert and

Perich 1995, Alexander and Maroli 2003, Orshan et al. 2006, Faiman et al. 2009, Claborn 2010, Coleman et al. 2011, Warburg and Faiman 2011, Müller et al. 2012, Calzada et al. 2013).

Insecticide History and Mode of Action. Organochlorines came to exist in 1939 when dichlorodiethyltrichloroethane (DDT) was discovered to be an insecticide by Paul Müller (Coats 1994). Pyrethroids, derivatives of natural pyrethrum insecticides from *Chrysanthemum* spp., were commercialized on a large-scale in the 1970s (Davies et al. 2008). Both DDT and pyrethroids are neurotoxic to insects and are ligands for insects' voltage-gated sodium channels in axons of nervous tissue involved in action potential propagation. When bound to the sodium channels, these insecticides cause changes in the channels' ion conductance, ion selectivity, and gating properties. Sodium channels remain open (activated) and are unable to inactivate, which causes a continued influx of sodium ions into the cell. The cell remains hyper-excited, also known as “knockdown,” a sublethal incapacitating effect on the insect. At the cellular level, nerve function is altered from continued action potential spasms and disrupted synaptic transmission. This causes a systemic failure of the nervous tissue and ultimately death. There are two groupings of pyrethroids that target sodium channels. Type I pyrethroids cause sodium channel modifications that can last up to tens of milliseconds and effective in causing knockdown in insects. Whereas, Type II pyrethroids cause sodium channel modifications that can last for many seconds and are effective in causing mortality in insects (Bloomquist and Miller 1986, Davies et al. 2007, Dong et al. 2014).

Organophosphates (OP) were developed by the Axis powers during World War II as nerve gases because of their strong toxicity to humans, and carbamates (CX) are patterned after physostigmine, which is the agent isolated from the calabar bean (Coats 1994). Both OPs and CXs are neurotoxins, but they have different targets than DDT and pyrethroids. OPs and CXs target the acetylcholinesterase enzyme, which clears saturated cholinergic synapses of acetylcholine following synaptic transmission in nervous tissue. OPs and CXs inhibit the

acetylcholinesterase's functional ability by phosphorylating (OPs) or carbamylating (CXs) the catalytic serine hydroxyl group within the enzyme's oxyanion hole. Unable to hydrolyze acetylcholine into choline, acetylcholine builds up in the synapse and overstimulates the postsynaptic membrane of the dendrite. For many OPs, the phosphorylation of acetylcholinesterases is irreversible, and the constant overstimulation of neurons leads to the insect's death (Toutant 1989; Fukuto 1990; Costa 2006).

Insecticide Resistance.

“In any event, the spread of the resistant strains constitutes probably the best proof of the effectiveness of natural selection yet obtained.” - Theodosius Dobzhansky (1937)

Genetics and the Origin of Species

Most species of phlebotomines remain susceptible to insecticides (Coleman et al. 2011); however, around the world, there is increasing evidence of insecticide resistance. The insecticide resistance phenotype is defined as a heritable, genetic change in response to insecticide exposure that allows for increased survival (Feyereisen 1995, Scott 1999, Hemingway et al. 2002). Organochlorines, pyrethroids, organophosphates, and carbamates are all contact insecticides (Kolbezen et al. 1954, Casida 1980), and so they must be applied frequently as they degrade. Often, delivering insecticides to the microhabitats where sand flies live, breed, and oviposit is difficult. Insecticide treatments targeting sand flies, while initially successful, can degrade quickly due to harsh environmental conditions, and re-applications are necessary (Karapet'ian et al. 1983, Coleman et al. 2011). The recurring application of insecticides can be indiscriminant, which has exerted tremendous selective pressure for insecticide resistance (Feyereisen 1995, WHO 2006). Increasing the insecticide dosage in response to resistance only exacerbates the problem by increasing the frequency of the genetic trait(s) in a vector population (Feyereisen

1995). Insecticide resistance continues to be a threat to the success of insect vector control programs that incorporate synthetic chemical insecticides (Rivero et al. 2010).

Today, resistant sand fly populations have been documented in the Middle East, southern Asia, and South America (Yaghoobi-Ershadi and Javadian 1995, Singh et al. 2001, Surendran et al. 2005, Alexander et al. 2009, Dinesh et al. 2010, Afshar et al. 2011, Faraj et al. 2012, Hassan et al. 2012, Saeidi et al. 2012, Singh et al. 2012, Hassan et al. 2015, Khan et al. 2015, Kumar et al. 2015, Singh and Kumar 2015). The number of documented cases of sand fly insecticide resistance is low compared to other insect vectors, such as mosquitoes, and this may be due to limited field resources, inadequate monitoring, challenges in collecting the necessary number of live flies for using in resistance testing bioassays, and because there is a lack of a standardized sand fly bioassays to assess resistance to fully evaluate the susceptibility status to insecticides in sand flies in many countries (Alexander and Maroli 2003, Saeidi et al. 2012, Li et al. 2015). It is important to understand the prevalence of insecticide resistance and its public health impact if resistance is more commonplace than currently understood.

Detecting Insecticide Resistance. Managing insecticide resistance requires timely, accurate data through resistance monitoring and insecticide evaluation to assess a vector species' susceptibility to insecticides (Surendran et al. 2005). The primary way to assess insecticide resistance in many vectors, including sand flies, is to use insecticide susceptibility bioassays. The two most commonly used bioassays worldwide are the WHO exposure kit bioassay and the Centers for Disease Control (CDC) bottle bioassay (Brogdon and Chan 2010, WHO 2013b).

The WHO exposure kit bioassay is a standardized protocol that consists of an exposure kit containing tubes lined with filter papers that are impregnated with a specific concentration of an insecticide (WHO 2013b). The CDC bottle bioassay protocol consists of exposing insects to concentrations of insecticide that are coated on the interior of glass bottles (Brogdon and Chan 2010). Both bioassays have been used to assess insecticide resistance in sand flies, but the WHO

bioassay is used more frequently (Santamaría et al. 2003, Surendran et al. 2005, Alexander et al. 2009, Henriquez et al. 2009, Dinesh et al. 2010, Afshar et al. 2011, Faraj et al. 2012, Hassan et al. 2012, Saeidi et al. 2012, Singh et al. 2012, Marceló et al. 2014, Coleman et al. 2015, Khan et al. 2015, Kumar et al. 2015, Singh and Kumar 2015, Karakuş et al. 2016). To assess resistance with the WHO and CDC bioassays, up-to-date diagnostic doses and diagnostic times are fundamental. A diagnostic dose of an insecticide is the lowest concentration that causes 100% mortality in a susceptible population in some time span, the diagnostic time (Brogdon and Chan 2010). There are very few insecticides for which diagnostic doses and diagnostic times have been determined for sand flies (Santamaría et al. 2003, Henriquez et al. 2009, Marceló et al. 2014).

One issue that has become apparent as the CDC bottle bioassay becomes more popular is defining resistance. The WHO bioassay considers resistance when there is less than 90% mortality in the tested population, and the CDC considers resistance if there is less than 100% mortality in a tested population (Brogdon and Chan 2010, WHO 2013b). These criteria for resistance for both bioassays are based on mosquitoes. Saeidi et al. (2012) recommend tailoring resistance criteria for sand flies because of the physiological, behavioral, and size differences between mosquitoes and sand flies. Synchronization of diagnostic doses, diagnostic times, and criteria for resistance in sand flies will certainly be needed as both the WHO and CDC bioassays continue to be used to assess resistance in sand fly populations (Owusu et al. 2015). Despite the recent findings of insecticide resistance in sand fly populations around the world, there remains a dearth of information about the genetic mechanisms of resistance in these populations.

Resistance Mechanisms. Insecticide resistance to synthetic insecticides has been found in many important insect vectors: mosquitoes, black flies, triatomines, lice, fleas, and sand flies (Hemingway and Ranson 2000, Rivero et al. 2010). Four mechanisms of resistance are known to exist in insects: reduced penetration, behavior avoidance, target-site insensitivity, and metabolic detoxification (Ffrench-Constant et al. 2004, Hemingway et al. 2004, Nauen 2007, Lilly et al.

2016, Nansen et al. 2016). Of the four, target-site insensitivity and metabolic detoxification are the two most geographically- and entomologically-widespread. Today, there is evidence of target-site insensitivity and metabolic detoxification resistance to the four main classes of synthetic insecticides in all major vector species (Mallet 1989, Brogdon and McAllister 1998, Nauen 2007, Rivero et al. 2010).

Target-Site Insensitivity. Target-site insensitivity resistance is caused by single nucleotide variants (SNVs) that cause nonsynonymous mutations and change the amino acid at that codon locus. These mutations alter a protein's conformation such that the insecticide is rendered ineffective because it can no longer interact with the target protein. Many TSI-conferring mutations in proteins are conserved across insect species, insect families, insect orders, Insecta, and Arthropoda (Soderlund and Knipple 2003, Hemingway et al. 2004, Dong et al. 2014, Douris et al. 2016). In many insect vectors, insensitivity resistance is conferred in three genes: paralytic (*para*), acetylcholinesterase-1 (*ace-1*), and resistance to dieldrin (*Rdl*). depending on the class of insecticide that resistance is targeted towards (Bloomquist 1996, Soderlund and Knipple 2003, Weill et al. 2003, Hemingway et al. 2004).

Pyrethroids and DDT target the α -subunits of voltage-gated sodium channels of nervous tissue, which are encoded by the paralytic (*para*) gene. Pyrethroids and DDT normally block the channels' inactivation; cause action potential spasms, involuntary movements, and muscle spasms known as knockdown; and eventually kill the insect (Martins et al. 2009). TSI in *para* that prevent knockdown is known as knockdown resistance (*kdr*). *Kdr* in the *para* protein decreases the channels' sensitivity to insecticides by decreasing ligand affinity and/or altering the kinetics of channels by favoring the closed-state and accelerating deactivation (Bloomquist and Miller 1986, Davies et al. 2007, Davies et al. 2008, Burton et al. 2011, Dong et al. 2014). The *para* protein has four domains, and each domain has six transmembrane helices. *Kdr* SNV mutations have been discovered in many insect species across several orders: Blattodea, Coleoptera,

Diptera, Hemiptera, Lepidoptera, Phthiraptera, Thysanoptera, Siphonaptera. Examples of insect *kdr* has been found in all four domains, and often there is convergent evolution at homologous loci, which highlights the importance of certain loci in their interaction with pyrethroids and DDT (Martinez-Torres et al. 1997, references in Dong et al. 2014)

Target-site insensitivity resistance to organophosphates and carbamates occurs in the acetylcholinesterase enzyme, which is encoded by the *ace-1* gene. Like *kdr*, SNVs have been discovered in *ace-1*, although not at nearly as many loci as in *para*. These SNVs code for bulkier amino acids that block the interaction of the OP or CX with acetylcholinesterase (Weill et al. 2002, Weill et al. 2003, Weill et al. 2004, Fournier 2005). Target-site insensitivity mutations in *ace-1* have been found in Diptera, Coleoptera, and Hemiptera (Fournier 2005).

The *Rdl* protein is the receptor for the neurotransmitter γ -aminobutyric acid (GABA) and is the target of cyclodiene insecticides (organochlorine). GABA ligand binding to the GABA receptor activates chlorine ion channels in nervous tissue. *Rdl* has the fewest known examples of target-site insensitivity of the three genes that have been discussed. Only a single codon has been shown to confer target-site insensitivity in *Rdl*, but there has been convergent evolution of mutations at this same across many orders of insects (Anthony et al. 1998, Hemingway et al. 2004, references in Nakao 2016).

Metabolic Detoxification. Metabolic detoxification (MD) resistance involves changes in the expression of specific enzymes [carboxylesterases (EST), cytochrome P450s (MFO), and glutathione S-transferases (GST)] that are capable of binding, sequestering, and metabolizing insecticides (Hemingway 2000, Hemingway and Ranson 2000, Ffrench-Constant et al. 2004). Increasing the numbers of these enzymes is achieved through gene amplification or through changes in gene expression (Rivero et al. 2010). It is also common for enzyme classes correlated with metabolic resistance to detoxify multiple insecticide classes. ESTs can detoxify organophosphates, carbamates, and pyrethroids; MFOs can detoxify all insecticide classes; and

GSTs can detoxify organophosphates, organochlorines, and pyrethroids (Hemingway and Karunaratne 1998, Hemingway 2000, Corbel et al. 2007, Perera et al. 2008, Che-Mendoza et al. 2009, David et al. 2013).

Assessing Resistance Mechanisms.

Target-Site Insensitivity. Linking TSI conferring genes and SNVs has its origins with *kdr* and *para* in *Drosophila melanogaster* (Loughney et al. 1989). Allelism assays were soon developed for *D. melanogaster* and other orthologous *para* genes in other insects (Doyle and Knipple 1991, Williamson et al. 1993, Knipple et al. 1994). Following those breakthroughs, associations were made between TSI and *ace-1* and *Rdl* (Ffrench-Constant et al. 1993, Zhu et al. 1996). Advances in sequencing technologies have allowed for an explosion of reverse transcription PCR, PCR, allele-specific PCR, and real-time PCRs to become the standards for identifying SNVs associated with TSI (Martinez-Torres et al. 1998, Brengues et al. 2003, Bass et al. 2004, Weill et al. 2004, Verhaeghen et al. 2006, Bass et al. 2007, Saavedra-Rodriguez et al. 2007, García et al. 2009).

One limitation of conventional assessment of TSI is that the genomic region being examined is very small compared to the entire genome, and this potentially constrains researchers from identifying other TSI mutations. Genome-wide association mapping can now be done with whole-genome techniques, such as genotype-by-sequencing, that identify thousands of SNVs associated with a trait of interest, such as insecticide resistance (Romay et al. 2013, Comeault et al. 2014, Comeault et al. 2015).

Metabolic Detoxification. Biochemical assays are used to assess for metabolic detoxification. These assays measure the activity levels of ESTs, MFOs, GSTs, *p*-nitrophenyl acetate (PNPA), and acetylcholinesterase. Biochemical assays are microplate colorimetric assays that quantify the activity of detoxifying enzymes (Valle et al. 2006). MD has been assessed using biochemical assays in several vector groups: mosquitoes, lice, triatomines, and sand flies

(Hemingway et al. 1999, Picollo et al. 2005, Corbel et al. 2007, Ghosh et al. 2015). Biochemical assays have assessed MD in sand flies in Brazil, Sudan, and Sri Lanka (Surendran et al. 2005, Alexander et al. 2009, Hassan et al. 2012). Biochemical assays are limited in that they only measure the activity of the enzymes, not the mechanism(s) controlling their activities (Ranson et al. 2011). Nonetheless, biochemical assays are useful in giving a useful profile of families of resistance-conferring enzymes (Surendran et al. 2005).

Overview of Chapters. Despite the recent findings of widespread insecticide resistance in phlebotomine sand fly populations around the world, there is limited information about the genetic mechanisms of resistance in these populations. One goal of this dissertation is to investigate both known and novel mechanisms of insecticide resistance in two sand fly species under artificial selection and to make inferences from an evolutionary perspective. The second goal was to provide worldwide phlebotomine researchers baseline insecticide susceptibilities in order to assist applied field research in identifying insecticide populations of sand flies. Being able to identify resistant populations of sand flies and the mechanisms of resistance in these populations will provide valuable insight for vector biologists and public health officials in making appropriate, informed, and effective decisions about sand fly control to lessen the burden of leishmaniasis around the world.

Chapter 2 provides a novel approach to blood-feeding the sand fly *Phlebotomus papatasi* in the laboratory. It reviews literature about the importance of working with laboratory colonies of sand flies and the type of basic and applied research that has resulted from it while discussing how to maintain these colonies in the laboratory. We compared the effectiveness of three blood-feeding methods in terms of the proportion of blood-fed female *P. papatasi*, and we looked at the importance of the number of females in the colony on blood-feeding success. We found that in

vivo blood-feeding feeds the largest proportion of female *P. papatasi*, but in vitro methods may be comparable in large-sized colonies.

[Denlinger, D. S., A. Y. Li, S. L. Durham, P. G. Lawyer, J. L. Anderson, and S. A. Bernhardt. 2016. Comparison of *in vivo* and *in vitro* methods for blood feeding of *Phlebotomus papatasi* (Diptera: Psychodidae) in the laboratory. *J. Med. Entomol.* 53: 1112-1116.]

Chapter 3 examines the insecticide susceptibility of *Phlebotomus papatasi* and *Lutzomyia longipalpis* sand flies using a hybrid of the CDC bottle bioassay and WHO exposure kit bioassay. The goal was to determine lethal concentration doses for ten different insecticides that caused 50%, 90%, and 95% mortality. The lethal concentration values causing 50% mortality for permethrin and malathion served as doses for our artificial selection experiments in Chapters 5 and 6.

[Denlinger, D. S., S. Lozano-Fuentes, P. G. Lawyer, W. C. Black IV, and S. A. Bernhardt. 2015. Assessing insecticide susceptibility of laboratory *Lutzomyia longipalpis* and *Phlebotomus papatasi* sand flies (Diptera: Psychodidae: Phlebotominae). *J. Med. Entomol.* 52: 1003-1012.]

Chapter 4 builds upon Chapter 3 by determining diagnostic doses and diagnostic times for the same ten insecticides tested in Chapter 3. These data represent the first large collection of diagnostics using the CDC bottle bioassay protocol for phlebotomine sand flies. These data will serve as important baselines for understanding the susceptibility status of sand flies from populations around the world.

[Denlinger, D. S., J. A. Creswell, J. L. Anderson, C. K. Reese, and S. A. Bernhardt. 2016. Diagnostic doses and times for *Phlebotomus papatasi* and *Lutzomyia longipalpis* sand flies (Diptera: Psychodidae: Phlebotominae) using the CDC bottle bioassay to assess insecticide resistance. *Parasit. Vectors.* 9: 212.]

Chapter 5 examines the mechanisms of insecticide resistance in laboratory colonies of *Phlebotomus papatasi* and *Lutzomyia longipalpis* under artificial resistance selection to permethrin and malathion. We document our process in artificially selecting for resistance over the course of several years. We tested for evidence of target-site insensitivity and metabolic detoxification in multiple early-exposed generations. No evidence of target-site insensitivity or metabolic detoxification was found. We conclude by discussing possible reasons for the lack of these mechanisms. The ability for these populations to survive continued insecticide exposure for multiple years suggests that other mechanisms may be responsible

Chapter 6 builds upon Chapter 5 by examining the standing genetic variation of insecticide-susceptible *Phlebotomus papatasi* and *Lutzomyia longipalpis*. Susceptible individuals of each species were exposed to permethrin or malathion. Genotype-by-sequencing identified single nucleotide variants throughout the genomes of each species. We found, in all four treatments, that insecticide survival is a heritable trait with a modest genetic architecture and polygenic mechanisms. Several variants were found that associated strongly with survival, and their genetic consequences were determined using VectorBase. To conclude, the evolutionary implications of monogenic and polygenic resistance are discussed.

Chapter 7 is the concluding chapter where I summarize the results found in Chapters 2-6. I finish by making evolutionary comparisons between the global issues of insecticide resistance and antimicrobial resistance, and I conclude by speaking to the social media movement in bringing global awareness to these critical issues that we face.

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CHAPTER 2
COMPARISON OF *IN VIVO* AND *IN VITRO* METHODS FOR BLOOD FEEDING
PHLEBOTOMUS PAPTASI (DIPTERA: PSYCHODIDAE) IN THE
LABORATORY¹

Abstract

Phlebotomus papatasi Scopoli is a medically-important insect that has been successfully colonized in the laboratory, and blood-feeding is critical for colony propagation. There has been much interest in developing established protocols for *in vitro* blood-feeding systems. The objective of this study was to determine if a Parafilm membrane and a hog's gut membrane could be successfully used with *in vitro* feeding systems. We evaluated percentages of *P. papatasi* females that blood-fed on different blood-feeding systems (a mouse, a Hemotek® feeder, or a glass feeder) used with either a Parafilm or a hog's gut membrane, with cohorts of 250 and 500 *P. papatasi* females, and with or without external exhalations. For all feeding system combinations, female *P. papatasi* blood-fed in higher percentages when in cohorts of 500 individuals and in the presence of exhalations. Higher percentages of *P. papatasi* fed on a mouse, but this study also demonstrates that *P. papatasi* will readily feed with *in vitro* feeding systems using a Parafilm membrane or a hog's gut membrane. This study suggests that female *P. papatasi* may use an invitation effect to blood-feed and are attracted to blood sources via chemical olfaction cues, both of which have been characterized in other blood-feeding arthropods. Our study demonstrates that a Parafilm membrane or a hog's gut membrane, in conjunction with the Hemotek® or glass

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feeder system, is potentially a viable alternative to live rodents to blood-feed a colony of *P. papatasi*.

The establishment of laboratory colonies is critical for understanding the biology of arthropods that vector disease agents (Yaghoobi-Ershadi et al. 2007). Researchers using laboratory colonies of phlebotomine sand flies (Diptera: Psychodidae) have been able to study sand fly systematics, physiology, insecticide efficacy, disease transmission, and vaccine development (Rowton et al. 2008, Volf and Volfova 2011). Fewer than 60 sand fly species have been successfully reared in the laboratory, and even fewer have been reared in large numbers (Maroli et al. 1987, Harre et al. 2001, Chelbi and Zhioua 2007, Ivović et al. 2007, Mann and Kaufman 2010, Alarcón-Elbal et al. 2011, Castillo et al. 2015, Oliveira et al. 2015, Goulart et al. 2015).

Phlebotomus papatasi Scopoli, the principal vector of *Leishmania major*, the agent of cutaneous leishmaniasis, is one sand fly species that has been successfully colonized in the laboratory (Chelbi and Zhioua 2007). Laboratory *P. papatasi* females blood feed on anesthetized rodents (e.g. mice, hamsters, guinea pigs) to acquire a bloodmeal. The blood of these rodents yields sufficient sand fly fecundity, and *P. papatasi* females are able to readily adapt to feeding on these laboratory hosts (Modi and Rowton 1999, Harre et al. 2001, Volf and Volfova 2011).

To maintain colonies of sand flies, a large number of rodents are required to meet the sand fly feeding demands. The cost and maintenance of supporting rodent colonies have advocated for alternative blood-feeding methods to be investigated (Ward et al. 1978, Harre et al. 2001). Rowton et al. (2008) showed that membrane-feeding was a viable alternative to anesthetized hamsters in terms of fecundity and the hatching success of eggs of *P. papatasi*.

The use of Parafilm has garnered little attention as a potential membrane for *in vitro* membrane-feeding. Ready (1978) found that *Lutzomyia longipalpis* fed more intensely through a chick skin membrane than a Parafilm membrane. In that same year, Ward et al. (1978) found that *Lu. flaviscutellata* did not successfully feed through Parafilm membrane. Overall, Parafilm has not been endorsed as a viable, alternative membrane (Volf and Volfova 2011). In addition, chicken membranes are often used with *in vitro* feeding systems for *Phlebotomus* and *Lutzomyia* species (Harre et al. 2001, Noguera et al. 2006, Rowton et al. 2008), but a hog's gut membrane has been used for feeding *Lu. shannoni* (Mann and Kaufman 2010). In this study, we demonstrate that *P. papatasi* females feed through a Parafilm membrane and a hog's gut membrane using a Hemotek® feeding system, as well as successfully demonstrating the use of a hog's gut membrane with a glass feeder system.

Materials and Methods

***Phlebotomus papatasi* Colony.** The *Phlebotomus papatasi* sand flies used in this study were from a laboratory colony at Utah State University (USU, Logan, UT). This colony was derived from a long-established *P. papatasi* colony maintained at Walter Reed Army Institute of Research (Silver Spring, MD). All stages were reared in an environmental growth chamber at 25°C, 85% relative humidity, and a photoperiod of 16:8 (L:D) h according to methods of Lawyer et al. (1991) and Modi and Rowton (1999). Larvae were fed a composted 1:1 mixture of rabbit feces and rabbit food (Young et al. 1981, Volf and Volfova 2011). Adults were provided 30% sucrose-water solution daily on saturated cotton balls.

Only female *P. papatasi* were used in this experiment. All females used were at least 2-d post-eclosion and had never blood-fed. The blood feed trials occurred on the same day and time (between 0900 and 1100 hours), and within the same growth chamber as the main laboratory sand fly colonies. Adult females used in the feeding trials were aspirated and counted from the main colony and released into 24x24x24" cages (BioQuip, Rancho Dominguez, CA).

Feeding Trials. Four replicates of each treatment combination (feeding system: membrane, 250 or 500 adult female *P. papatasi*, and with or without external exhalations) were completed. For trials with exhalations, the investigator exhaled in the direction of the feeder unit ten times every five minutes. The same investigator exhaled for all the replicates. The exhalations were performed to simulate natural carbon dioxide emissions from an animal host. Female sand flies that had blood-fed were visually confirmed and were counted as blood-fed if they were fully engorged or if they had any blood that was visible in the gut.

***In vitro* Membranes.** Two membranes for *in vitro* blood-feeding were used in this experiment: hog's gut and Parafilm. Hog's gut was cleaned with de-ionized water and stored at -20°C until used. On the day a piece was to be used, the membrane was brought to 25°C and blotted dry before being used with the feeder units. For Parafilm (Neenah, WI), on the day a piece was to be used, the piece was cut, stretched, and wrapped around an investigator's arm for 10 minutes. Wrapping the Parafilm around the investigator's arm was an attempt to allow sweat and odorants to adsorb onto the Parafilm to lure the females to the heated blood source. The Parafilm was then removed from the arm such that the surface in contact with the skin was the outer membrane and in direct contact with the probing sand flies. The Parafilm was further stretched, tightened, and sealed to an artificial feeding unit.

***In vitro* Artificial Feeders.** Two *in vitro* artificial feeding systems were used for this experiment: the Hemotek® PS5 electrical feeder (Discovery Workshops, Accrington, United Kingdom) and glass feeders (Kontes Custom Glass, Vineland, NJ). For the Hemotek® feeder, the Parafilm or hog's gut membrane was secured to the feeder unit using an O-ring, and 1.5 ml of defibrinated bovine blood was added. The now-ready Hemotek® unit was attached to the heating source and set to 38°C, placed inside the sand fly cage on a stand, and female sand flies were allowed to blood-feed across the membrane for 1 h (Fig. 2.1A.). For the glass feeders, the Parafilm or hog's gut membrane was secured to the open end of the feeder using a rubber band,

and 1.5 ml of defibrinated bovine blood was added. One feeder was placed inside the sand fly cage, horizontally secured to a stand, and the blood was heated to 38°C from circulating water using a peristaltic pump (Woessner 2007) and a Fisher Scientific Isotemp™ model 2340 water bath (Fisher Scientific, Marietta, OH). For 1 h, female sand flies were allowed to blood-feed across the membrane.

Mouse Blood-Feed. One hairless mouse was anesthetized via intraperitoneal injection of a cocktail of ketamine/xylazine/acepromazine maleate. Once immobilized, the mouse was placed on its side inside the middle of the cage. Female sand flies were allowed to blood-feed on the mouse for 1 h (Fig. 2.1B.).

Statistical Analyses. Statistical analyses were performed using SAS/STAT 14.1 in the SAS System for Windows 9.4 TS1M3 using the GLIMMIX procedure (SAS Institute 2015). We conducted analyses of two data subsets to accommodate the fact that the mouse feeding system cannot be combined with “no exhalations” as it is a living organism.

The effects of an *in vitro* feeder (Hemotek® feeder or glass feeder), membrane (Parafilm or hog’s gut), exhalation (presence or absence), and number of females in the cage (250 or 500)



Fig. 2.1. Images of an aggregation of female *P. papatasi* sand flies feeding through a Parafilm membrane with the Hemotek® PS5 *in vitro* blood-feeder (A) and on anesthetized hairless mouse (B).

on the percentage of female *P. papatasi* that blood-fed were analyzed using a four-way factorial in a completely randomized design. The effects of feeding system:membrane (mouse, Hemotek® feeder:Parafilm membrane, Hemotek® feeder:hog's gut membrane, glass feeder:Parafilm membrane, glass feeder:hog's gut membrane) and number of females (250 or 500) on the percentage of female *P. papatasi* that blood-fed, only with exhalations, were assessed using a two-way factorial in a completely randomized design.

Both analyses used a generalized linear model with a binomial distribution and a logit link, with observation-level variance estimated to address overdispersion. Pairwise comparisons among means were adjusted for inflated Type I error using the Tukey method. A threshold of $\alpha = 0.05$ was used for all analyses.

Results

Feeding trials. Mean percentages of female *P. papatasi* that blood-fed in each trial combination are shown in Table 2.1. The mouse system had the highest observed mean percentage of females that blood-fed of any of the treatment combinations (38.3% with 500 females). The glass feeder with a hog's gut membrane, 500 females, and exhalations had the highest observed mean percentage of females that blood-fed of any *in vitro* combination (26.5%). The Hemotek® system with a Parafilm membrane, 250 females, and no exhalations had the lowest observed mean percentage of females that blood fed of any *in vitro* combination (0.8%).

***In vitro* Feeding Outcomes.** A higher percentage of female *P. papatasi* blood-fed in larger cohorts of 500 than in cohorts of 250 ($P = 0.011$). Presence of exhalations increased the percentage of female *P. papatasi* that blood-fed ($P < 0.001$), as well as the increase was more pronounced with the Hemotek® feeder ($P = 0.028$). The percentage of female *P. papatasi* that blood-fed was higher with hog's gut membranes than Parafilm ($P < 0.001$), particularly in the absence of exhalations ($P < 0.001$).

***In vivo* and *in vitro* Feeding Outcomes in the Presence of Exhalations.** The effect of

Table 2.1. Mean percentage (\pm SD) (n=4 per treatment combination) of female *P. papatasi* that blood-fed.

	Exhalations			
	No		Yes	
	No. of flies in replicate		No. of flies in replicate	
	250	500	250	500
Feeder:Membrane				
Mouse			26.4% (\pm 9.2%)	38.3% (\pm 3.4%)
Glass Feeder:Parafilm	1.0% (\pm 1.0%)	3.6% (\pm 0.6%)	7.0% (\pm 1.7%)	21.0% (\pm 3.1%)
Glass Feeder:Hog's Gut	15.6% (\pm 5.9%)	18.2% (\pm 3.9%)	14.9% (\pm 6.7%)	26.5% (\pm 10.9%)
Hemotek®:Parafilm	0.8% (\pm 0.7%)	1.3 % (\pm 0.7%)	8.4% (\pm 4.6%)	22.3% (\pm 2.7%)
Hemotek®:Hog's Gut	6.1% (\pm 3.1%)	5.5% (\pm 2.8%)	23.7% (\pm 12.6%)	23.6 % (\pm 7.5%)

cohort size on percentage of female *P. papatasi* that blood-fed was not the same for all five system:membrane combinations ($P = 0.041$). With a cohort of 500 female *P. papatasi*, the percentage of females that blood-fed with the mouse system was higher than the percentage with any *in vitro* feeding system (Hemotek® feeder:Parafilm membrane system, $P = 0.004$; Hemotek® feeder:hog's gut membrane system, $P = 0.010$; glass feeder:Parafilm membrane system, $P = 0.002$; and glass feeder:hog's gut membrane system, $P = 0.055$). With a cohort of 250 females, the percentage of females that blood-fed with the mouse system was larger than any *in vitro* feeding system using Parafilm (Hemotek® feeder:Parafilm membrane, $P = 0.008$; glass feeder:Parafilm membrane, $P = 0.004$). The mouse feeding system was not distinguishable from either hog's gut membrane feeding system (Hemotek® feeder:hog's gut membrane, $P = 0.987$; glass feeder:hog's gut membrane, $P = 0.185$). With the mouse system, the percentage of females that blood-fed was higher with cohorts of 500 than with 250 ($P = 0.028$).

Discussion

The findings from this study demonstrate that an *in vitro* feeding system using Parafilm and/or hog's gut membrane can be successfully used to feed female *P. papatasi* a blood meal, which counters previous reports that suggest that *in vitro* feeding systems with a Parafilm membrane will not adequately provide the required blood meal to sand flies (Ready 1978; Ward et al. 1978). This option reduces the cost burden, potential safety hazards, and the need for additional protocols associated with maintaining and handling live animals and controlled substances (Costa-da-Silva et al. 2014).

Our initial efforts with the Hemotek® feeder and a Parafilm membrane involved numerous iterations to find an effective blood feeding method. Preliminary attempts included the use of baited lures with octenol to attract female *P. papatasi* to the blood source, but these were not very effective at increasing the feeding rate. Using arm-wrapped Parafilm with intermittent exhaling into the cage near the Hemotek® feeder was a successful combination to lure *P. papatasi* females to the blood source. Many hematophagous Diptera are attracted to some combination of chemicals including carbon dioxide, water vapor, and host odors (Gibson and Torr 1999). Pinto et al. (2001) found that the closer carbon dioxide traps and human-baited traps were positioned to one another, the fewer *Lutzomyia* sand flies were trapped in carbon dioxide traps compared to human-bait traps. Bernier et al. (2008) found that traps baited with carbon dioxide and human hair captured more sand flies, although not significantly more, than traps with only carbon dioxide or with carbon dioxide plus octenol. Kline et al. (2011) discovered that black traps with body heat, moisture, and carbon dioxide captured roughly 40 times more *P. papatasi* than equivalent traps without carbon dioxide. The feasibility of humans to provide carbon dioxide in the form of human exhalants may not be deemed practical for long-term, large-scale mass rearing of *P. papatasi* in the laboratory. Other sources of carbon dioxide, such as compressed carbon dioxide, or less frequent intervals of human exhalations, should be considered when using

in vitro systems of blood-feeding. The research findings from this study though, suggest and support a combination of body odorants with carbon dioxide as a potent lure for sand flies.

Higher percentages of female *P. papatasi* blood-fed when in cohorts of 500 compared to 250. This effect may be explained by an aggregation behavior on hosts or blood feeding sites (Tripet et al. 2009). We observed that an aggregation would initiate when a single female probed the Parafilm membrane, hog's gut membrane, or mouse until the sand fly found a suitable location to blood-feed (Fig. 2.1A, 2.1B). Schlein et al. (1984) was able to characterize the invitational effect for *P. papatasi* via a pheromone released from the palps of females. The invitational effect has been characterized in another sand fly species, *Lutzomyia longipalpis*, as well as ceratopogonids, simuliids, and *Amblyomma* ticks (Norval et al. 1989, Blackwell et al. 1994, McCall and Lemoh 1997, Tripet et al. 2009). Aggregations of *Lu. longipalpis* during blood feeds have been suggested to benefit individual females by needing to produce less saliva, truncating the time needed complete blood-feeding, and having higher fecundity (Tripet et al. 2009).

The use of biological membranes with *in vitro* feeding systems has been demonstrated to be effective in blood-feeding sand flies (Harre et al. 2001, Noguera et al. 2006, Rowton et al. 2008, Mann and Kaufman 2010). Even with the seemingly low *P. papatasi* blood-feeding rates demonstrated in this study, a Hemotek® feeder with a Parafilm membrane has been used successfully at Utah State University to establish new colonies, as well as to maintain longstanding colonies. For example, we used a Hemotek® with Parafilm membrane system to obtain sufficiently large quantities of flies for the analysis of insecticide resistance (Denlinger et al. 2015, Denlinger et al. 2016). Even with an *in vitro* system feeding rate ranging from 8% to 22% (Table 2.1.), researchers working to establish a newly formed colony are capable of successfully feeding and capturing substantial numbers of female sand flies needed to oviposit on a weekly basis through multiple blood-feeds, thereby establishing a colony within a few

generations. We hypothesize that a larger colony (e.g. 750, 1000, or 2000 females) would increase the percentage of female *P. papatasi* that blood-feed. A limitation of this study was that fecundity rates were not evaluated for all feeding system combinations. It is important to note though, that during the initial months of establishing a *P. papatasi* colony at Utah State University when Parafilm was discovered to be an effective membrane, the colony consistently yielded sufficient numbers of viable eggs from generation to generation.

The *P. papatasi* colony used in this study originally derived from a 30-year established colony maintained at the Walter Reed Army Institute of Research. That colony has a history of blood-feeding using hamsters and was not pre-adapted for feeding across a membrane used with an *in vitro* feeding system. This history suggests that host-seeking traits in laboratory *P. papatasi* can be quickly selected for and that feeding on a different host or membrane (i.e. mouse, Parafilm membrane, or hog's gut membrane) does not have significant detrimental effects on fecundity.

Further studies could be developed to understand the success, utility, and impacts of an *in vitro* feeding system, with a Parafilm or hog's gut membrane, with respects to fecundity and hatching rates as a viable alternative where live animals are not feasible as a blood-source. For example, studies could include analyzing impact on fecundity and survival of recently field-collected sand flies, its utility in mass-rearing other laboratory-colonized sand fly species capable of vectoring *Leishmania*, and its ability for initiating and maintaining sand fly species that are not yet successfully colonized in the laboratory. Our study demonstrates that *in vitro* feeding system combinations were effective for a single colony of *P. papatasi*. Feeding success may vary for different geographic *P. papatasi* collections from around the world. In addition, *in vitro* blood-feeding systems, especially with a Parafilm membrane, may be viable for other laboratory uses, like vector competence analysis. For example, in studies examining sand fly vector competence, chick skin membranes have been used with *in vitro* blood-feeding systems for many types sand fly species with *Leishmania*-infected blood (Hlavacova et al. 2013; Pruzinova and Volf 2013;

Sadlova et al. 2013). The effect of Parafilm in lieu of a biological membrane needs to be investigated as a potential membrane in vector competence studies.

The findings from this study suggest that a Parafilm or hog's gut membrane used with either the Hemotek® or glass feeder system is well-suited for maintaining large *P. papatasi* colonies. These combinations can be considered as alternative feeding systems in lieu of rodents if the costs and maintenance of keeping rodents is prohibitive. This option could also potentially be used to conduct additional studies to further the understanding of vector competence and the sand fly's contribution to disease transmission.

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CHAPTER 3
ASSESSING INSECTICIDE SUSCEPTIBILITY OF LABORATORY *LUTZOMYIA*
LONGIPALPIS AND *PHLEBOTOMUS PAPATASI* SAND FLIES (DIPTERA:
PSYCHODIDAE: PHLEBOTOMINAE)²

Abstract

Chemical insecticides are effective for controlling *Lutzomyia* and *Phlebotomus* sand fly (Diptera: Psychodidae) vectors of *Leishmania* parasites. However, repeated use of certain insecticides has led to tolerance and resistance. The objective of this study was to determine lethal concentrations (LCs) and lethal exposure times (LTs) to assess levels of susceptibility of laboratory *Lutzomyia longipalpis* (Lutz and Neiva) and *Phlebotomus papatasi* (Scopoli) to 10 insecticides using a modified version of the WHO exposure kit assay and Centers for Disease Control (CDC) bottle bioassay. Sand flies were exposed to insecticides coated on the interior of 0.5-gallon and 1,000-ml glass bottles. Following exposure, the flies were allowed to recover for 24 h, after which mortality was recorded. From dose-response survival curves for *L. longipalpis* and *P. papatasi* generated with the QCal software, LC's causing 50, 90, and 95% mortality were determined for each insecticide. The LCs and LTs from this study will be useful as baseline reference points for future studies using the CDC bottle bioassays to assess insecticide susceptibility of sand fly populations in the field. There is a need for a larger repository of sand fly insecticide susceptibility data from the CDC bottle bioassays, including a range of LCs and

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LTs for more sand fly species with more insecticides. Such a repository would be a valuable tool for vector management.

Since their introduction in the 1940s, synthetic chemical insecticides remain an effective tool for controlling insects that are vectors of disease agents (Hemingway and Ranson 2000, World Health Organization [WHO] 2006). Unfortunately, insecticides have been used indiscriminately, exerting tremendous selective pressure for insecticide resistance (Feyereisen 1995, WHO 2006). The insecticide resistance phenotype is defined as a heritable, genetic change in response to insecticide exposure (Feyereisen 1995, Scott 1999, Hemingway et al. 2002). Increasing the insecticide dosage in response to resistance only exacerbates the problems of resistance by increasing the frequency of the genetic trait(s) in a vector population (Feyereisen 1995). Two resistance phenotypes observed in the field are target-site insensitivity and metabolic-detoxification resistance (Mallet 1989, Brogdon and McAllister 1998a, Rivero et al. 2010). Today, there is evidence of target-site insensitivity and metabolic-detoxification resistance to all classes of synthetic insecticides in all major vector species (Nauen 2007, Rivero et al. 2010). Acquiring data on vector species' susceptibility to insecticides will support the strategies directed at effectively managing these vector populations (Surendran et al. 2005). The following two techniques are commonly used to measure a vector species' susceptibility to insecticides: 1) the WHO exposure kit bioassay and 2) the Centers for Disease Control (CDC) bottle bioassay (CDC 2010, WHO 2013).

The WHO exposure kit bioassay is widely accepted because it can measure insecticide susceptibility in many species of insect vectors worldwide (Braverman et al. 2004, Ocampo et al. 2011, Faraj et al. 2012, Aizoun et al. 2013, Chen et al. 2013). The assays can be run with live insects collected in the field or with their progeny reared in the laboratory. The WHO bioassay is

a standardized protocol that consists of an exposure kit containing tubes lined with filter papers that are impregnated with a specific concentration of an insecticide (WHO 1998; 2013). Despite its accepted use, the WHO bioassay is expensive, filter papers are not available for some insecticides, and there is a limited range of concentrations that can be purchased for some insecticides (Perea et al. 2009, Aïzoun et al. 2013).

The CDC bottle bioassay is an inexpensive and portable alternative to the WHO bioassay, especially in regions where there is little money to implement the WHO bioassay (Perea et al. 2009, Aïzoun et al. 2013). The CDC bottle bioassay requires fewer test insects than the WHO bioassay (Aïzoun et al. 2013). The protocol consists of coating the interior of a glass bottle with an insecticide that has been diluted in a solvent. The solvent is then allowed to evaporate, leaving the insecticide coated to the glass surface. Once the bottles are treated, insects are introduced into the bottles and exposed to the insecticide for a specified amount of time (Brogdon and McAllister 1998b, CDC 2010, Aïzoun et al. 2013). Insect mortality can be scored at distinct time intervals during the exposure test (e.g., every 15 min for 1-h), and percent mortality at each time interval is plotted (Brogdon and McAllister 1998b). The CDC bottle bioassay can also be used as an end-point assay where mortality is only measured at the end of the exposure test. Susceptibility is measured by simply comparing mortality rates between insect populations (Perea et al. 2009).

Sand flies (Diptera: Psychodidae: Phlebotominae) are among the insect vectors that require resistance monitoring because they have been actively targeted with insecticides. Many sand fly species in the genera *Lutzomyia* and *Phlebotomus* are capable of vectoring *Leishmania* parasites, infection with which causes leishmaniasis, a disease currently infecting millions of people world-wide (Guerin et al. 2002, Rutledge and Gupta 2009). To control sand flies, populations around the world have been exposed to the four main classes of insecticides- 1) organochlorines 2) organophosphates, 3) carbamates, and 4) pyrethroids- via residual spraying, ultra-low volume spraying, insecticide-treated clothing, and insecticide-treated nets. These

exposures are either intentional in directed vector control efforts or are inadvertent as part of vector control efforts targeted against other insect vectors (Alexander and Maroli 2003, Surendran et al. 2005, Alexander et al. 2009, Henriquez et al. 2009, Rutledge and Gupta 2009, Dinesh et al. 2010, Faraj et al. 2012, Hassan et al. 2012, Saeidi et al. 2012).

Some sand fly populations have been found to be tolerant or resistant to the insecticides used in the Middle East, southern Asia, and South America. In Montes Claros, Brazil, 29 of 80 (36.3%) *Lutzomyia longipalpis* (Lutz and Neiva) survived a 0.05% deltamethrin exposure (Alexander et al. 2009). In a Delft Island population from Sri Lanka, 11 of 80 *Phlebotomus argentipes* (Annandale & Brunetti) (14%) had insensitive acetylcholinesterase, and 20 (25%) had elevated esterases, of which both of these findings are associated with resistance to malathion (Surendran et al. 2005). *P. argentipes* was found to be DDT-resistant throughout the Muzaffarpur, Vaishali, and Patna districts of the Bihar state, India, and in the Amahibelha village of the Sunsari district, Nepal, as only 43% and 62% of populations died from DDT exposure, respectively (Dinesh et al. 2010). In the Surogia village of Khartoum State, Sudan, 51 *Phlebotomus papatasi* (Scopoli) (79.7%) had insensitive acetylcholinesterase, which are associated with malathion and propoxur resistance. Both of these insecticides have been extensively used in this region as part of the anti-malaria mosquito control program (Hassan et al. 2012).

Many of the examples demonstrating reduced insecticide susceptibility in sand flies have been determined using the WHO bioassay. However, a few studies have used the CDC bottle bioassay to measure the susceptibility status of sand fly populations to insecticides (Santamaría et al. 2003, Alexander et al. 2009, Henriquez et al. 2009). These studies have been completed entirely in the New World. The CDC bottle bioassay is preferred over the WHO bioassay because the susceptibility results can be generated quickly, the bottles can be prepared with any

insecticide, the results are reproducible with fewer insects and fewer replicates, and the results allow one to infer the detoxification mechanism conferring resistance (Santamaría et al. 2003).

It is imperative to develop expansive baseline susceptibility data to different insecticides in different sand fly species and in flies from different geographic regions (CDC 2010). In addition, these bioassays require baseline data from known susceptible sand fly populations in order to assess insecticide-susceptibility in field populations and for the calculation of relative risk ratios (e.g., lethal concentration causing 50% mortality [LC₅₀] in a field population / LC₅₀ control population). These data will provide vector management programs the information necessary to ensure appropriate and effective insecticide application (Maharaj 2011). Potentially, CDC bottle bioassay is one tool that could be incorporated into sand fly surveillance programs to a greater extent worldwide, especially in regions where *Leishmania* transmission is a concern.

The objective of this study was to quantify, using a modified version of the WHO exposure kit assay and the CDC bottle bioassay, the susceptibility of laboratory *L. longipalpis* and *P. papatasi* to 10 insecticides that are incorporated globally in vector control efforts. Specifically, for each insecticide, a dose-response survival curve was produced. From each curve, LC₅₀, LC₉₀, and LC₉₅ values were determined. These doses can now be used for comparison in future studies to assess sand fly susceptibility to insecticides.

Materials and Methods

Sand Flies. Insecticide-susceptible *L. longipalpis* and *P. papatasi* sand fly colonies at Utah State University (USU) were derived from long-established colonies maintained at the Walter Reed Army Institute of Research (Silver Spring, MD). The original colonies are > 30 years old and have never been exposed to insecticides. All life stages were reared at USU at 25°C, 85% relative humidity, and a photoperiod of 16:8 (L:D) h according to methods of Lawyer et al. (1991) and Modi and Rowton (1999). Larvae were fed a composted 1:1 mixture of rabbit feces and rabbit food (Young et al. 1981; Volf and Volfova 2011). Adults were provided 30% sucrose-

water solution daily on saturated cotton balls, and adult female *L. longipalpis* and *P. papatasi* were blood-fed on anesthetized mice placed inside holding cages twice weekly.

Insecticides. Ten technical-grade insecticides were used in this study: four pyrethroids [cypermethrin (Sigma-Aldrich, St. Louis, MO), deltamethrin (Sigma-Aldrich, St. Louis, MO), lambda(λ)-cyhalothrin (Sigma-Aldrich, St. Louis, MO), and permethrin (Chem Service, Inc., West Chester, PA)]; three organophosphates [chlorpyrifos (Sigma-Aldrich, St. Louis, MO), fenitrothion (Sigma-Aldrich, St. Louis, MO), and malathion (Chem Service, Inc., West Chester, PA)]; two carbamates [bendiocarb (Sigma-Aldrich, St. Louis, MO) and propoxur (Sigma-Aldrich, St. Louis, MO)]; and the organochlorine DDT (Sigma-Aldrich, St. Louis, MO). The concentrations of each insecticide to which *L. longipalpis* and *P. papatasi* were exposed are provided in Table 3.1. The diagnostic doses for *Anopheles* and *Aedes* mosquitoes were used as starting reference points for initial insecticide exposure (CDC 2010). Concentrations higher and lower than these diagnostic doses were determined to derive the dose-response survival curves for the two sand fly species. All insecticide dilutions were prepared in acetone, stored in glass bottles, wrapped in aluminum foil, and refrigerated while not being used (CDC 2010).

Preparation of Exposure Bottles. On the day prior to exposing the sand flies, 0.5-gallon glass bottles (1,892.5 ml) (unknown maker) or 1,000-ml glass bottles (Fisher Scientific, Pittsburgh, PA) were prepared by coating them with insecticide. For both bottle sizes, the concentration of insecticide in each bottle was determined to be X μg per bottle (CDC 2010). For a 250-ml bottle, 1 ml of insecticide at 10 μg insecticide/ml acetone gives a concentration of 10 μg /250 ml bottle. To maintain an equivalence of 10 μg insecticide/250 ml bottle to compensate for the larger bottle sizes, 4.0 ml of 10 μg insecticide/ml acetone is needed to coat the interior of the 1,000-ml bottle, and 7.57 ml of 10 μg insecticide/ml acetone is needed to coat the interior of the 0.5-gallon bottle. The bottles were coated with insecticide by swirling the acetone:insecticide solution on the bottom, on the sides, and on the lid. The bottle was then placed on a mechanical

Table 3.1. Concentrations of ten insecticides used in the CDC bottle bioassays to expose *L. longipalpis* and *P. papatasi* sand flies.

Insecticide Class	Insecticide	Species	Concentration (μg insecticide per bottle)
Pyrethroid	Cypermethrin	<i>L. longipalpis</i>	1, 5, 10, 25, 50, 100, 125, 150
		<i>P. papatasi</i>	0.5, 1, 5, 10, 25, 50, 75, 100, 125, 150, 175, 200, 225, 250
	Deltamethrin	<i>L. longipalpis</i>	0.01, 0.1, 1, 5, 10, 25, 50, 75
		<i>P. papatasi</i>	5, 10, 25, 50, 100, 125, 150, 200
	λ -Cyhalothrin	<i>L. longipalpis</i>	0.01, 0.05, 0.1, 0.5, 1, 5, 10, 25, 50, 75
		<i>P. papatasi</i>	0.01, 0.05, 0.1, 0.5, 1, 5, 10, 25, 50
Permethrin	<i>L. longipalpis</i>	1, 5, 10, 25, 50, 75, 100	
	<i>P. papatasi</i>	5, 10, 25, 50, 75, 100, 125, 150, 175, 200, 225, 250	
Organophosphate	Chlorpyrifos	<i>L. longipalpis</i>	0.01, 0.05, 0.1, 0.5, 1, 5, 10
		<i>P. papatasi</i>	0.001, 0.01, 0.05, 0.1, 0.5, 1, 5, 10
	Fenitrothion	<i>L. longipalpis</i>	0.1, 0.5, 1, 3, 5, 10, 25, 50
		<i>P. papatasi</i>	0.1, 0.5, 1, 3, 5, 10, 25, 50
	Malathion	<i>L. longipalpis</i>	5, 8, 10, 12, 15, 25, 50, 100
		<i>P. papatasi</i>	10, 25, 50, 100, 125, 150
Carbamate	Bendiocarb	<i>L. longipalpis</i>	0.01, 0.1, 1, 5, 10, 25, 50, 75, 100, 125, 150, 175, 200
		<i>P. papatasi</i>	0.01, 0.1, 0.5, 1, 5, 10, 25
	Propoxur	<i>L. longipalpis</i>	0.01, 0.1, 0.5, 1, 5, 10, 25, 50, 75, 100
		<i>P. papatasi</i>	0.01, 0.1, 0.5, 1, 5, 10, 25, 50, 75
Organochlorine	DDT	<i>L. longipalpis</i>	10, 25, 50, 75, 100, 150, 200, 300, 350, 450
		<i>P. papatasi</i>	5, 10, 25, 50, 75, 100, 150, 200, 300, 350

bottle roller under a chemical hood for 30 minutes to dry. During this time, the lids were slowly loosened to allow the acetone to evaporate. After 30 minutes, the caps were removed, and the bottles were rolled until all of the acetone had evaporated. The bottles were then left open to dry overnight. For each test replicate, one bottle serving as a control was coated with either 7.57 ml or 4.0 ml of acetone depending on its volume. All bottles were re-used throughout the duration of the experiment. To clean a bottle with residual insecticide, the bottle and lid was first triple-rinsed with acetone; filled with warm, soapy water; drained; rinsed and filled with cold water; drained; and autoclaved for at least 20 minutes. After being autoclaved, the bottles were left to dry for at least one day before being used again (CDC 2010). Each cleaned bottle also underwent testing to determine the presence of residual insecticide. Ten sand flies were aspirated into each

bottle and were left in the bottle for at least 3 h. If no mortality was observed at the end of the 3 h, the bottles were cleared and allowed to be re-used. If mortality was observed, the bottles were cleaned again and re-tested until no mortality was observed.

Insecticide Exposure Tests. Approximately 12 h after the bottles were prepared with insecticide, adult sand flies at least 2 d post-eclosion were aspirated from the main colony and gently blown into each bottle: 40-50 flies into each 0.5-gallon bottle and 20-30 flies into each 1,000-ml bottle. Approximately equal numbers of un-fed female and male flies were used for each replicate. At least three replicates were completed for each concentration of every insecticide.

Both species were exposed for the same length of time to each insecticide. In preliminary tests, exposure time for all 10 insecticides was 60 min, but it was soon discovered that for some insecticides, 60 min of exposure was either too short or too long because sand fly survival was nearly 0 or 100% for most of the insecticide concentrations (Brogdon and McAllister 1998b). Therefore, the range of exposure times was adjusted to 30 min or to 120 min depending on unexpected and actual sand fly survival rates (Table 3.2.) (CDC 2010).

The sand flies were captured after insecticide exposure via mechanical aspiration,

Table 3.2. Length of exposure of *L. longipalpis* and *P. papatasi* to ten insecticides with the CDC bottle bioassay.

Insecticide	Exposure Time (Minutes)
Cypermethrin	60
Deltamethrin	60
λ -cyhalothrin	60
Permethrin	60
Chlorpyrifos	60
Fenitrothion	30
Malathion	60
Bendiocarb	30
Propoxur	30
DDT	120

released into 1-pint cardboard containers with a fine mesh screen top, and kept under the same temperature, light, and humidity environment as the main, untreated colonies. A cotton ball saturated with 30% sugar-water was placed on the top of each container as an energy/water source. Using procedures established for mosquitoes, sand flies were held in these containers for 24 h prior to mortality being recorded (Saavedra-Rodriguez et al. 2008). Mortality was scored as a complete cessation of movement (Perea et al. 2009). A 24 h holding period was used because in some preliminary experiments, many of the sand flies that appeared physically affected, and would have been scored as dead at the end of a 30, 60, or 120 min exposure period as described in Brogdon and McAllister (1998b), recovered after this 24-h period.

If mortality in the control group ranged between 5 and 20%, mortalities in the experimental bottles of that test group were corrected using Abbott's formula (CDC 2010). Abbott's formula was not used to correct experimental mortalities if the control group mortality was <5%. If control group mortalities exceeded 20%, the entire testing replicate was not used (Saeidi et al. 2012).

Survival Curves. Using the QCal software, a dose-response survival curve was created for each insecticide (Lozano-Fuentes et al. 2012). This software can be used for any insect vector with data from insecticide bioassays. The QCal software also uses a logistic regression model to generate LC_{50s} , LC_{90s} , and LC_{95s} for each insecticide. Mortalities corrected with Abbott's formula were rounded to the nearest whole fly. For example, a cohort of 30 flies had an empirical mortality of 80% (24 flies died). If 80% was Abbott's-corrected to 78.1% mortality, then 23.43 flies died. In QCal, a mortality of 23 flies of 30 was recorded.

Results

Physical Observations. Both *L. longipalpis* and *P. papatasi* sand fly species shed their legs when exposed to cypermethrin, deltamethrin, lambda-cyhalothrin, and permethrin during and after exposure. This was observed predominantly at the higher concentrations of each insecticide. Neither species shed its legs when exposed to organophosphates, carbamates, or DDT. In addition, for the pyrethroids, both *L. longipalpis* and *P. papatasi* experienced the “knockdown effect,” evident by involuntary movements and muscle spasms, during insecticide exposure and during the initial recovery time in the holding containers (Martins et al. 2009). At lower concentrations of the four pyrethroids, many sand flies were able to recover from the knockdown (no convulsions or erratic movements) by the completion of the 24-h holding period. At higher pyrethroid concentrations, sand flies succumbed to muscle spasms, convulsions, and paralysis.

It was also observed that the time required for the carbamates, organophosphates, and organochlorine (DDT) to cause mortality differed. The carbamates were lethal very quickly, causing death only a few minutes after the sand flies were aspirated into the bottles. This quick lethality necessitated a reduction in the exposure time of both sand fly species to the carbamates (Table 3.2.). On the other hand, the three organophosphates and DDT caused delayed mortality. Many sand flies appeared physically healthy after exposure to these insecticides, but died during the 24-h holding period.

Survival Curves. A dose-response survival regression analysis was performed for *L. longipalpis* and *P. papatasi* to estimate LC₅₀, LC₉₀, and LC₉₅ for all 10 insecticides. Figure 3.1. shows each species’ survival curve for cypermethrin (pyrethroid), chlorpyrifos (organophosphate), propoxur (carbamate), and DDT (organochlorine). These graphs were produced in GraphPad Prism (version 6.0, GraphPad Software Inc., San Diego, CA). Table 3.3

shows the QCal logistic regression parameters and the extrapolated LC₅₀, LC₉₀, and LC₉₅ values for each insecticide for both species. For many insecticides, the LC₉₅ was substantially greater than the LC₉₀ (e.g., *P. papatasi*'s LC₉₀ for cypermethrin was 73.279 µg cypermethrin per bottle, while its LC₉₅ for cypermethrin was 150.010 µg cypermethrin per bottle), which may be attributed to the sigmoidal shape of the logistic curve, where it takes much higher doses to reach a smaller percentage change in mortality (i.e., LC₉₀ to LC₉₅) nearing the 100% mortality asymptote.

Pyrethroids. *Lutzomyia longipalpis* and *P. papatasi* have very similar LC₅₀'s for cypermethrin, roughly 9.0 µg cypermethrin per bottle; however, *P. papatasi* has an LC₉₅ more

Table 3.3. QCal logistic regression parameters and lethal concentration (LC) values causing 50, 90, and 100% mortality in *L. longipalpis* and *P. papatasi* exposure to ten insecticides with the CDC bottle bioassay.

Insecticide	Species	LC50 (µg insecticide per bottle) [LL, UL]*	LC90 (µg insecticide per bottle) [LL, UL]*	LC95 (µg insecticide per bottle) [LL, UL]*
Cypermethrin	<i>L. longipalpis</i>	8.955 [7.888, 10.167]	41.851 [35.499, 49.338]	70.704 [57.530, 86.886]
	<i>P. papatasi</i>	8.897 [7.499, 10.556]	73.279 [61.313, 87.584]	150.010 [120.265, 187.354]
Deltamethrin	<i>L. longipalpis</i>	0.922 [0.637, 1.334]	28.707 [18.291, 45.056]	92.434 [51.594, 165.571]
	<i>P. papatasi</i>	9.907 [8.165, 12.020]	90.244 [67.938, 119.869]	191.290 [130.804, 279.779]
λ-Cyhalothrin	<i>L. longipalpis</i>	0.232 [0.189, 0.284]	5.001 [3.627, 6.895]	14.215 [9.487, 21.298]
	<i>P. papatasi</i>	0.269 [0.217, 0.334]	3.654 [2.625, 5.087]	8.873 [5.863, 13.430]
Permethrin	<i>L. longipalpis</i>	17.069 [14.889, 19.570]	82.402 [65.957, 102.946]	140.752 [105.890, 187.073]
	<i>P. papatasi</i>	41.344 [37.233, 45.906]	188.579 [162.796, 218, 438]	315.955 [261.648, 381.572]
Chlorpyrifos	<i>L. longipalpis</i>	0.458 [0.377, 0.557]	5.734 [4.058, 8.099]	13.538 [11.695, 29.020]
	<i>P. papatasi</i>	0.327 [0.256, 0.419]	6.417 [4.102, 10.037]	17.653 [10.135, 30.774]
Fenitrothion	<i>L. longipalpis</i>	0.347 [0.277, 0.434]	2.655 [1.933, 3.647]	5.306 [3.549, 7.934]
	<i>P. papatasi</i>	1.368 [1.173, 1.595]	7.334 [5.684, 9.489]	13.007 [9.478, 17.850]
Malathion	<i>L. longipalpis</i>	8.432 [8.004, 8.883]	13.815 [12.914, 14.779]	16.340 [14.957, 17.852]
	<i>P. papatasi</i>	20.011 [17.277, 23.176]	77.008 [63.459, 93.447]	121.778 [94.869, 156.319]
Bendiocarb	<i>L. longipalpis</i>	0.986 [0.737, 1.318]	38.961 [29.312, 52.159]	136.047 [94.292, 196.311]
	<i>P. papatasi</i>	0.289 [0.232, 0.359]	2.507 [1.875, 3.353]	5.229 [3.632, 7.529]
Propoxur	<i>L. longipalpis</i>	3.837 [2.860, 5.148]	75.446 [46.150, 123.347]	207.763 [112.101, 385.060]
	<i>P. papatasi</i>	5.502 [4.524, 6.692]	39.135 [28.126, 54.451]	76.264 [50.729, 114.652]
DDT	<i>L. longipalpis</i>	28.364 [23.173, 34.716]	218.581 [166.052, 287.724]	437.685 [303.506, 631.249]
	<i>P. papatasi</i>	15.047 [11.321, 19.997]	295.979 [196.684, 445.412]	815.173 [463.219, 1434.541]

*LL = lower 95% confidence limit; UL = upper 95% confidence limit

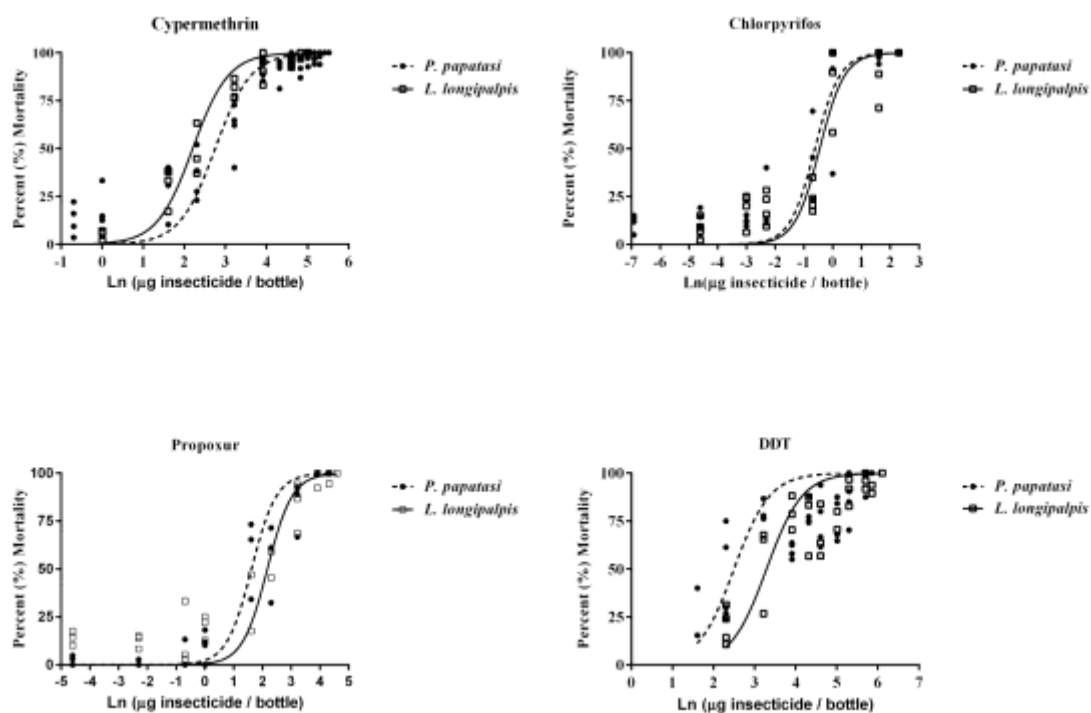
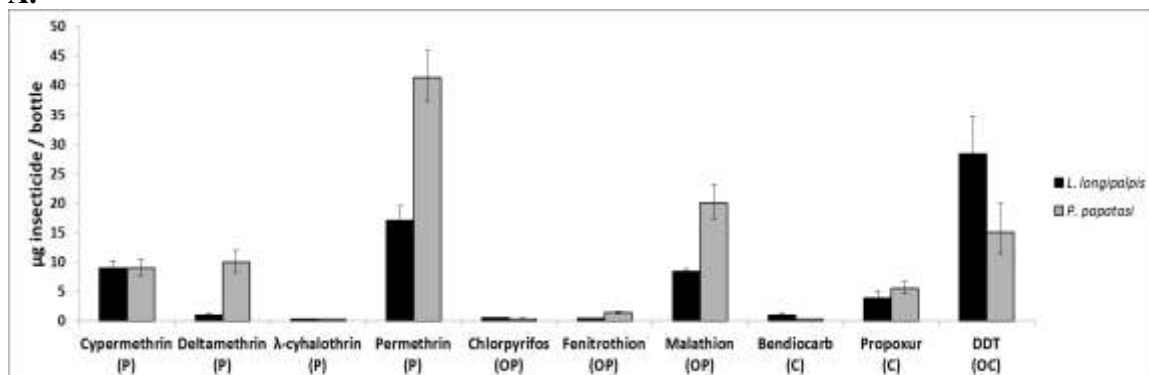


Fig. 3.1. *L. longipalpis* and *P. papatasi* dose-response survival curves to cypermethrin (pyrethroid), chlorpyrifos (organophosphate), propoxur (carbamate), and DDT (organochlorine).

than twice as large as *L. longipalpis* (Table 3.3.). For deltamethrin, *L. longipalpis* has a 10-fold lower LC_{50} than *P. papatasi* (Fig. 3.2A.) and a much lower LC_{90} and LC_{95} than *P. papatasi* (Fig. 3.2B.; Table 3.3.). *Lutzomyia longipalpis* and *P. papatasi* have very similar lethal concentration values for lambda-cyhalothrin, and both species are very susceptible as their LC_{50} , LC_{90} , and LC_{95} values are <20.0 μg lambda-cyhalothrin per bottle, which are the lowest LC_{95} values for of the four pyrethroid insecticides (Table 3.3.). For permethrin, *P. papatasi* has a LC_{50} , LC_{90} , and LC_{95} that are at least twice as large compared with those same LC values of *L. longipalpis*.

Organophosphates. Both sand fly species are highly susceptible to chlorpyrifos and fenitrothion. The LC_{95} 's for both *L. longipalpis* and *P. papatasi* are <20.0 μg per bottle. Besides *P. papatasi*'s LC_{95} for bendiocarb and both species LC_{95} 's for lambda-cyhalothrin, these are the lowest LC_{95} 's for all 10 insecticides (Table 3.3.). In addition, the LC_{50} 's for both species to

A.



B.

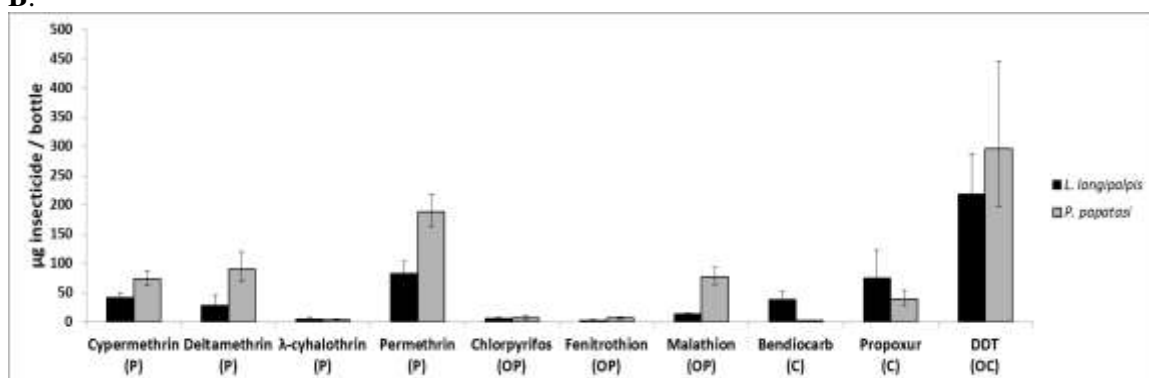


Fig. 3.2. Bar graphs of *L. longipalpis* and *P. papatasi* lethal concentrations causing 50% mortality (LC₅₀) (2A) and 90% mortality (LC₉₀) (2B) for 10 insecticides. Error bars represent the lower and upper 95% confidence intervals as determined by QCal. Letters in parentheses below each insecticide represent the insecticide class: P = pyrethroid; OP = organophosphate; C = carbamate; OC = organochlorine.

chlorpyrifos are <0.5 µg chlorpyrifos per bottle. Like chlorpyrifos, both *L. longipalpis* and *P. papatasi* are highly susceptible to fenitrothion (Table 3.3.) even with exposure times of 30 minutes. *P. papatasi* has a LC₉₅ malathion that is approximately eight times larger than *L. longipalpis*' LC₉₅ for malathion.

Carbamates. *Lutzomyia longipalpis* has a smaller LC₉₅ than *P. papatasi* to all of the pyrethroids and to all of the organophosphates except lambda-cyhalothrin. For the carbamates, *P. papatasi* is more susceptible than *L. longipalpis* to bendiocarb and propoxur. The exposure time for both species is 30 minutes. In preliminary tests for the carbamates, ~ 100% mortality was observed for all of the insecticide doses with a 60-min exposure time. Therefore, the duration of

exposure was reduced to 30 min, which was a sufficient amount of time to obtain 50, 90, and 95% mortality (Table 3.3.). *P. papatasi* has a LC₉₅ for bendiocarb that is 26 times lower than *L. longipalpis*' bendiocarb LC₉₅ (Table 3.3), and *P. papatasi* has a LC₉₀ of bendiocarb that is ~15 times lower than *L. longipalpis*' bendiocarb LC₉₀ (Fig. 3.2B.). Both species have a LC₅₀ <1.0 µg bendiocarb per bottle (Fig. 3.2A.). *P. papatasi* has a much lower LC₉₅ for propoxur (LC₉₅ = 76.264 µg propoxur per bottle) than *L. longipalpis* (LC₉₅ = 207.763 µg propoxur per bottle). However, *P. papatasi* does have a greater LC₅₀ to propoxur than does *L. longipalpis* (Fig. 3.2A.).

Organochlorine. In preliminary tests with DDT, 60 minutes was insufficient to quantify 50, 90, and 95% mortality with all of the insecticide doses. Therefore, the duration of exposure was increased to 120 minutes to allow sufficient time to obtain these values for both *L. longipalpis* and *P. papatasi*. Even with this extended exposure period, both species have very high LC₉₅'s (437.729 µg DDT per bottle and 815.173 µg DDT per bottle for *L. longipalpis* and *P. papatasi*, respectively). These are the highest LC₉₅'s for any of the 10 insecticides evaluated in this study (Table 3.2.).

Discussion

The objective of this study was to quantify insecticide susceptibility in laboratory *L. longipalpis* and *P. papatasi* to 10 insecticides comprising four chemical classes using a modified version of the CDC bottle bioassay. It was demonstrated that this modified CDC bottle bioassay is an effective tool for measuring the susceptibility of these two sand fly species to pyrethroid, organophosphate, carbamate, and organochlorine insecticides.

One important observation of this study was that different insecticide classes have different LTs. Organophosphate insecticides caused delayed mortality, while carbamate insecticides caused mortality extremely quickly, although both insecticide classes have similar modes of action: inhibiting the acetylcholinesterase enzyme from hydrolyzing acetylcholine (Fukuto 1990). Despite the differences in kill rates for carbamates and organophosphates, *L.*

longipalpis and *P. papatasi* are most susceptible to the carbamates bendiocarb and propoxur and to the organophosphate fenitrothion. A 30-minute exposure to these insecticides is sufficient to cause 100% mortality in these sand fly species. *Aedes* and *Anopheles* mosquitoes both have diagnostic LTs of 30 min for bendiocarb and fenitrothion using the CDC bottle bioassay (CDC 2010). For vector control programs aimed at targeting sand flies with synthetic insecticides, bendiocarb, propoxur, and fenitrothion deserve attention for their efficacy.

Conversely, of the 10 insecticides tested, both *L. longipalpis* and *P. papatasi* are least susceptible to DDT. Even with an exposure time of 120 min, the longest exposure time of the 10 insecticides, both species' LC₉₅'s are very large: at least 400 µg DDT per bottle. Unlike pyrethroids, which inhibit the sodium channels involved in action potential propagation in the central nervous system and in the peripheral nervous system, DDT only blocks the sodium channels in the peripheral nervous system (Davies et al. 2007). Only affecting the peripheral nervous system requires more time and higher doses to cause excitatory paralysis that leads to death (Davies et al. 2007). Similar results have been found in insecticide-susceptible Italian *P. perniciosus* and *P. papatasi*, where the LT₅₀'s and LT₉₀'s for DDT were longer compared with permethrin and lambda-cyhalothrin (Maroli et al. 2002). Also, Saeidi et al. (2012) found both insecticide-susceptible male and female *P. papatasi* to have much longer LT₅₀'s and LT₉₀'s to DDT than to permethrin, deltamethrin, cyfluthrin, and lambda-cyhalothrin.

For many years, DDT has been used worldwide to control sand flies by direct intervention or inadvertently as a collateral benefit of anti-malaria campaigns (Kaul et al. 1994, Alexander and Maroli 2003, Surendran et al. 2005, Kishore et al. 2006, Dinesh et al. 2010, Afshar et al. 2011, Faraj et al. 2012, Saeidi et al. 2012). Our results suggest that laboratory colonies of insecticide-susceptible sand flies are not very susceptible to DDT. Despite reports of sand fly tolerance and resistance to DDT in India, Iran, Nepal, and Turkey (WHO 1986, Kaul et al. 1994, Yaghoobi-Ershadi and Javadian 1995, Dinesh et al. 2010, Afshar et al. 2011), DDT's use for

indoor residual spraying is still permitted (WHO 2007). The data from this study suggest that large doses of DDT are required, which may produce strong selection pressure for resistance if it not applied correctly or at appropriate times (Maharaj 2011). Compounded with years of DDT use, and the potential for underlying low levels of tolerance and resistance, field populations of sand flies may be able to develop resistance to DDT more quickly than to other insecticides.

The shedding of legs in response to exposure to the four pyrethroids used in this study was evident for both *L. longipalpis* and *P. papatasi*. A similar phenomenon was observed in *L. longipalpis* from Brazil when exposed to permethrin, deltamethrin, and lambda-cyhalothrin (Alexander et al. 2009). It is suggested that sand flies lacking one or more legs will be unable to blood-feed effectively, which could subsequently reduce the potential to vector *Leishmania* parasites (Alexander et al. 2009). However, we have consistently observed that laboratory *L. longipalpis* and *P. papatasi* exposed to pyrethroids that have shed one or more legs are still capable of blood-feeding on anesthetized mice (unpublished data). Female sand flies with shed legs, and with a mature *Leishmania* infection, which probe the skin of a vertebrate host have also been shown to transmit *Leishmania* parasites without a complete blood-meal. During probing, *Leishmania* metacyclic promastigotes are regurgitated in attempt of the female sand fly to clear her alimentary canal of the *Leishmania*-secreted promastigote secretory gel (PSG) (“blocked-fly hypothesis”) (Bates 2007).

One future study could quantify and evaluate the ability of surviving sand flies with shed legs that have been routinely exposed to pyrethroids or DDT with the persistence of probing vertebrate hosts. Rogers and Bates (2007) demonstrated that female sand flies infected with *Leishmania* metacyclic promastigotes are manipulated by the *Leishmania* to increase their biting persistence, leading to an increase in the number of parasites transmitted to the vertebrate host. We have observed that a loss of legs is a potential physical challenge for the female sand fly. When other sand flies are in the vicinity of the female with shed legs during a blood feeding

event, the female with shed legs would often lose her balance and would need to relocate to find a suitable position to probe and blood-feed. Increased probing because of a physical challenge, in combination with *Leishmania* manipulation could theoretically increase probing and the number of parasites vectored to a host. These hypothetical scenarios apply to pyrethroid and DDT insecticides. Future studies with organophosphate and carbamate insecticides, which do not cause sand flies to shed their legs, and their effect on surviving flies' ability to probe and transmit *Leishmania* warrant investigation as well.

Another observation of this study is the difference between the LC values of the Type I and Type II pyrethroid insecticides. Type I pyrethroids, including permethrin, have been described to cause sodium channel modifications that can last up to tens of milliseconds and are better at causing knockdown in insects. Whereas Type II pyrethroids, including cypermethrin, deltamethrin, and lambda-cyhalothrin, cause sodium channel modifications that can last for many seconds and are better at causing mortality in insects (Davies et al. 2007). In this study, permethrin LC₅₀'s for both *L. longipalpis* and *P. papatasi* were greater than cypermethrin, deltamethrin, and lambda-cyhalothrin LC₅₀'s (Fig 2A; Table 3). These findings at the LC₅₀ support previous research and are consistent with the physiological differences between the two types of pyrethroids in that it takes a higher concentrations of permethrin (Type I pyrethroid) to cause 50% mortality than it does cypermethrin, deltamethrin, or lambda-cyhalothrin (Type II pyrethroids) (Fletcher and Axtell 1993, Jirakanjanakit et al. 2007).

One potential limitation of this study is that we used well-established, laboratory-adapted strains of *L. longipalpis* and *P. papatasi*. All the female sand flies used in this experiment were nulliparous. Comparisons of the efficacy of the 10 insecticides between parous and nulliparous females would be extremely difficult. Through several years of laboratory observation, the percent survival of gravid females after oviposition is extremely low. This low survivorship presents a challenge to replicate this experiment in parous females. In addition, lethal

concentrations and lethal times from insecticide-susceptible laboratory and field-collected sand flies may differ. This is why determining LCs and LTs for susceptible laboratory strains are imperative for using a bioassay on field populations. Due to the highly variable conditions in nature, wild sand flies may exhibit different development times, body sizes, longevity, behaviors, and physiologies that make them more or less susceptible to insecticides (Rivero et al. 2010).

In the initial development of the bottle bioassay by Brogdon and McAllister (1998b), 250-ml Wheaton bottles were used. These sized glass bottles are now recommended for all bottle assays (CDC 2010), although Alexander et al. (2009) used 200-ml Wheaton glass bottles.

Another potential limitation of this study is that owing to availability, 0.5-gallon and 1,000-ml glass bottles were used. For both *L. longipalpis* and *P. papatasi*, the deltamethrin, fenitrothion, chlorpyrifos, propoxur, and DDT exposure trials were completed using both the 0.5-gallon and the 1,000-ml bottles. In these situations, when the bottles of one size were temporarily unavailable (e.g., being cleaned for re-use), the other-size bottles were used. Therefore, the survival curves for these insecticides were generated by combining the mortalities from the 0.5-gallon and from the 1,000-ml bottles. Comparatively, the mortalities between the bottle sizes were similar, but often the percent mortality was higher in the smaller 1,000-ml bottles than in the 0.5-gallon bottles. Despite an equal concentration of insecticide and the even coating of insecticide, an unequal density of sand flies exposed, 20-30 and 40-50 in the 1,000-ml and 0.5-gallon bottles, respectively, or potential differences in air volume to bottle surface area may explain the differing mortalities.

Using a modified bioassay that combines aspects of the CDC bottle bioassay and the WHO exposure kit bioassay allowed us to manipulate insecticide concentrations to collect dose-response survival curve data and to determine LCs and LTs. In our experiments, to determine LCs and LTs, a 24-h holding period was incorporated for all ten insecticides after insecticide exposure (Saavedra-Rodriguez et al. 2008, Norris and Norris 2011). A 24-h holding period was

used because many of the sand flies that scored as dead following the insecticide exposure were able to completely recover. We suggest that the additional 24 h of recovery time provided more precise susceptibility data than seen immediately at the end of the insecticide exposure period. Using the data from this study, a future direction could still be to determine diagnostic doses and diagnostic times for *L. longipalpis* and *P. papatasi* using the CDC bottle bioassay for these same 10 insecticides. With these future data, researchers and public health administrators will have diagnostic doses and diagnostic times comparable with what is available for *Aedes* and *Anopheles* mosquitoes (CDC 2010). Having diagnostic doses and diagnostic times for phlebotomine sand flies will enable field researchers to assess the insecticide susceptibility status of sand fly populations in the wild using the CDC bottle bioassay.

The CDC recommends determining diagnostic concentrations and diagnostic times from *time-response* mortality curves (CDC 2010). To assess an insect populations' insecticide susceptibility status, diagnostic concentrations and diagnostic times are used (CDC 2010). A diagnostic dose is the dose of an insecticide that kills 100% of susceptible insects within a given time, the diagnostic time. Because we used our assays to produce *dose-response* survival curves, we were insufficiently able to determine diagnostic doses and diagnostic times, even though doses causing 100% mortality were discovered. QCal cannot determine LC_{100} values (diagnostic doses) because an insecticide concentration causing empirical 100% mortality cannot be determined with a logistic regression because 100% mortality is the upper asymptote. When put into the model, doses causing 100% mortality empirically are adjusted to causes mortality <100%. In *time-response* mortality curves, mortality from an insecticide dose is measured at distinct time intervals during the exposure test. Percent mortality is then plotted at each time interval (Brogdon and McAllister 1998b). A *time-response* diagnostic dose is the lowest concentration of insecticide that causes 100% mortality in a specified exposure time period, between 30 and 60 min (CDC 2010). A diagnostic dose and diagnostic time can both serve as

reference points to understand the insecticide susceptibility of a population of insects (WHO 1998).

The baseline LCs and LTs for each insecticide were determined for laboratory *L. longipalpis* and *P. papatasi* and can now be incorporated as comparative reference points in field assays measuring the insecticide susceptibility of sand flies. The CDC recommends determining diagnostic doses and diagnostic times for an insecticide for each vector species in a specific geographic region (CDC 2010). Similarly, the LCs and LTs from this experiment should not be considered universal for *L. longipalpis* or *P. papatasi*. The data from this study should be used only as a reference point for future determinations of diagnostic doses and diagnostic times for different populations of *Phlebotomus* and *Lutzomyia* around the world.

Insecticide resistance management requires control programs to monitor for resistance (Surendran et al. 2005; Badolo et al. 2012). Insecticide resistance resulting from poor timing of insecticide application or from incorrect dosage applications can lead to ineffective vector control programs. Where insecticides are used, resistance monitoring will ensure that appropriate insecticides and dosages are applied at times when they will most effectively control the target vectors (Maharaj 2011). This modified version of the CDC bottle bioassay and the WHO exposure kit assay can help to inform researchers and epidemiologists of sand fly populations that are resistant to specific insecticides or to entire insecticide classes. It is vital to continue to further develop integrated public health management programs that include effective vector surveillance and control.

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CHAPTER 4

DIAGNOSTIC DOSES AND TIMES FOR *PHLEBOTOMUS PAPATASI* AND *LUTZOMYIA LONGIPALPIS* SAND FLIES (DIPTERA: PSYCHODIDAE: PHLEBOTOMINAE) USING THE CDC BOTTLE BIOASSAY TO ASSESS INSECTICIDE RESISTANCE³**Abstract**

Background: Insecticide resistance to synthetic chemical insecticides is becoming a worldwide concern in phlebotomine sand flies (Diptera: Psychodidae), the vectors of *Leishmania* spp. parasites. The CDC bottle bioassay assesses resistance by testing populations against verified diagnostic doses and diagnostic times for an insecticide, but the assay has been used limitedly with sand flies. The objective of this study was to determine diagnostic doses and diagnostic times for laboratory *Lutzomyia longipalpis* (Lutz and Neiva) and *Phlebotomus papatasi* (Scopoli) to ten insecticides, including pyrethroids, organophosphates, carbamates, and DDT, that are used worldwide to control vectors.

Methods: Bioassays were conducted in 1,000-ml glass bottles each containing 10-25 sand flies from laboratory colonies of *L. longipalpis* or *P. papatasi*. Four pyrethroids, three organophosphates, two carbamates, and one organochlorine, were evaluated. A series of concentrations were tested for each insecticide, and four replicates were conducted for each concentration. Diagnostic doses were determined only during the exposure bioassay for the organophosphates and carbamates. For the pyrethroids and DDT, diagnostic doses were determined for both the exposure bioassay and after a 24-hour recovery period.

³ Coauthored by David S. Denlinger, Joseph A. Creswell, Joseph L. Anderson, Conor K. Reese, and Scott A. Bernhardt. Reprinted from *Parasites & Vectors*. Vol. 9. Pages 212.

Results: Both species are highly susceptible to the carbamates as their diagnostic doses are under 7.0 µg/ml. Both species are also highly susceptible to DDT during the exposure assay as their diagnostic doses are 7.5 µg/ml, yet their diagnostic doses for the 24-h recovery period are 650.0 µg/ml for *L. longipalpis* and 470.0 µg/ml for *P. papatasi*.

Conclusions: Diagnostic doses and diagnostic times can now be incorporated into vector management programs that use the CDC bottle bioassay to assess insecticide resistance in wild populations of *L. longipalpis* and *P. papatasi*. These findings provide initial starting points for determining diagnostic doses and diagnostic times for other sand fly vector species and wild populations using the CDC bottle bioassay.

Background

Insecticide resistance continues to be a threat to the success of insect vector control programs that incorporate synthetic chemical insecticides [1]. Insecticide resistance is a heritable phenotype that allows arthropods to survive an exposure to an insecticide that would normally kill a susceptible population [2-4]. Today, insecticide resistance to all classes of synthetic insecticides has been found in the major insect vectors [1, 5]. Managing insecticide resistance requires timely, accurate data through resistance monitoring and insecticide evaluation to assess a vector species' susceptibility to insecticides. These aspects can be used to develop effective strategies at managing vector populations [6]. The primary way to assess insecticide resistance is to use insecticide susceptibility bioassays.

The Centers for Disease Control and Prevention (CDC) bottle bioassay is one technique used to measure a vector species' susceptibility to insecticides [7, 8]. This bioassay is an economical and portable alternative to the World Health Organization's (WHO) exposure kit bioassay, especially in geographic regions where the WHO bioassay cannot be implemented [9-11]. Another benefit of the CDC bottle bioassay is that the materials, including the glass bottles, can be locally acquired and prepared on site [12].

Sand flies (Diptera: Psychodidae: Phlebotominae) require resistance monitoring because they have been, and continue to be, actively targeted with insecticides [13-16]. Fewer than seventy species of sand flies, including *Lutzomyia longipalpis* (Lutz and Neiva) and *Phlebotomus papatasi* Scopoli, are capable of vectoring *Leishmania* parasites, infection with which causes leishmaniasis, a world-wide disease currently infecting millions of people [17, 18]. Sand fly populations around the world have been exposed to the four main classes of insecticides: organochlorines, organophosphates, carbamates and pyrethroids. Insecticide exposure has been both intentional in directed vector control efforts and inadvertent as part of vector control efforts targeted against other insects [6, 13, 17, 19-24]. Populations of sand flies have been found to be tolerant or resistant, using the WHO exposure kit bioassay and diagnostic doses derived for mosquitoes, to the insecticides used worldwide [6, 19-30]. Despite these examples, there is a gap in understanding the prevalence of insecticide resistance in sand fly populations throughout the world. This has been attributed to challenges in collecting the necessary number of live flies for the bioassays and because there is a lack of a standardized sand fly bioassay [31].

To test an insect vector species' susceptibility status to an insecticide using the CDC bottle bioassay, a diagnostic dose and diagnostic time are needed for that insecticide [8]. A diagnostic dose is the lowest dose of an insecticide that causes 100% mortality in a susceptible population between 30 and 60 minutes, the diagnostic time [8]. There have been few published studies that have determined diagnostic doses for phlebotomine sand flies using the CDC bottle bioassay. In Colombia, Santamaría et al. [32] determined the diagnostic dose of lambda(λ)-cyhalothrin to be 10.0 $\mu\text{g/ml}$ for *Lu. longipalpis*. One concern of this finding is that Santamaría et al. [32] only tested three concentrations of lambda(λ)-cyhalothrin (10.0, 50.0, and 100.0 $\mu\text{g/ml}$), which makes it difficult to identify a precise diagnostic dose and diagnostic time because of the large differences between the doses tested [33]. Also working with *Lu. longipalpis*, Marceló et al. [33] determined the diagnostic doses and diagnostic times for malathion, deltamethrin, and

lambdacyhalothrin to be 75.0 µg/ml in 25 minutes, 10.0 µg/ml in 35 minutes and 15.0 µg/ml in 30 minutes, respectively. Diagnostic doses and diagnostic times for field-collected *Lu. evansi*, an important vector of *Le. infantum* in the Americas, have been previously described as 7.0 µg/ml in 10 minutes for deltamethrin and 3.5 µg/ml for in 10 minutes for lambda(λ)-cyhalothrin [20].

Dose-response survival curves to determine lethal concentrations causing 50 %, 90 %, and 95 % mortality for laboratory colonies of *Lu. longipalpis* and *P. papatasi* to ten insecticides were previously determined using a modified version of the CDC bottle bioassay and the WHO exposure kit [34]. These concentrations can serve as starting points for determining diagnostic doses and diagnostic times from time-response survival curves for a susceptible population of any sand fly species. Recently, Li et al. [31] also describes a bottle bioassay using 20 ml glass scintillation vials to determine lethal times causing 50 % mortality for *P. papatasi* and *P. duboscqi* exposed to ten pyrethroid and organophosphate insecticides. While not diagnostic doses, these data can be used for comparative purposes for future insecticide resistance studies for *P. papatasi* and *P. duboscqi*, two important Old World *Leishmania* vectors.

The objective of this study is to define and establish diagnostic doses and diagnostic times using the CDC bottle bioassay for *Lu. longipalpis* and *P. papatasi* to ten insecticides. No standardized diagnostic doses exist for insecticides using the CDC bottle bioassay. These diagnostic doses and diagnostic times determined in this study can now be incorporated into future studies assessing insecticide resistance from field-collected sand fly populations.

Methods

Sand Flies. Laboratory strains of insecticide-susceptible *Lu. longipalpis* and *P. papatasi* sand flies at Utah State University were derived from 30-year established colonies maintained at the Walter Reed Army Institute of Research (WRAIR) (Silver Spring, MD). The original colonies from Walter Reed have never been exposed to insecticides. All life stages were reared and maintained at USU [34-38].

Insecticides. Ten technical-grade insecticides were used in this study: four pyrethroids [cypermethrin (Sigma-Aldrich, St. Louis, MO), deltamethrin (Sigma-Aldrich, St. Louis, MO), lambda(λ)-cyhalothrin (Sigma-Aldrich, St. Louis, MO), and permethrin (Chem Service, Inc., West Chester, PA)]; three organophosphates [chlorpyrifos (Sigma-Aldrich, St. Louis, MO), fenitrothion (Sigma-Aldrich, St. Louis, MO), and malathion (Chem Service, Inc., West Chester, PA)]; two carbamates [bendiocarb (Sigma-Aldrich, St. Louis, MO) and propoxur (Sigma-Aldrich, St. Louis, MO)]; and the organochlorine dichlorodiphenyltrichloroethane (DDT) (Sigma-Aldrich, St. Louis, MO). All insecticide dilutions were prepared in acetone, stored in glass bottles, wrapped in aluminum foil, and kept at 4°C while not being used [8]. The concentrations of each insecticide used in these experiments are listed in (Table 4.1.). Whole-value lethal concentrations causing 50 % and 90 % mortality for each insecticide and for each sand fly species from Denlinger et al. [34] were used as initial concentrations tested for determining diagnostic doses.

Preparation of Exposure Bottles. The day before exposing the sand flies, four 1,000-ml glass bottles (Fisher Scientific, Pittsburgh, PA) were prepared by coating them with insecticide, as described in Denlinger et al. [34]. Following Brogdon & Chan [8], for a 250-ml bottle, 1.0 ml of insecticide at 10.0 μg insecticide/ ml acetone gives a concentration of 10.0 μg / 250-ml bottle. To compensate for these larger bottle sizes, and to maintain an equivalence of X μg insecticide / 250-ml bottle [8], 4.0 ml of X μg insecticide was used to coat the interior of the 1,000-ml bottle [34]. The bottles were coated with insecticide by swirling the acetone:insecticide solution on the bottom, on the sides, and on the lid. The bottle was then placed on a mechanical bottle roller for 30 minutes to dry and reduce the potential for bubble formation. During this time, the lids were slowly loosened to allow the acetone to evaporate. After 30 minutes, the caps were removed, and the bottles were rolled until all of the acetone had evaporated. The bottles were then left open to dry overnight in the dark to prevent photodegradation of the insecticides.

Table 4.1. Concentrations of ten insecticides used to expose *L. longipalpis* and *P. papatasi* sand flies.

Insecticide (Insecticide Class ^a)	Species	Concentration (μg insecticide/bottle)
Cypermethrin (PYR)	<i>Lu. longipalpis</i>	5.0, 10.0, 15.0, 20.0
	<i>P. papatasi</i>	20.0, 25.0, 30.0, 35.0, 40.0, 45.0, 50.0, 55.0, 60.0, 65.0, 70.0, 75.0, 90.0, 95.0
Deltamethrin (PYR)	<i>Lu. longipalpis</i>	5.0, 10.0, 15.0, 20.0, 25.0, 30.0, 35.0, 40.0, 45.0, 50.0, 75.0, 100.0
	<i>P. papatasi</i>	5.0, 10.0, 15.0, 20.0, 25.0, 30.0, 35.0, 40.0, 50.0, 75.0, 100.0
λ -Cyhalothrin (PYR)	<i>Lu. longipalpis</i>	1.0, 2.0, 3.0, 4.0, 10.0, 20.0, 30.0, 40.0
	<i>P. papatasi</i>	1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 10.0, 20.0, 30.0, 40.0
Permethrin (PYR)	<i>Lu. longipalpis</i>	5.0, 10.0, 12.5, 15.0, 20.0
	<i>P. papatasi</i>	10.0, 20.0, 25.0, 30.0, 35.0, 40.0, 45.0, 50.0
Chlorpyrifos (OP)	<i>Lu. longipalpis</i>	5.0, 10.0, 15.0, 20.0, 25.0, 30.0
	<i>P. papatasi</i>	20.0, 25.0, 30.0, 35.0, 40.0, 45.0
Fenitrothion (OP)	<i>Lu. longipalpis</i>	2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0, 16.0, 18.0, 20.0, 22.0, 24.0, 26.0, 28.0, 30.0, 32.0
	<i>P. papatasi</i>	5.0, 10.0, 15.0, 20.0, 25.0, 30.0, 35.0, 40.0, 45.0
Malathion (OP)	<i>Lu. longipalpis</i>	5.0, 10.0, 15.0, 20.0, 25.0, 30.0, 35.0, 40.0, 45.0
	<i>P. papatasi</i>	50.0, 75.0, 100.0, 125.0, 130.0, 135.0, 140.0, 145.0
Bendiocarb (CX)	<i>Lu. longipalpis</i>	1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0
	<i>P. papatasi</i>	1.0, 2.0, 3.0, 4.0, 5.0, 6.0,
Propoxur (CX)	<i>Lu. longipalpis</i>	1.0, 2.0, 3.0, 4.0, 10.0
	<i>P. papatasi</i>	1.0, 2.0, 3.0, 7.0, 15.0
DDT (OC)	<i>Lu. longipalpis</i>	2.5, 5.0, 7.5, 10.0, 15.0, 20.0, 50.0, 100.0, 150.0, 200.0, 250.0, 300.0, 350.0, 400.0, 450.0, 500.0, 550.0, 600.0, 630.0, 635.0, 640.0, 645.0, 650.0, 700.0
	<i>P. papatasi</i>	2.5, 5.0, 7.5, 10.0, 50.0, 100.0, 150.0, 200.0, 350.0, 400.0, 450.0, 455.0, 460.0, 465.0, 470.0, 480.0, 490.0, 500.0, 550.0

^aPYR = pyrethroid, OP = organophosphate, CX = carbamate, OC = organochlorine

For each test replicate, one bottle serving as a control was coated with 4.0 ml of acetone depending on its volume [8]. All bottles were re-used throughout the duration of the experiment. To clean a bottle with residual insecticide, the bottle and lid was first triple-rinsed with acetone; filled with warm, soapy water; drained; rinsed and filled with cold water; drained; and autoclaved for at least 20 minutes. After being autoclaved, the bottles were left to dry for at least one day before being used again [34].

Insecticide Exposure Tests. Approximately 12 hours after the bottles were prepared with insecticide, 10-25 adult sand flies at least two days post-eclosion were aspirated from the

main colony and gently blown into each bottle [8]. Approximately equal numbers of un-fed female and male flies were used for each insecticide-coated bottle, while only females were used in the control bottle [8]. Sand flies were aspirated into the control bottle first followed by the four insecticide-coated bottles. Once sand flies had been aspirated into all five bottles, the timer was initiated and recorded as time zero. At time zero, the total number of flies in each bottle was recorded. The number of alive or dead sand flies was recorded at each time point, depending on which was easier to visually determine [8]. All bottles were held horizontally for the duration of the experiment. During initial replicates with the largest doses of DDT, the authors infrequently observed that the legs of some sand flies would become stuck to the interior surface of the bottles during the 60-minute exposure. These flies were unable to be removed from the bottles via aspiration. These replicates were not used. To remedy this issue at these high concentrations, the bottles were rotated every few minutes to promote limited hopping and movement of the sand flies. This movement reduced extended surface contact in one place and eliminated the issue of sand flies becoming fixed on the insecticide surface.

The percent mortality at each time point was the average of the percent mortalities of the four replicates. The percent mortality at a time point in the insecticide-treated bottles was corrected with Abbott's formula if mortality in the control bottle ranged between 5% and 20%. Abbott's formula was not used to correct experimental mortalities if the control group mortality was less than 5%. If control group mortalities exceeded 20%, the entire testing replicate was not used [24].

Organophosphates and Carbamates. Mortality was recorded at 0, 15, 30, 35, 40, 45, 60, 75, 90, 105, and 120 minutes by gently rotating the bottle (time-to-knockdown) [8]. Sand flies were scored as "dead" if they had difficulty flying, could not fly altogether, or had trouble righting themselves [8]. If all sand flies were scored as dead before 120 minutes, the flies were kept in the bottles and continued to be observed until 120 minutes was reached.

Pyrethroids and DDT. Mortality was scored during the exposure test (time-to-knockdown) to create survival curves as well after 24-hours of recovery time (24-h mortality) [8]. During the exposure test, mortality was recorded at 0, 15, 30, 35, 40, 45, and 60 minutes by gently rotating the bottle. Scoring mortality was equivalent to the criteria used for the carbamate and organophosphate insecticides. If all sand flies were scored as dead before 60 minutes, the flies were kept in the bottles until 60 minutes was reached. At the end of the 60 minutes, the sand flies were captured via mechanical aspiration, released into 1-pint cardboard containers with a fine mesh screen top, and kept under the same temperature, light, and humidity environment as the main, untreated colonies. A cotton ball saturated with 30% sugar-water was placed on the top of each container. Sand flies were held in these containers for 24-hours prior to mortality being recorded. Mortality was corrected with Abbott's formula using the same criteria described above for both the time-to-knockdown and 24-h mortality.

Survival Curves. Time-response survival curves were made for each insecticide for each sand fly species by plotting time on the X-axis against percent mortality on the Y-axis [8]. For each insecticide dose, the percent mortality at each time point is the average mortality between all four insecticide-treated bottles. A diagnostic dose was determined to be the lowest dose tested that caused 100 % mortality between 30 and 60 minutes, the diagnostic time [8].

Results

Survival Curves. A time-response survival curve for each of the ten insecticides for both *Lu. longipalpis* and *P. papatasi* was created following Brogdon & Chan [8]. For all the time-to-knockdown survival curves, the time to reach 100% mortality decreased with increasing insecticide concentrations. Diagnostic doses and diagnostic times for the organophosphates and carbamates are presented in (Table 4.2.). Diagnostic doses and diagnostic times for time-to-knockdown and for 24-h mortality for the pyrethroids and DDT are presented in (Table 4.3.). Representative survival curves for bendiocarb, fenitrothion, permethrin, and DDT are presented

in (Figs. 4.1 and 4.2). For some insecticides, multiple diagnostic doses and diagnostic times were observed. Whereas for other insecticides, only one diagnostic dose and diagnostic time were observed because all of the other doses that were tested for that specific insecticide either did not cause 100 % mortality between 30 minutes and 60 minutes or they were saturated doses.

Organophosphates. Two diagnostic doses for *Lu. longipalpis* have been determined for chlorpyrifos: 20.0 µg/ml at 45 minutes and 25.0 µg/ml at 30 minutes. Only one diagnostic dose was determined for *P. papatasi* to chlorpyrifos: 30.0 µg/ml at 60 minutes. Both *Lu. longipalpis* and *P. papatasi* have identical diagnostic doses and diagnostic times for fenitrothion: 30.0 µg/ml at 60 minutes. *Lutzomyia longipalpis* has an additional diagnostic dose for fenitrothion of 32.0 µg/ml at 45 minutes. For malathion, however, the diagnostic doses between species are markedly different. *Lutzomyia longipalpis*' diagnostic dose is 40.0 µg/ml at 60 minutes, and *P. papatasi*'s diagnostic dose is 130.0 µg/ml at 60 minutes.

Carbamates. Similar to the small LC values from Denlinger et al. [31], both *Lu.*

Table 4.2. Diagnostic Doses and Diagnostic Times for organophosphate and carbamate insecticides at the time-to-knockdown.

Insecticide (Insecticide Class ^a)	Species	Diagnostic Dose and Diagnostic Time (for time-to-knockdown)
Chlorpyrifos (OP)	<i>Lu. longipalpis</i>	25.0 µg/ml (30 min) 20.0 µg/ml (45 min)
	<i>P. papatasi</i>	30.0 µg/ml (60 min)
Fenitrothion (OP)	<i>Lu. longipalpis</i>	32.0 µg/ml (45 min) 30.0 µg/ml (60 min)
	<i>P. papatasi</i>	30.0 µg/ml (60 min)
Malathion (OP)	<i>Lu. longipalpis</i>	40.0 µg/ml (60 min)
	<i>P. papatasi</i>	130.0 µg/ml (60 min)
Bendiocarb (CX)	<i>Lu. longipalpis</i>	6.0 µg/ml (40 min) 5.0 µg/ml (60 min)
	<i>P. papatasi</i>	2.0 µg/ml (30 min) 1.0 µg/ml (40 min)
Propoxur (CX)	<i>Lu. longipalpis</i>	3.0 µg/ml (30 min) 2.0 µg/ml (40 min)
	<i>P. papatasi</i>	3.0 µg/ml (30 min) 2.0 µg/ml (35 min)

^aOP = organophosphate, CX = carbamate

Table 4.3. Diagnostic Doses and Diagnostic Times for pyrethroid and DDT insecticides at time-to-knockdown and after 24-hours.

Insecticide (Insecticide Class ^a)	Species	Diagnostic Dose and Diagnostic Time (for time-to-knockdown)	Diagnostic Dose after 24 hours for mortality
Cypermethrin (PYR)	<i>Lu. longipalpis</i>	20.0 µg/ml (40 min) 10.0 µg/ml (60 min)	20.0 µg/ml
	<i>P. papatasi</i>	95.0 µg/ml (45 min) 65.0 µg/ml (60 min)	60.0 µg/ml
Deltamethrin (PYR)	<i>Lu. longipalpis</i>	45.0 µg/ml (35 min) 15.0 µg/ml (40 min) 5.0 µg/ml (60 min)	30.0 µg/ml
	<i>P. papatasi</i>	45.0 µg/ml (35 min) 25.0 µg/ml (40 min) 15.0 µg/ml (45 min) 5.0 µg/ml (60 min)	25.0 µg/ml
λ-Cyhalothrin (PYR)	<i>Lu. longipalpis</i>	4.0 µg/ml (40 min) 3.0 µg/ml (45 min) 1.0 µg/ml (60 min)	1.0 µg/ml
	<i>P. papatasi</i>	4.0 µg/ml (40 min) 2.0 µg/ml (60 min)	6.0 µg/ml
Permethrin (PYR)	<i>Lu. longipalpis</i>	15.0 µg/ml (30 min)	15.0 µg/ml
	<i>P. papatasi</i>	60.0 µg/ml (40 min) 50.0 µg/ml (60 min)	55.0 µg/ml
DDT (OC)	<i>Lu. longipalpis</i>	7.5 µg/ml (30 min)	650.0 µg/ml
	<i>P. papatasi</i>	7.5 µg/ml (30 min)	470.0 µg/ml

^aPYR = pyrethroid, OC = organochlorine

longipalpis and *P. papatasi* have very small diagnostic doses. *Lutzomyia longipalpis* has a diagnostic dose and diagnostic time for bendiocarb of 6.0 µg/ml at 40 minutes or 5.0 µg/ml at 60 minutes. For propoxur, the diagnostic dose and diagnostic time is 3.0 µg/ml at 30 minutes or 2.0 µg/ml at 40 minutes. *Phlebotomus papatasi* has smaller diagnostic doses and diagnostic times for bendiocarb than *Lu. longipalpis*: 2.0 µg/ml at 30 minutes or 1.0 µg/ml at 40 minutes. For propoxur, the diagnostic dose is 3.0 µg/ml at 30 minutes or 2.0 µg/ml at 35 minutes, which is almost identical to the diagnostic dose and diagnostic time for *Lu. longipalpis*.

Pyrethroids. *Phlebotomus papatasi* has a larger time-to-knockdown and 24-h mortality for cypermethrin than *Lu. longipalpis*. *Phlebotomus papatasi* has two time-to-knockdown diagnostic doses of 65.0 µg/ml at 60 minutes and 95 µg/ml at 45 minutes, and its 24-h mortality

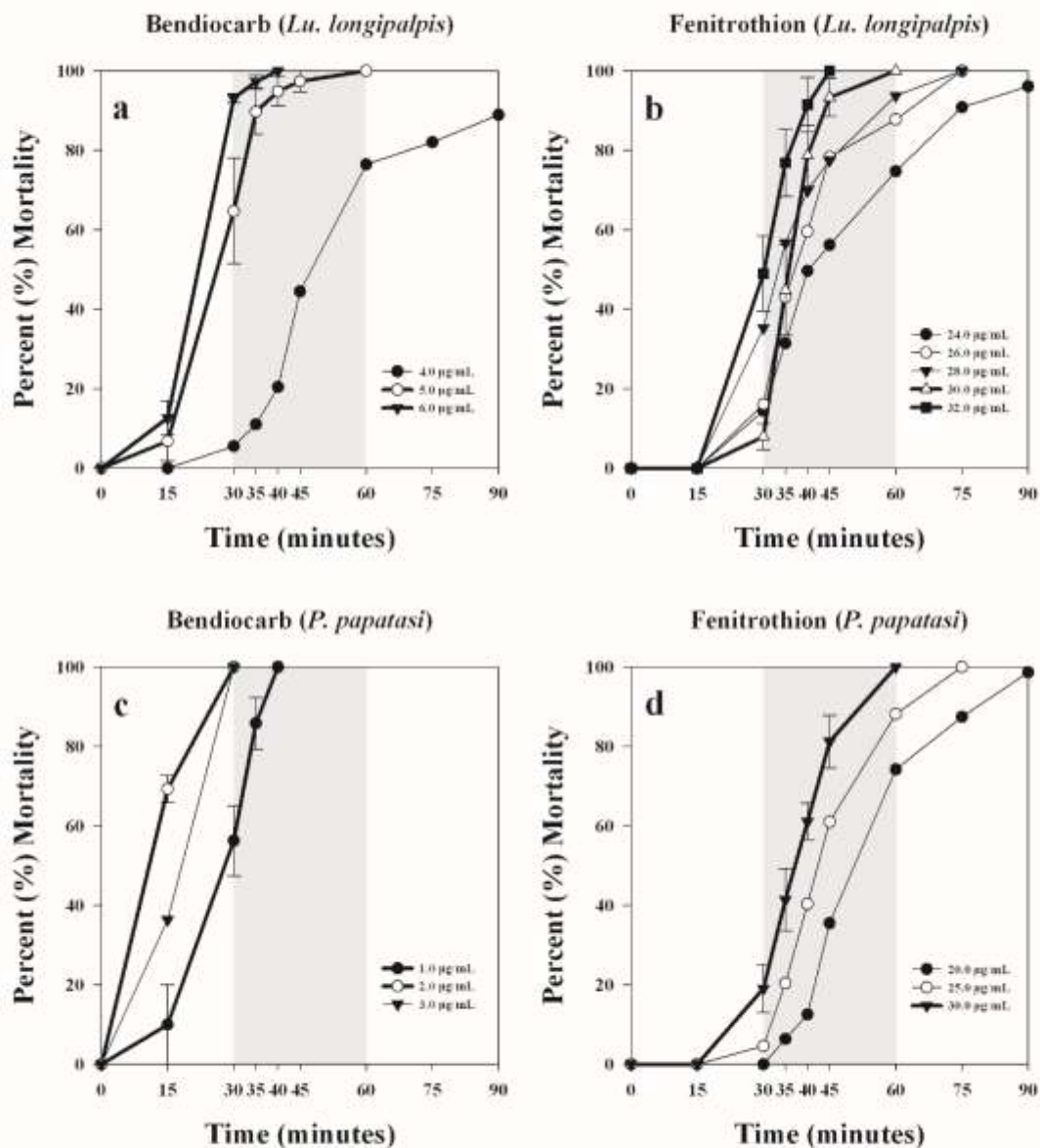


Fig. 4.1. Time-to-knockdown survival curves for *Lu. longipalpis* to bendiocarb (A) and fenitrothion (B) and *P. papatasi* to bendiocarb (C) and fenitrothion (D). For each graph, bolded lines represent the time-response for doses that are considered diagnostic doses. At each time point of the bolded lines the error bars show the standard error, of the mean percent mortality, across the four bottle replicates. Error bars are only displayed on the diagnostic dose lines for visual clarity. The shaded region of each graph designates a window of time (30, 35, 40, 45, or 60 minutes) that can be considered diagnostic times for diagnostic doses.

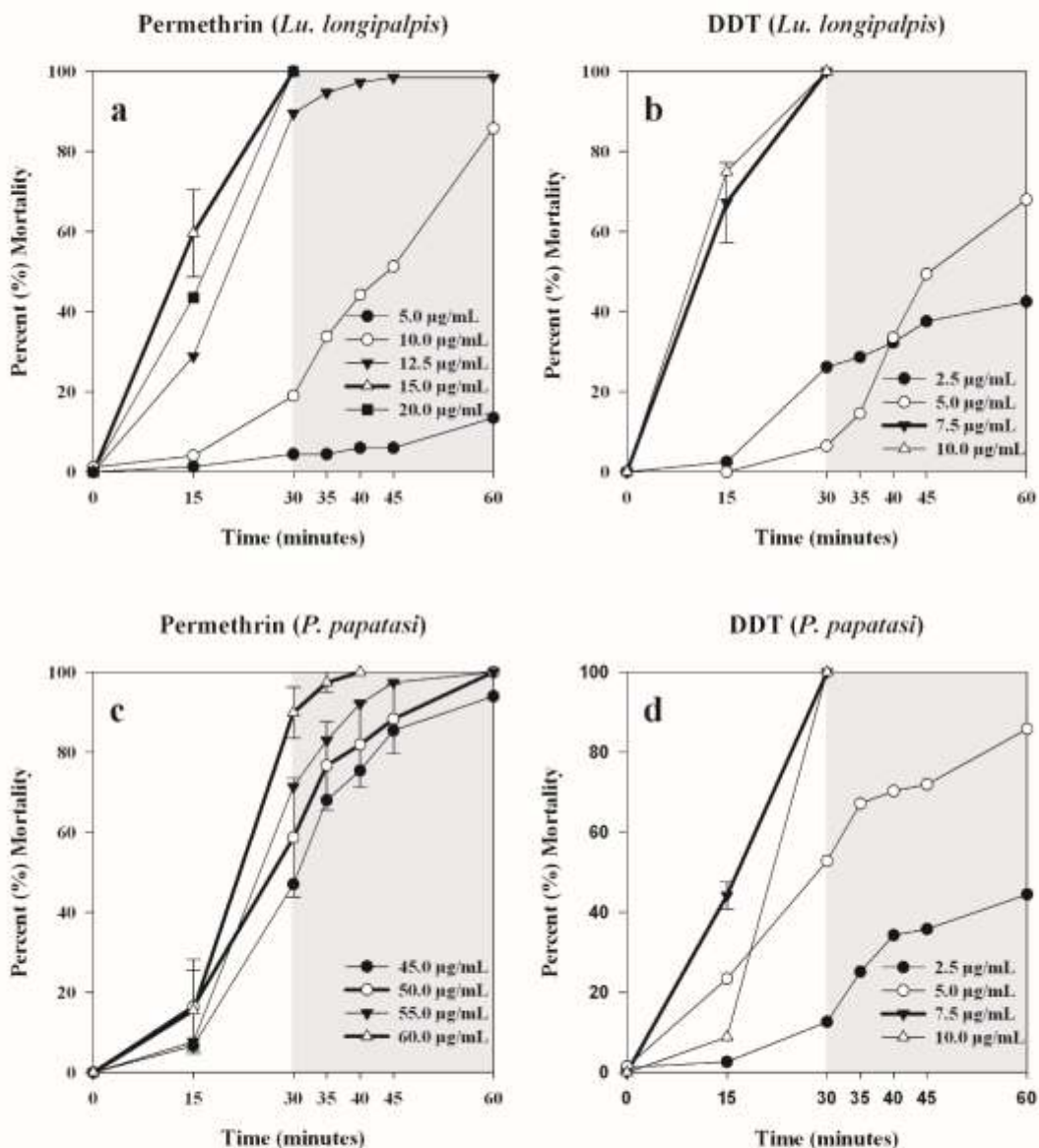


Fig. 4.2. Time-to-knockdown survival curves for *Lu. longipalpis* to permethrin (A) and DDT (B) and *P. papatasi* to permethrin (C) and DDT (D). For each graph, bolded lines represent the time-response for doses that are considered diagnostic doses. At each time point of the bolded lines the error bars show the standard error, of the mean percent mortality, across the four bottle replicates. Error bars are only displayed on the diagnostic dose lines for visual clarity. The shaded region of each graph designates a window of time (30, 35, 40, 45, or 60 minutes) that can be considered diagnostic times for diagnostic doses.

diagnostic dose is 60.0 µg/ml. Comparatively, *Lu. longipalpis*' time-to-knockdown diagnostic doses are 10.0 µg/ml at 60 minutes and 20.0 µg/ml at 40 minutes, and its 24-h mortality diagnostic dose is 20.0 µg/ml. *Lutzomyia longipalpis* and *P. papatasi* have the same time-to-knockdown diagnostic doses of 5.0 µg/ml at 60 minutes and 45.0 µg/ml at 35 minutes. *Lutzomyia longipalpis* has an additional diagnostic dose of 15.0 µg/ml at 40 minutes, and *P. papatasi* has two additional diagnostic doses of 15.0 µg/ml at 45 minutes and 25.0 µg/ml at 40 minutes. Both species have almost equivalent 24-h mortality diagnostic doses to deltamethrin. *Lutzomyia longipalpis* requires 30.0 µg/ml and *P. papatasi* requires 25.0 µg/ml. Besides the carbamates, the time-to-knockdown diagnostic doses for lambda-cyhalothrin are the lowest for all ten insecticides. Both *Lu. longipalpis* and *P. papatasi* have a diagnostic dose of 4.0 µg/ml at 40 minutes. *Lutzomyia longipalpis* has two additional diagnostic doses of 1.0 µg/ml at 60 minutes and 3.0 µg/ml at 45 minutes. *Phlebotomus papatasi* has one additional diagnostic dose of 2.0 µg/ml at 60 minutes. Noticeably, *P. papatasi* has a lambda-cyhalothrin 24-h mortality diagnostic dose of 6.0 µg/ml, while it only required 1.0 µg/ml to cause 100% mortality after 24 hours for *Lu. longipalpis*. For permethrin, *P. papatasi*'s time-to-knockdown diagnostic doses are 50.0 µg/ml at 60 minutes and 60.0 µg/ml at 40 minutes, and *Lu. longipalpis* has a diagnostic dose of 15.0 µg/ml in 30 minutes. There is a large difference between the two sand fly species permethrin 24-h mortality diagnostic doses: 55.0 µg/ml and 15.0 µg/ml for *P. papatasi* and *Lu. longipalpis*, respectively.

Organochlorine. Both *Lu. longipalpis* and *P. papatasi* have small time-to-knockdown diagnostic doses of 7.5 µg/ml at 30 minutes when exposed to DDT. However, both species required very large 24-h mortality diagnostic doses: 650.0 µg/ml of DDT was needed for *Lu. longipalpis* and 470.0 µg/ml of DDT for *P. papatasi*.

Discussion

The objective of this study was to develop baseline data of ten insecticide diagnostic doses and diagnostic times for laboratory *Lu. longipalpis* and *P. papatasi* using the CDC bottle bioassay. We have demonstrated that the CDC bioassay can be used to determine diagnostic doses for phlebotomine sand flies to pyrethroid, organophosphate, carbamate, and organochlorine insecticides. This work strengthens the collection of diagnostic doses and diagnostic times that are available for sand flies using the CDC bottle bioassay by presenting for the first time concentrations and times for *Phlebotomus* spp. [20, 32, 33]. The present study provides precise time-to-knockdown diagnostic doses for all ten insecticides for both sand fly species. In addition, for the first time, diagnostic doses for the 24-h recovery period are presented for sand flies to four pyrethroids and DDT.

There have been few studies that have determined diagnostic doses and diagnostic times for *Lu. longipalpis* using the CDC bottle bioassay. With the results presented in this study, comparisons can now be made for the insecticides malathion, deltamethrin, and lambda-cyhalothrin. For our *Lu. longipalpis* colony, a dose of malathion of 40.0 µg/ml caused 100 % mortality in 60 minutes, while for the *Lu. longipalpis* tested by Marceló et al. [33], 75.0 µg/ml caused 100 % mortality in 25 minutes. Against our colony of *Lu. longipalpis*, 45.0 µg/ml deltamethrin was needed to cause 100 % mortality in 35 minutes compared to 10.0 µg/ml in 35 minutes [33]. All currently published studies for lambda(λ)-cyhalothrin have found *Lu. longipalpis* to have low diagnostic doses. In the present study, a dose of 4.0 µg/ml was sufficient to cause 100 % mortality in 40 minutes. A dose of 15.0 µg/ml caused 100 % mortality in 30 minutes [33], and Santamaría et al. [32] found 10.0 µg/ml to cause 100 % mortality in approximately 60 minutes, although only three doses were tested and no precise diagnostic time was provided.

The only direct comparison that can be made for *Lu. longipalpis* is for deltamethrin as both colonies (present work and [33]) had equal diagnostic times of 35 minutes. Our colony needed 45.0 $\mu\text{g/ml}$ to cause 100 % mortality, and the *Lu. longipalpis* from [33] only needed 10.0 $\mu\text{g/ml}$. The CDC bottle bioassay protocol designates that a diagnostic dose needs to cause 100 % mortality in the 30 minute – 60 minute window of exposure (specifically at 30, 35, 40, 45, and 60 minutes) [8]. Some of the diagnostic times determined from Henriquez et al. [20] and Marceló et al. [33] for *Lu. evansi* and *Lu. longipalpis* do not fall into this window, and we are therefore not able to make direct comparisons. Future studies using the CDC bottle bioassay need to have comparable diagnostic times to be able to compare diagnostic doses between different populations of a sand fly species. In addition, the CDC bottle bioassay protocol could potentially be amended to include a larger time window (e.g. 10 minutes and 25 minutes) of potential diagnostic times.

In accordance with the recommendations provided by Brogdon & Chan [8], as small as 5 $\mu\text{g/ml}$ dose increments were used initially when determining diagnostic doses. It was necessary for lambda-cyhalothrin, fenitrothion, bendiocarb, propoxur, and DDT to work in increments as small as 1.0 $\mu\text{g/ml}$, 2.0 $\mu\text{g/ml}$, or 2.5 $\mu\text{g/ml}$ because increments of 5.0 $\mu\text{g/ml}$ were too large to effectively determine appropriate diagnostic doses. For insecticides requiring larger doses, initial testing used increments larger than 5.0 $\mu\text{g/ml}$ and then adjusted down to 5.0 $\mu\text{g/ml}$ increments as we approached the diagnostic dose. The small dose increments ensure that diagnostic doses are precise. An inaccurate diagnostic dose that is too low in concentration has the potential of displaying false-positives of resistance because individuals will survive during the bioassay. An inaccurate diagnostic dose that is too high will potentially display false-negatives of resistance because resistant individuals will be killed even if they are demonstrating a quantifiable level of resistance [8].

One potential limitation of this study was the use of 1,000-ml bottles, not the standard 250-ml bottles [7, 8], although non-standard volume bottles have been used to assess insecticide susceptibility and determine diagnostic doses and diagnostic times with the CDC bottle bioassay [12, 31]. The 1000-ml bottles are the same bottles used in Denlinger et al. [34]. We were unable to use a larger quantity of flies in each bottle (> 10-25 of the required number of flies per 250-ml bottle [8]) to account for the larger bottle size because the sand fly demand throughout the entirety of the experiment would have exhausted our main colonies. The use of the same number of required flies (10-25) in the larger sized bottles potentially may have influenced the diagnostic doses that we observed. Despite an equivalent concentration of insecticide, a smaller density of sand flies exposed per bottle volume (10-25 flies/ 1,000-ml bottle compared to 10-25 flies / 250-ml bottle) and/or potential differences in air volume to bottle surface area may be a factor in the determination of our calculated diagnostic doses and diagnostic times. However, the ten insecticides used are contact insecticides, and the sand flies were regularly observed to be in contact with the interior surface of bottle due to them being poor fliers. The authors suggest that the diagnostic concentrations and times would be very similar for sand flies, regardless of these limited volume differences.

Diagnostic doses and diagnostic times of insecticides for susceptible populations of vector species are fundamentally required when assessing resistance in test field populations [39-43]. Accordingly, the diagnostic doses and diagnostic times presented for *Lu. longipalpis* and *P. papatasi* in this study should be used as an initial reference point for determining diagnostic doses and diagnostic times for other insecticide-susceptible populations. The criteria differ between the WHO exposure kit bioassay and the CDC bottle bioassay. The most recent criterion for resistance for mosquito vectors by the WHO [11] states that resistance is present if there is less than 90 % mortality, while the criterion for resistance by the CDC states that resistance is present if there is less than 100 % mortality [8]. Using the CDC bottle bioassay to test mosquito

populations for resistance, there are examples of employing both the WHO's criterion for resistance [40, 44-47] and the CDC's criterion for resistance [48, 49]. Recommendations from Saeidi et al. [24] suggest tailoring the WHO's resistance criterion for sand flies because of the physiological, behavioral, and size differences between mosquitoes and sand flies. We suggest that if the CDC bottle bioassay is used to assess the insecticide susceptibility status of a sand fly population, established diagnostic dose and times specific to sand flies and the CDC's criterion for resistance should be used.

One important aspect of the CDC bottle bioassay is the 24-h holding period used for pyrethroids and DDT to allow insects to recover from "knockdown" [39, 41, 44, 50-52]. An imperative question with the CDC bottle bioassay is to determine which mortality endpoint to use when assessing resistance: at the time-to-knockdown or at the of the 24-h mortality [53, 54]. Both the knockdown endpoint and the 24-h mortality endpoint communicate different resistance mechanisms: knockdown resistance (*kdr*) via target-site insensitivity or metabolic detoxification. *Kdr* will cause knockdown to be lower than mortality, but metabolic detoxification resistance can cause mortality to be lower than knockdown [53]. Without the 24-h recovery period, the CDC bioassay could miss evidence of metabolic resistance because the lack of a 24-h recovery period does not allow resistant insects to recover; they may be scored as dead during the time-to-knockdown but would have recovered if allowed the 24-h recovery period [53]. In our experiments, the importance of the 24-hour recovery period as part of the CDC bottle bioassay protocol is evident for DDT. The time-to-mortality diagnostic doses were 63-87 fold greater than the time-to-knockdown diagnostic doses for *P. papatasi* and *Lu. longipalpis*, respectively (Table 3). This demonstrates that while sand flies, even from laboratory colonies, may have small time-to-knockdown diagnostic doses, large concentrations are need to cause 100 % mortality after 24 hours.

The CDC bottle bioassay and WHO exposure kit bioassay are mutually used to detect insecticide resistance. However, a literature search of other studies conducted by [53] found differences in agreement between the two assays in detecting resistance in mosquitoes at both the time-to-knockdown and after 24 hours both at the 90 % and 98 % mortality cutoffs. Several studies have utilized the WHO exposure kit bioassay to assess insecticide resistance in sand flies [19, 21-27]. If future monitoring of insecticide resistance in sand fly populations is to utilize the CDC bottle bioassay, there will need to be a calibration of both the WHO exposure kit bioassay and CDC bottle bioassay. A synchronization of the diagnostic doses and diagnostic times for both assays will need to use the same population of sand flies, such that the same level of mortality can be derived from each assay [53].

The CDC bottle bioassay has been used for many years to track the spread of insecticide resistance in mosquitoes; however, this assay does not assess the intensity of insecticide resistance [54]. The CDC bottle bioassay intensity rapid diagnostic tests (I-RDT's), developed by Bagi et al. [54], follows the CDC bottle bioassay protocol but measures insecticide concentrations 1x, 2x, 5x and 10x the known diagnostic doses. The intended goal is not so much with understanding the prevalence of insecticide resistance but to quantify the intensity of resistance [54]. For sand flies, I-RDT's are not yet necessary because the prevalence of resistance is low and baseline data from field collections are limited. Resistance prevalence for sand flies may be initially low because it has not been assessed very frequently or because it may not be very prevalent [13, 31, 55]. Regardless, knowing the speed with which resistance has developed and spread in mosquito populations demonstrates the need to continue to assess insecticide resistance prevalence in wild sand fly populations and to prepare I-RDT's in areas where resistance is already present. The diagnostic doses and diagnostic times presented in this study provides necessary baseline data for developing CDC bottle bioassay I-RDT's for sand flies.

Conclusions

Evidence of insecticide resistance in worldwide populations of phlebotomine sand flies is a threat to the success of control programs that aim to mitigate the spread of leishmaniasis. It is crucial to have timely insecticide susceptibility data for different sand fly populations. The CDC bottle bioassay is one method to assess insecticide resistance, but it has been used infrequently with sand flies. With the diagnostic doses and diagnostic times presented here, the CDC bottle bioassay has great potential to be assimilated into sand fly control programs where other resistance-assessing methods are not feasible. The data presented in this study can serve as starting points for determining the susceptibility of field-collected and laboratory-reared *L. longipalpis* and *P. papatasi*, and for determining diagnostic doses and diagnostic times for other sand fly species of public health concern. Knowing if a population of sand flies is resistant to an insecticide or insecticide class is critical because it allows control strategies to be effectively implemented while not exacerbating the prevalence of insecticide resistance.

Ethical Considerations

The maintenance of SKH1 hairless mice (Charles River, Wilmington MA) and the experimental animal-use protocol was approved by Utah State University's Institutional Animal-Care and Use Committee.

Competing Interests

The author(s) declare that they have no competing interests.

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Authors' Contributions

DSD and SAB conceived and designed the experiment. DSD, JAC, JLA, and CKR contributed to data collection. DSD, JAC, and SAB wrote the manuscript. CKR, DSD, and SAB contributed to the figure designs. All authors read and approved the final manuscript.

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CHAPTER 5

EVALUATING TARGET-SITE INSENSITIVITY AND METABOLIC DETOXIFICATION
INSECTICIDE RESISTANCE MECHANISMS IN LABORATORY POPULATIONS OF
SAND FLIES (DIPTERA: PSYCHODIDAE: PHLEBOTOMINAE) UNDER ARTIFICIAL
SELECTION TO PYRETHROIDS AND ORGANOPHOSPHATES**Abstract**

Synthetic insecticides are used to kill insect vectors to reduce disease transmission, and since the middle of the twentieth century vectors have been forced to adapt to incredible selection pressures imposed by these insecticides. Resistance is now a worldwide pandemic threatening the utility of insecticides as tools to lessen the burden of disease. Phlebotomine sand flies (Diptera: Psychodidae) transmit the protozoans that cause leishmaniasis to humans, causing tens of thousands of deaths each year. Despite evidence of resistance in sand fly populations, there is little knowledge about their genetic and molecular mechanisms of resistance. We hypothesized that resistance in laboratory populations of *Phlebotomus papatasi* and *Lutzomyia longipalpis* sand flies would be convergent to the mechanisms found in other insects. Over the course of several years (~ 20 generations), two populations from each species were exposed to sublethal doses of permethrin or malathion. We looked for evidence of target-site insensitivity and metabolic detoxification, two well-studied mechanisms of insecticide resistance, in several generations of each population. No evidence of target-site insensitivity in the paralytic or acetylcholinesterase genes was found in any resistance-selected colony. Additionally, except for a few cases, all four colonies had decreased activities of enzymes associated with metabolic detoxification, which would be expected to increase in resistant individuals. The evolutionary reasons and implications for a lack of evidence of target-site insensitivity and metabolic detoxification, and ideas for other mechanisms, are discussed.

Leishmaniasis is a lethal and disfiguring worldwide neglected tropical disease [World Health Organization (WHO) 2010]. Endemic transmission of leishmaniasis is found in almost one hundred countries spanning five continents, and there is an annual incidence of 1.3 million new cases and 20,000-40,000 deaths (Alvar et al. 2012, WHO 2013). Leishmaniasis is endemic where there is poor housing and sanitation. In addition, there are incredible social, cultural, familial, and economic stigmas associated with this disease (Hotez 2008, Kassi et al. 2008, WHO 2013, Hotez 2016).

Leishmaniasis is caused by infection with *Leishmania* protozoans (Trypanosomatida: Trypanosomatidae). *Leishmania* are dioxenous parasites of mammals, including humans, and phlebotomine sand flies (Maslov et al. 2013). At least twenty species of *Leishmania* are known to be pathogenic to humans (Bañuls et al. 2007, Antinori et al. 2012). Only females in the genera *Phlebotomus* and *Lutzomyia* are the competent, putative vectors of these parasites (Akhoundi et al. 2016).

In the Old World, the peridomestic species *P. papatasi* (Scopoli) is the incriminated vector for transmitting *Leishmania major*, the agent causing zoonotic cutaneous leishmaniasis (ZCL), from gerbil rodents to humans (Reithinger et al. 2007, Gramiccia and Gradoni 2005, Ready 2013). This disease is found in humans in xeric and arid regions of northern Africa, the Middle East, the Caucasus, and central Asia (Maroli et al. 2013). Despite being non-fatal, the disfiguring effects of this disease incapacitates people in terms of social and economic status. ZCL has resurged recently in northern Africa and in the Middle East because of the recent political instability and refugee movement (Al-Salem et al. 2016, Du et al. 2016, Mondragon-Shem and Acosta-Serrano 2016).

In the Americas, the peridomestic *Lu. longipalpis* species-complex (Lutz and Neiva) is the most important vector of American visceral leishmaniasis (AVL). AVL is caused by infection with *Leishmania infantum chagasi* (Lutz and Neiva 1912, Soares and Turco 2003,

Jochim et al. 2008). Pathologically, AVL presentation ranges from asymptomatic to severely viscerotropic and is lethal if untreated (Maingon et al. 2008). AVL in the Americas ranges from Mexico to northern Argentina. Its burden remains largely unknown and is probably under-recognized because of the lack of public health disease surveillance infrastructure (Arrivillaga et al. 2002, Bern et al. 2008, Romero and Boelaert 2010, Belo et al. 2013).

A vaccine for ZCL or AVL does not currently exist (Gillespie et al. 2016), and therefore, public health authorities focus on integrated vector management solutions. Vector control for leishmaniasis has historically relied on the use of synthetic insecticides including pyrethroids, organophosphates, carbamates, and organochlorines either directly or inadvertently as part of anti-malarial campaigns (Alexander and Maroli 2003, Kishore et al. 2006, Sharma and Singh 2008, Amóra et al. 2009). Often, though, sand flies live and breed in sylvatic or arid microhabitats that prove challenging to deliver insecticides to, and the effects of initially successful treatments are often transient, making frequent re-applications necessary (Alexander and Maroli 2003, Coleman et al. 2011, Mascari et al. 2011).

Through continued application of insecticides on a multitude of species, tremendous selective pressures for resistance have been exerted. Today, resistant sand fly populations have been documented in the Middle East, southern Asia, and South America (Yaghoobi-Ershadi and Javadian 1995, Singh et al. 2001, Surendran et al. 2005, Alexander et al. 2009, Dinesh et al. 2010, Afshar et al. 2011, Faraj et al. 2012, Hassan et al. 2012, Saeidi et al. 2012, Singh et al. 2012, Coleman et al. 2015, Hassan et al. 2015, Khan et al. 2015, Kumar et al. 2015, Singh and Kumar 2015).

Despite the recent findings of widespread insecticide resistance in sand fly populations around the world, there is little information about the genetic and molecular mechanisms of resistance in these populations. Insecticide resistance to synthetic insecticides has been found in many insect vectors (Hemingway and Ranson 2000, Rivero et al. 2010). Target-site insensitivity

and metabolic detoxification are the most geographically- and entomologically-widespread mechanisms that have been found and studied (Ffrench-Constant et al. 2004, Hemingway et al. 2004, Nauen 2007).

Target-site insensitivity (TSI) results from single nucleotide variant (SNV) nonsynonymous mutations that substitute amino acids in a protein, which causes a conformational change that alters the proteins' structure such that the insecticide can no longer perform its function (Hemingway et al. 2004). TSI-conferring resistance is found in many genes depending on the class of insecticide (Bloomquist 1996, Soderlund and Knipple 2003, Weill et al. 2003, Hemingway et al. 2004). For pyrethroids/DDT and organophosphates/carbamates, TSI is found in the paralytic (*para*) and acetylcholinesterase-1 (*ace-1*) genes, respectively. *Para* encodes the α -subunits of voltage-gated sodium ion channel proteins that surround axons, which are targeted by pyrethroids and DDT. *Ace-1* encodes the acetylcholinesterase enzyme, which clears saturated synapses of acetylcholine following synaptic transmission (Toutant 1989). Organophosphate and carbamate insecticides target the acetylcholinesterase enzyme (Fukuto 1990).

Metabolic detoxification (MD) resistance involves changes in the expression of specific enzymes [carboxylesterases (EST), cytochrome P450s (MFO), and glutathione S-transferases (GST)] that are capable of binding, sequestering, and metabolizing insecticides (Hemingway 2000, Hemingway and Ranson 2000, Ffrench-Constant et al. 2004). Increasing the numbers of these enzymes is achieved through gene amplification or through changes in gene expression (Rivero et al. 2010). It is also common for enzyme classes correlated with metabolic resistance to detoxify multiple insecticide classes: ESTs can detoxify organophosphates, carbamates, and pyrethroids; MFOs can detoxify all insecticide classes; and GSTs can detoxify organophosphates, organochlorines, and pyrethroids (Hemingway and Karunaratne 1998, Hemingway 2000, Corbel et al. 2007, Perera et al. 2008, Che-Mendoza et al. 2009, David et al. 2013).

The hypothesis of this research is that laboratory colonies of *P. papatasi* and *Lu. longipalpis* would evolve resistance over multiple, successive generations of exposure to sublethal doses of pyrethroids and organophosphates. We predicted that sand flies would adapt to surviving insecticide exposure via TSI or MD mechanisms. Specifically, we looked for evidence of convergent evolution of TSI or MD resistance that have been found in other arthropods.

Materials and Methods

Sand Fly Colonies. Laboratory colonies of insecticide-susceptible *P. papatasi* and *Lu. longipalpis* was maintained at Utah State University (Logan, UT). These colonies were obtained in 2012 from 30-year established colonies maintained at the Walter Reed Army Institute of Research (WRAIR) (Silver Spring, MD) that had been originally collected from Jordan and Jacobina, Brazil. All life stages were maintained and reared following established protocols and novel blood-feeding techniques (Denlinger et al. 2015, Denlinger et al. 2016a, and Denlinger et al. 2016b).

Development of Insecticide-Resistant Colonies. Approximately 500 adult *P. papatasi* and *Lu. longipalpis* (generation P) each, including both females and males, were exposed to a sub-lethal dose of either permethrin or malathion to initiate laboratory-bred permethrin-resistant and malathion-resistant colonies. This was done using lethal concentrations (LC) that caused X% mortality of permethrin or malathion in a modified CDC bottle bioassay protocol (Denlinger et al. 2015). Fifty $\mu\text{g/ml}$ permethrin and twenty-five $\mu\text{g/ml}$ malathion served as the LC_{51} and LC_{57} , respectively, for *P. papatasi*, and twenty-five $\mu\text{g/ml}$ permethrin and ten $\mu\text{g/ml}$ malathion served as the LC_{63} and LC_{68} respectively, for *Lu. longipalpis*. Twenty-four hours after insecticide exposure, the surviving females were blood-fed and allowed to oviposit. This process was repeated at successive generations (F_1 - F_n). The resistant-selected colonies were housed in the same environmental growth chamber and reared under the same conditions as the main insecticide-susceptible colony.

Assessing Target-Site Insensitivity (TSI). DNA from ten *P. papatasi* that had survived insecticide exposure from the F₃, F₄, F₆, F₁₁, and F₁₂ generations of permethrin-resistant-selected colony, and DNA from ten *P. papatasi* from the F₃, F₇, and F₈ generations of the malathion-resistant-selected colony were stored at -80°C. DNA from ten *Lu. longipalpis* that had survived insecticide exposure from the F₁-F₄, F₆, F₇, and F₉ generations of the permethrin-resistant-selected colony, and DNA from ten *Lu. longipalpis* from the F₁, F₃, F₄, and F₆ generations from the malathion-resistant-selected colony were stored at -80°C. Among the four colonies, individuals from disparate generations were saved for assessing TSI because of challenges in also sacrificing flies for use in the biochemical assays and in maintaining the propagation of the colony.

Additionally, DNA from ten insecticide-susceptible *P. arabicus* (Israel), *P. argentipes* (India), *P. duboscqi* (Mali), *P. longicuspis* (Tunisia), *P. perfiliewi* (Tunisia), *P. perniciosus* (Tunisia), *P. sergenti* (Israel), *P. sergenti* (South Sinai, Egypt), and *Lu. longipalpis* from Cavunji, Brazil was also extracted. Individuals from all species were provided by (WRAIR). Prior to initiating the resistant-selected colonies, DNA was also extracted from ten *P. papatasi* and *Lu. longipalpis* of our insecticide-susceptible colonies.

Total DNA was extracted from individual sand flies using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA). Each sand fly was initially macerated in 180µl PBS with three solid glass beads (Fisher Scientific) at 25Hz for five minutes using at Retsch® MM 400 (Retsch, Haan, Germany).

TSI was assessed in the *para* gene for the permethrin-selected *P. papatasi* and *Lu. longipalpis* colonies, and TSI was assessed in the *ace-1* gene for the malathion-selected *P. papatasi* and *Lu. longipalpis* colonies. *Para* and *ace-1* gene fragments were amplified in all susceptible species, and in the above-mentioned generations of the *P. papatasi* and *Lu. longipalpis* selected colonies, using PCR. For both genes, the primers and primer sequences used

for each *Phlebotomus* and *Lutzomyia* species, thermal cycler protocols, and thermal cycler conditions for each primer set pair are provided in Tables 5.1., 5.2., 5.3., and 5.4.

All PCR fragments were visually analyzed using gel electrophoresis with 1% TAE gels and purified using Qiagen's QIAquick PCR Purification Kit. Bi-directional sequencing reactions were prepared at Utah State University in 20 µl reactions in a Bio-Rad T100™ thermal cycler. Samples were then sent out for automated Sanger sequencing. DNA sequences were quality trimmed, analyzed, and aligned using DNASTAR Lasergene® version 10.0.1 SeqMan Pro™ (DNASTAR, Madison, WI). For all species, and each generation of the resistant-selected colonies, the DNA sequences from the ten individuals were aligned in Clustal Omega (Sievers et al. 2011) to form a consensus sequence.

The *para* and *ace-1* primers used for all species were derived from *para* and *ace-1* cDNA sequence and primers from the sand fly *Lutzomyia longipalpis* (Coutinho-Abreu et al. 2007, Lins et al. 2008- accession numbers DQ898276 and DQ914434, respectively). Initial *para* and *ace-1*

Table 5.1. *Para* gene primer pairs, thermal cycler protocol name, number of protocol cycles, and annealing temperature during the cycles.

Sand Fly Species*	Primer Pairs	Thermal Cycler Protocol	Number of Thermal Cycler Cycles	Annealing Temperature (°C)
PAIN	Para Nested	SFPARAN	40	60
PAIS	Para Nested-2	SFAGRADN	35	50
PDMA	Para Nested-2	SFAGRADN	35	50
PFTN	Para Nested-2	SFAGRADN	35	50
PLTN	Para Nested-2	SFAGRADN	35	50
PPJO	Para Nested-2	SFAGRADN	35	50
PRTN	Para Nested-2	SFAGRADN	35	50
PSIS	Para Nested-2	SFAGRADN	35	50
PSSS	Para Nested	SFPARAN	35	60
LLJB	Para Nested-2	SFAGRADN	35	50
LLCV	Para Nested-2	SFAGRADN	35	50

*PAIN = *P. argentipes*, India; PAIS = *P. arabicus*, Israel; PDMA = *P. dubosqui*, Mali; PFTN = *P. perfiliewi*, Tunisia; PLTN = *P. longicuspis*, Tunisia; PPJO = *P. papatasi*, Jordan; PRTN = *P. perniciosus*, Tunisia; PSIS = *P. sergenti* Israel; PSSS = *P. sergenti*, South Sinai; LLJB = *Lu. longipalpis*, Jacobina, Brazil; LLCV = *Lu. longipalpis*, Cavunji, Brazil

Table 5.2. *Ace-1* gene primer pairs, thermal cycler protocol name, number of protocol cycles, and annealing temperature during the cycles.

Sand Fly Species*	Primer Pairs	Thermal Cycler Protocol	Number of Thermal Cycler Cycles	Annealing Temperature (°C)
PAIN	DegF-NestR	SFAGRADN	35	50
	F12-R8	SFAGRADN	35	50
PAIS	F14-R8	SFAGRADN	35	50
	F18-R18	SFAGRADN	35	52
PDMA	F12-R8	SFAGRADN	35	52
	F6-R6	SFAGRADN	35	52
PFTN	F12-R8	SFAGRADN	35	50
	F6-NestR	SFAGRADN	40	52
PLTN	F12-R8	SFAGRADN	35	50
	F6-NestR	SFAGRADN	35	52
PPJO	F12-R8	SFAGRADN	35	52
	F6-R6	SFAGRADN	35	52
PRTN	DegF-NestR	SFAGRADN	35	56.5
	F12-R8	SFAGRADN	35	50
PSIS	F14-R8	SFAGRADN	35	50
	F6-R6	SFAGRADN	35	50
PSSS	F14-R8	SFAGRADN	35	50
	F6-R6	SFAGRADN	35	50
LLJB	NewF-NestR	SFAGRADN	35	50
	F14-R8	SFAGRADN	35	56.5
LLCV	NewF-NestR	SFAGRADN	35	50
	F14-R8	SFAGRADN	35	56.5

*PAIN = *P. argentipes*, India; PAIS = *P. arabicus*, Israel; PDMA = *P. dubosqui*, Mali; PFTN = *P. perfiliewi*, Tunisia; PLTN = *P. longicuspis*, Tunisia; PPJO = *P. papatasi*, Jordan; PRTN = *P. perniciosus*, Tunisia; PSIS = *P. sergenti* Israel; PSSS = *P. sergenti*, South Sinai; LLJB = *Lu. longipalpis*, Jacobina, Brazil; LLCV = *Lu. longipalpis*, Cavunji, Brazil

sequence underwent BLAST analysis in VectorBase (VectorBase.org) with the annotated *P. papatasi* and *Lu. longipalpis* genomes. This allowed us to troubleshoot, develop our own primers for *para* and *ace-1*, and expand our *ace-1* coverage by using the cDNA sequence provided in Temeyer et al. (2013). For each species, at least two primer sets were used that sequenced overlapping fragments of the *para* and *ace-1* genes, and the fragments were overlaid and combined to produce one sequence using Clustal Omega (Sievers et al. 2011). The *para* and

Table 5.3. Thermal cycler protocols to amplify the *para* and *ace-1* gene fragments. The “X” cycles and “X°C” annealing temperature match the number of thermal cycler cycles and annealing temperatures found for each sand fly species in Tables 5.1. and 5.2.

Gene	Thermal Cycler Protocol	Thermal Cycler Protocol Conditions
<i>Para</i>	SF paran	95°C - 180 seconds
		X cycles of
		95°C - 30 seconds
		X°C - 30 seconds
		72°C - 30 seconds
		72°C - 300 seconds
<i>Para/Ace-1</i>	SF agrADN	95°C - 120 seconds
		X cycles of
		95°C - 30 seconds
		X°C - 30 seconds
		72°C - 60 seconds
		72°C - 300 seconds

Table 5.4. Primer names and sequences used to amplify *para* and *ace-1* gene fragments. The primer names are associated with the primer pairs listed in Tables 5.1. and 5.2.

Gene	Primer	Sequence 5' - 3'
<i>Para</i>	Para Nested - Forward	ACGGACTTCATGCATTCATTC
	Para Nested - Reverse	TGGTGCTGATSSSCTTGACG
	Para Nested-2 - Forward	GTRTTCCGTGTGYTGTGC
	Para Nested-2 - Reverse	ATCCGAAATTGCTCAAAA
<i>Ace-1</i>	DegF	GCSACYATGTGGAAYCCSAA
	NestR	GTCCAGTCTGTGTA CT CGAA
	F6	GGTATCKATGCAGTATCG
	R6	AATTCCTTCTCTTCGTCC
	F12	CAACGGATAAGGGGAAGG
	R8	AAACCTGTGATCGTACAC
	F14	GAAGGTGAGAGGTGTTAC
	F18	ATGTTTAGGACCTTGGTG
	R18	CGAACAGCCTTTGGAATA
	NewF	TGTCGCAGTACCACATCCGC

ace-1 DNA sequences from the insecticide-susceptible *P. papatasi* and *Lu. longipalpis* served as a baseline to be able to identify TSI-conferring SNVs in the *P. papatasi* and *Lu. longipalpis*

resistant-selected colonies.

Assessing Metabolic Detoxification (MD). Forty non-blood-fed females each from the F₆ and F₁₁ generations of the *P. papatasi* permethrin-resistant-selected colony, F₃ and F₇ generations of the *P. papatasi* malathion-resistant-selected colony, F₂-F₇ generations of the *Lu. longipalpis* permethrin-resistant-selected colony, and F₃ and F₄ generations of the *Lu. longipalpis* malathion-resistant-selected colony were flash-frozen and stored at -80°C for assessing metabolic detoxification. Disparate generations were used because of the availability for flies to be sacrificed for assessing TSI and for maintaining the propagation of the colony.

The established biochemical assay procedures from Valle et al. (2006) were used to assess protein activity and MD in this experiment. These procedures are similar to the procedures used to assess MD in sand flies (Surendran et al. 2005, Alexander et al. 2009, Hassan et al. 2012). Each adult sand fly was individually homogenized in 300 µl of Milli-Q water in 1.5 ml Eppendorf® tubes. Mixed functional oxidases (MFO), acetylcholinesterase (ACE), alpha-esterase (ALPHA), beta-esterase (BETA), ρ -nitrophenyl acetate (PNPA), and glutathione-S-transferase (GST) expression were measured. The total protein assay provided the necessary foundation for the other enzymatic assays. Enzyme levels were analyzed using a Bio-Rad XMark micro plate absorbance reader (Hercules, CA). A standard curve for total proteins, MFO, ALPHA, and BETA were used to convert the optical density for each sample to a protein concentration.

Statistical analyses. For each protein, differences among generations in protein activity were assessed using a one-way ANOVA. Because variances often were unequal among treatment generations, we specified heterogenous variances; the number and composition of generations with unique variance estimates were selected using AICc as a measure of model fit. Pairwise mean comparisons among all generations were adjusted for inflated Type I error using the Tukey method. A significance level of $\alpha = 0.05$ was used for all analyses. Statistical analyses were

performed using SAS/STAT 14.1 in the SAS System for Windows 9.4 TS1M3 using the GLIMMIX procedure (SAS Institute 2001).

Results

Colony Survival.

Phlebotomus papatasi. The percent survival of the permethrin-selected and malathion-selected are provided in Table 5.5. The permethrin-selected colony reached the F₂₁ generation, and it had 50.2% survival, which is an increase in percent survival from the 14.7% survival of the initial insecticide-susceptible generation (P). The malathion-selected colony reached the F₁₈ generation, and it had 42.5% survival, which is an increase from the 14.6% survival of the initial insecticide-susceptible generation (P). Between some generations, there were swings in percent survival (e.g. 79.6% survival in the F₁₀ generation to 37.1% survival in the F₁₁ generation back to 71.9% survival in the F₁₂ generation). For some generations of each resistant-selected colony, we did not expose the population to insecticide in order to boost the population size. This was done because either the population had a low number of individuals or because at multiple times over the duration of this research project we had a mite infestation that forced us to cull larval pots.

Lutzomyia longipalpis. The percent survival of the permethrin-selected and malathion-selected are provided in Table 5.5. The permethrin-selected colony reached the F₁₈ generation, and it had 58.7% survival, which is a decrease in percent survival from the 71.4% survival of the initial insecticide-susceptible generation (P). The malathion-selected colony reached the F₁₄ generation, and it had 22.8% survival, which is a decrease from the 32.9% survival of the initial insecticide-susceptible generation (P). Similar to the *P. papatasi* colonies, there were swings in percent survival (e.g. 28.7% survival in the F₁ generation to 9.8% survival in the F₂ generation back to 53.5% survival in the F₃ generation in the malathion-resistant-selected colony). For the same reasons as the *P. papatasi* colonies, some generations of each resistant-selected colony were not exposed to insecticide.

Table 5.5. Percent survival and the number of *P. papatasi* and *Lu. longipalpis* exposed for each generation of the permethrin-selected and malathion-selected colonies. For generations with “no exposure” the colony was not exposed to insecticide but still allowed to propagate.

Generation	Percent (%) Survival (Number of Flies Exposed)			
	<i>Phlebotomus papatasi</i>		<i>Lutzomyia longipalpis</i>	
	Permethrin-Selected Colony	Malathion-Selected Colony	Permethrin-Selected Colony	Malathion-Selected Colony
P	14.7 (468)	14.6 (522)	71.4 (836)	32.9 (2575)
F₁	68.8 (125)	37.0 (446)	63.3 (2456)	28.7 (2512)
F₂	79.7 (171)	5.3 (946)	57.5 (3651)	9.8 (1791)
F₃	94.8 (524)	64.0 (164)	63.6 (1471)	53.5 (1560)
F₄	65.2 (682)	42.9 (163)	39.6 (2095)	45.2 (3006)
F₅	63.3 (1456)	No exposure	44.7 (1161)	58.7 (702)
F₆	56.7 (1252)	No exposure	48.8 (3309)	43.3 (3160)
F₇	44.7 (805)	87.8 (2011)	61.3 (1862)	7.3 (3559))
F₈	No exposure	51.2 (1164)	82.5 (1632)	11.1 (1716)
F₉	No exposure	54.9 (1401)	77.1 (3693)	40.0 (6621)
F₁₀	43.2 (3096)	79.6 (765)	60.7 (7399)	6.2 (2386)
F₁₁	83.6 (2051)	37.1 (998)	60.8 (4636)	No exposure
F₁₂	77.1 (2008)	71.9 (1141)	60.3 (8776)	3.0 (1870)
F₁₃	51.2 (1855)	No exposure	50.9 (6794)	No exposure
F₁₄	47.0 (1331)	24.5 (261)	No exposure	22.8 (1617)
F₁₅	75.2 (1761)	No exposure	56.2 (3384)	
F₁₆	79.6 (1609)	No exposure	No exposure	
F₁₇	No exposure	No exposure	No exposure	
F₁₈	77.1 (813)	42.5 (2018)	58.7 (3050)	
F₁₉	No exposure			
F₂₀	No exposure			
F₂₁	50.2 (1766)			

Target-Site Insensitivity (TSI).

Paralytic Gene (para). In all the insecticide-susceptible *Phlebotomus* species and *Lu. longipalpis* populations, we successfully amplified portions of the *para* gene surrounding three codons of interest that have been associated with TSI in other insect species. One intron was identified that spanned the 1,016th codon (Table 5.6.). We amplified a fragment that ranged from 344-437bp, before intron removal, (143-206bp after intron removal) that spanned the 1,011th, 1,014th, and 1,016th codons. All species have an ATT at the 1,011th codon (isoleucine), TTA at

the 1,014th codon (leucine), and a GTT or GTC at the 1,016th codon (valine) (Table 5.6.). All ten *P. papatasi* and ten *Lu. longipalpis* individuals of each permethrin-selected generation had no change in these codons, demonstrating no evidence of TSI at these loci.

Acetylcholinesterase-1 gene (ace-1). We amplified a fragment of the *ace-1* gene surrounding the 119th codon, which has been associated with TSI in other insect species, in all the insecticide-susceptible *Phlebotomus* species and *Lu. longipalpis* populations. A fragment that ranged from 911-1,254bp before intron removal was sequenced (911-1,188bp after intron removal). All *Phlebotomus* species genotyped to a GGA at the 119th codon (glycine), and both *Lu. longipalpis* populations have a GGC (glycine) (Table 5.7.). All ten *P. papatasi* and ten *Lu. longipalpis* individuals of each malathion-selected generation had no change in these codons, demonstrating no evidence of TSI at these loci.

Table 5.6. Intron-removed fragments of *para* sequence of different insecticide-susceptible *Phlebotomus* and *Lutzomyia* species and populations. The 1,011, 1,014, and 1,016 codons are bolded and underlined.

Sand Fly Species*	<i>Para</i> DNA sequence containing 1,011, 1,014, and 1,016 th codons (bolded and underlined)**	Fragment Size (bp)
PAIN	...AGTAGTA <u>ATT</u> GGGAAT <u>TTA</u> GTCG [^] <u>TT</u> CTCAATCTTTTCTT...	203
PAIS	...AGTAGTA <u>ATT</u> GGGAAT <u>TTA</u> GTCG [^] <u>TT</u> CTCAATCTCTTCTT...	147
PDMA	...AGTAGTA <u>ATT</u> GGGAAT <u>TTA</u> GTCG [^] <u>TT</u> CTCAATCTTTTCTT...	157
PFTN	...TGTAGTA <u>ATT</u> GGGAAT <u>TTA</u> GTCG [^] <u>TT</u> CTCAATCTCTTCTT...	154
PLTN	...AGTAGTA <u>ATT</u> GGGAAT <u>TTA</u> GTCG [^] <u>TC</u> CTCAATCTCTTCTT...	154
PPJO	...AGTAGTA <u>ATT</u> GGGAAT <u>TTA</u> GTCG [^] <u>TT</u> CTCAATCTTTTCTT...	154
PRTN	...TGTAGTA <u>ATT</u> GGGAAT <u>TTA</u> GTCG [^] <u>TT</u> CTCAATCTCTTCTT...	158
PSIS	...AGTAGTA <u>ATT</u> GGGAAT <u>TTA</u> GTCG [^] <u>TT</u> CTCAATCTTTTCTT...	153
PSSS	...AGTAGTA <u>ATT</u> GGGAAT <u>TTA</u> GTCG [^] <u>TT</u> CTCAATCTTTTCTT...	206
LLJB	...AGTAGTA <u>ATT</u> GGGAAT <u>TTA</u> GTCG [^] <u>TT</u> CTCAATCTTTTCTT...	143
LLCV	...AGTAGTA <u>ATT</u> GGGAAT <u>TTA</u> GTCG [^] <u>TT</u> CTCAATCTTTTCTT...	150

*PAIN = *P. argentipes*, India; PAIS = *P. arabicus*, Israel; PDMA = *P. dubosqui*, Mali; PFTN = *P. perfiliewi*, Tunisia; PLTN = *P. longicuspis*, Tunisia; PPJO = *P. papatasi*, Jordan; PRTN = *P. perniciosus*, Tunisia; PSIS = *P. sergenti* Israel; PSSS = *P. sergenti*, South Sinai; LLJB = *Lu. longipalpis*, Jacobina, Brazil; LLCV = *Lu. longipalpis*, Cavunji, Brazil

**The “^” represents where an intron was identified, and it has been removed for this table.

Table 5.7. Fragments of *ace-1* sequence from insecticide-susceptible *Phlebotomus* and *Lutzomyia* species and populations. The 119th codon is bolded and underlined.

Sand Fly Species*	<i>Ace-1</i> DNA sequence containing 119 th codon (bolded and underlined)	Fragment Size (bp)
PAIN	...TCTTCGGTGGT <u>GGATT</u> CTACTCAGGAACATCCACAC...	1052
PAIS	...TCTTCGGTGGT <u>GGATT</u> CTACTCAGGAACATCCACAC...	1013
PDMA	...TCTTCGGTGGT <u>GGATT</u> CTACTCAGGAACATCCACAC...	1156
PFTN	...TCTTCGGTGGT <u>GGATT</u> TTTACTCAGGGACGTCCACAC...	911
PLTN	...TCTTCGGTGGT <u>GGATT</u> TTTACTCAGGGACGTCCACGC...	960
PPJO	...TCTTCGGTGGT <u>GGATT</u> CTACTCAGGAACATCCACAC...	1111
PRTN	...TCTTCGGTGGT <u>GGATT</u> TTTACTCAGGGACGTCCACAC...	1061
PSIS	...TCTTCGGTGGT <u>GGATT</u> CTACTCAGGAACATCCACAC...	1188
PSSS	...TCTTCGGTGGT <u>GGATT</u> CTACTCAGGAACATCCACAC...	1146
LLJB	...TCTTTGGTGGT <u>GGCTT</u> TTTACTCAGGAACATCCACAC...	1081
LLCV	...TCTTTGGTGGT <u>GGCTT</u> TTTACTCAGGAACATCCACAC...	1017

*PAIN = *P. argentipes*, India; PAIS = *P. arabicus*, Israel; PDMA = *P. dubosqui*, Mali; PFTN = *P. perfiliewi*, Tunisia; PLTN = *P. longicuspis*, Tunisia; PPJO = *P. papatasi*, Jordan; PRTN = *P. perniciosus*, Tunisia; PSIS = *P. sergenti* Israel; PSSS = *P. sergenti*, South Sinai; LLJB = *Lu. longipalpis*, Jacobina, Brazil; LLCV = *Lu. longipalpis*, Cavunji, Brazil

Metabolic Detoxification (MD).

P. papatasi Permethrin-Exposed Colony. Mean enzyme activity with standard deviations for the six enzymes are reported in Table 5.8. For ACE, permethrin susceptible sand flies had an 85.903% inhibition, which was not significantly different from the F₆ generation (85.677% inhibition); however, by the F₁₁ generation, there was significantly less ACE inhibition (80.364%). For ALPA, there was a significant increase in enzyme activity from the susceptible population (19.453 nmol α -naphthol consumed/mg sand fly protein/min) to both the F₆ generation (44.512 nmol α -naphthol consumed/mg sand fly protein/min) and F₁₁ generation (24.186 nmol α -naphthol consumed/mg sand fly protein/min). Opposite results from ALPHA were observed for BETA: the susceptible population (31.614 nmol β -naphthol consumed/mg sand fly protein/min) had significantly more enzyme activity than the F₆ generation (23.492 nmol β -naphthol consumed/mg sand fly protein/min) and the F₁₁ generation (13.457 nmol β -naphthol consumed/mg sand fly protein/min). GST enzyme activity results were similar to BETA results.

The susceptible population (2.915 mmol reaction product/mg sand fly protein/min) had significantly more GST activity than the F₆ generation (0.678 mmol reaction product/mg sand fly protein/min) and the F₁₁ generation (0.702 mmol reaction product/mg sand fly protein/min). For MFO enzyme activity, the susceptible population (5.126 µg cytochrome C) did not have significantly different enzyme activity than the F₆ generation (5.496 µg cytochrome C), but both the susceptible population and F₆ generation had significantly more MFO activity than the F₁₁ generation (2.238 µg cytochrome C). Last, there was no significant change in PNPA activity between the susceptible population (2.912 Δ absorbance/min), the F₆ generation (0.274 Δ absorbance/min), and the F₁₁ generation (-0.66 Δ absorbance/min).

P. papatasi Malathion-Exposed Colony. Mean enzyme activity with standard deviations for the six enzymes are reported in Table 5.9. For ACE, both the susceptible population and F₇

Table 5.8. *P. papatasi* mean enzyme activity (± standard deviation) for the susceptible generation, F₆ permethrin-resistant-selected generation, and F₁₁ permethrin-resistant-selected generation for the acetylcholinesterase (ACE), alpha-esterase (ALPHA), beta-esterase (BETA), glutathione-S-transferase (GST), mixed functional oxidases (MFO), and p-nitrophenyl acetate (PNPA). The mean enzyme activity for the susceptible population, F₆ generation, and F₁₁ generation for each enzyme are statistically different from other generations if they have different [boxed letters] at $\alpha = 0.05$.

Enzyme	Generation		
	Susceptible	F6	F11
ACE (% Inhibition)	85.903 (7.202) [A]	85.677 (2.126) [A]	80.364 (6.671) [B]
ALPHA (nmol α-naphthol consumed/mg sand fly protein/min)	19.453 (9.396) [A]	44.512 (17.037) [B]	24.186 (5.373) [C]
BETA (nmol β-naphthol consumed/mg sand fly protein/min)	31.614 (16.588) [A]	23.492 (8.056) [B]	13.457 (3.042) [C]
GST (mmol reaction product/mg sand fly protein/min)	2.915 (1.684) [A]	0.678 (0.547) [B]	0.702 (0.566) [B]
MFO (µg cytochrome C)	5.126 (3.334) [A]	5.496 (3.042) [A]	2.238 (0.715) [B]
PNPA (Δ absorbance/min)	2.912 (1.876) [A]	0.274 (32.553) [A]	-0.66 (9.169) [A]

generation had statistically similar enzyme inhibition, 85.903% and 82.363%, respectively. In the F₃ generation there had been a spike in inhibition to 95.957%. For the ALPHA enzyme, there was a decrease in activity from 19.453 nmol α -naphthol consumed/mg sand fly protein/min in the susceptible population to 16.909 nmol α -naphthol consumed/mg sand fly protein/min in the F₃ generation to 13.168 nmol α -naphthol consumed/mg sand fly protein/min in the F₇ generation. The change from the susceptible population to the F₃ generation was not significant, but both the susceptible population and F₃ generation had significantly more ALPHA enzyme activity than in the F₇ generation. BETA enzyme activity also saw a statistically significant drop off from the susceptible population (31.614 nmol β -naphthol consumed/mg sand fly protein/min) to the F₃ generation (11.543 nmol β -naphthol consumed/mg sand fly protein/min) and to the F₇ generation (13.037 nmol β -naphthol consumed/mg sand fly protein/min). Similarly, there was a statistically significant decrease in GST activity from the susceptible population (2.915 mmol reaction product/mg sand fly protein/min) to the F₃ generation (0.814 mmol reaction product/mg sand fly protein/min) and to the F₇ generation (0.973 mmol reaction product/mg sand fly protein/min). Likewise, the same trend was observed for MFO activity where we observed approximately half of MFO activity in the F₃ generation (2.854 μ g cytochrome C) and F₇ generation (2.555 μ g cytochrome C) compared to the susceptible population (5.126 μ g cytochrome C). Lastly, similar to the PNPA activity of the permethrin-selected colony, there were no statistically significant differences in PNPA activity among the susceptible population (2.912 Δ absorbance/min) and the F₃ generation (0.295 Δ absorbance/min) and the F₇ generation (1.894 Δ absorbance/min), although there was a decrease in both selected generations from the susceptible population.

Lu. longipalpis *Permethrin-Exposed Colony*. Mean enzyme activity with standard deviations for the six enzymes are reported in Table 5.10. For ACE, permethrin susceptible sand flies had an 92.077% inhibition, which was not significantly different from the F₇ generation

Table 5.9. *P. papatasi* mean enzyme activity (\pm standard deviation) for the susceptible generation, F₃ malathion-resistant-selected generation, and F₇ malathion-resistant-selected generation for the acetylcholinesterase (ACE), alpha-esterase (ALPHA), beta-esterase (BETA), glutathione-S-transferase (GST), mixed functional oxidases (MFO), and p-nitrophenyl acetate (PNPA). The mean enzyme activity for the susceptible population, F₃ generation, and F₇ generation for each enzyme are statistically different from other generations if they have different [boxed letters] at $\alpha = 0.05$.

Enzyme	Generation		
	Susceptible	F3	F7
ACE (% Inhibition)	85.903 (7.202) [A]	95.957 (2.075) [B]	82.363 (7.813) [A]
ALPHA (nmol α -naphthol consumed/mg sand fly protein/min)	19.453 (9.396) [A]	16.909 (7.28) [A]	13.168 (5.298) [B]
BETA (nmol β -naphthol consumed/mg sand fly protein/min)	31.614 (16.588) [A]	11.543 (12.016) [B]	13.037 (3.882) [B]
GST (mmol reaction product/mg sand fly protein/min)	2.915 (1.684) [A]	0.814 (0.697) [B]	0.973 (0.525) [B]
MFO (μ g cytochrome C)	5.126 (3.334) [A]	2.854 (3.456) [B]	2.555 (0.615) [B]
PNPA (Δ absorbance/min)	2.912 (1.876) [A]	0.295 (2.335) [A]	1.894 (9.577) [A]

(90.467% inhibition); but there was significantly less ACE inhibition in the F₂, F₅, and F₆ generations. For ALPHA, there was a significant decrease in enzyme activity from the susceptible population (31.366 nmol α -naphthol consumed/mg sand fly protein/min) to the F₂ generation (23.437 nmol α -naphthol consumed/mg sand fly protein/min) through the F₇ generation (19.890 nmol α -naphthol consumed/mg sand fly protein/min). There was no significant difference in BETA enzyme activity from the susceptible population (18.656 nmol β -naphthol consumed/mg sand fly protein/min) and the F₇ generation (19.942 nmol β -naphthol consumed/mg sand fly protein/min), although the F₂, F₄, F₅, and F₆ generations had a significant decrease in activity. GST enzyme activity was not significantly different from the susceptible population (0.409 mmol reaction product/mg sand fly protein/min) and the F₇ generation (0.283 mmol reaction product/mg sand fly protein/min), and the only increase in GST enzyme activity

was during the F₃ generation (1.247 mmol reaction product/mg sand fly protein/min). MFO enzyme activity saw a significant decrease from the susceptible population (4.047 µg cytochrome C) in the F₃ (2.329 µg cytochrome C), F₄ (2.598 µg cytochrome C), F₅ (1.717 µg cytochrome C), and F₆ (2.630 µg cytochrome C) generations; although the F₇ generation had a not significant difference in MFO enzyme activity (3.880 µg cytochrome C). Last, the F₂-F₄ and F₆ generations all had a significant decrease in PNPA enzyme activity from the susceptible population (2.2 Δ absorbance/min), although no significant decrease in PNPA enzyme activity was found in the F₇ generation (0.710 Δ absorbance/min).

Lu. longipalpis Malathion-Exposed Colony. Mean enzyme activity with standard deviations for the six enzymes are reported in Table 5.11. For ACE, there was a significant decrease in inhibition from the susceptible population (92.077%) to the F₃ generation and F₄ generation, 85.690% and 82.114% inhibition, respectively, but there was not a statistical difference between the F₃ and F₄ inhibitions. A similar observation was seen for the ALPHA and BETA enzymes. The susceptible population had an activity of 31.366 nmol α-naphthol consumed/mg sand fly protein/min and 18.656 nmol β-naphthol consumed/mg sand fly protein/min. There was a significant decrease in the F₃ generation, which had 17.947 nmol α-naphthol consumed/mg sand fly protein/min and 11.000 nmol β-naphthol consumed/mg sand fly protein/min. The F₄ generation activities were also significantly different from the susceptible generation: 17.461 nmol α-naphthol consumed/mg sand fly protein/min and 9.507 nmol β-naphthol consumed/mg sand fly protein/min. For GST activity, there was no significant difference between the susceptible population (0.409 mmol reaction product/mg sand fly protein/min) and the F₃ generation (0.402 mmol reaction product/mg sand fly protein/min), but the F₄ generation had a significant decrease in activity from both the susceptible population and the F₃ generation (0.174 mmol reaction product/mg sand fly protein/min). MFO activity saw a significant decrease from the susceptible population (4.047 µg cytochrome C) to the F₃

Table 5.10. *Lu. longipalpis* mean enzyme activity (\pm standard deviation) for the susceptible generation and F₂-F₇ permethrin-resistant-selected generations for the acetylcholinesterase (ACE), alpha-esterase (ALPHA), beta-esterase (BETA), glutathione-S-transferase (GST), mixed functional oxidases (MFO), and p-nitrophenyl acetate (PNPA). The mean enzyme activity for the susceptible population and F₂-F₇ generations are statistically different from other generations if they have different [boxed letters] at $\alpha = 0.05$. Some multiple comparison tests were unable to be made with the “lines” statement in GLIMMIX because the variances between the differences were unequal, in which case, the comparison is conservative and pairs of means are inferred to be significantly different by the test (SAS Institute 2001).

Enzyme	Generation						
	Susceptible	F2	F3	F4	F5	F6	F7
ACE (% Inhibition)	92.077 (2.633) [A]	85.915 (6.472) [C]	93.099 (6.424) [A]	92.481 (3.427) [A]	86.163 (4.037) [C]	87.963 (2.643) [BC]	90.467 (6.316) [AB]
ALPHA (nmol α -naphthol consumed/mg sand fly protein/min)	31.366 (11.549) [A]	23.437 (8.216) [B]	22.616 (6.525) [B]	20.314 (3.867) [B]	15.294 (2.398) [C]	22.765 (3.849) [B]*	19.890 (4.291) [B]
BETA (nmol β -naphthol consumed/mg sand fly protein/min)	18.656 (6.235) [A]	13.480 (4.309) [B]	19.741 (8.860) [A]	13.489 (3.260) [B]	10.494 (2.175) [C]	11.236 (3.629) [BC]	19.942 (4.670) [A]
GST (mmol reaction product/mg sand fly protein/min)	0.409 (0.201) [B]	0.257 (0.185) [B]	1.247 (0.289) [A]	0.239 (0.166) [B]**	0.214 (0.196) [B]	0.542 (0.999) [AB]	0.283 (0.165) [B]
MFO (μ g cytochrome C)	4.047 (1.704) [A]	3.179 (0.997) [A]	2.329 (0.477) [B]	2.598 (0.615) [B]	1.717 (0.291) [C]	2.630 (0.573) [B]	3.880 (1.401) [A]
PNPA (Δ absorbance/min)	2.200 (1.695) [A]	-0.407 (1.748) [BC]	-0.175 (1.804) [B]	-1.325 (1.320) [C]	0.695 (2.270) [AB]	-1.008 (6.270) [BC]	0.710 (3.372) [AB]

* F₆ and F₇ are inferred to be significantly different

** F₄ and Susceptible are inferred to be significantly different

generation (2.667 μ g cytochrome C) and F₄ generation (2.731 μ g cytochrome C), both the latter two not having a significant difference in activity. Lastly, like GST activity, PNPA activity was not significantly different between the susceptible population (2.200 Δ absorbance/min) and the F₃ generation (1.704 Δ absorbance/min), but the F₄ generation had a significant decrease in activity (-1.345 Δ absorbance/min) from both the susceptible population and F₃ generation.

Table 5.11. *Lu. longipalpis* mean enzyme activity (\pm standard deviation) for the susceptible generation, F₃ malathion-resistant-selected generation, and F₄ malathion-resistant-selected generation for the acetylcholinesterase (ACE), alpha-esterase (ALPHA), beta-esterase (BETA), glutathione-S-transferase (GST), mixed functional oxidases (MFO), and p-nitrophenyl acetate (PNPA). The mean enzyme activity for the susceptible population, F₃ generation, and F₄ generation for each enzyme are statistically different from other generations if they have different [boxed letters] at $\alpha = 0.05$.

Enzyme	Generation		
	Susceptible	F3	F4
ACE (% Inhibition)	92.077 (2.633) [A]	85.690 (10.809) [B]	82.114 (12.455) [B]
ALPHA (nmol α -naphthol consumed/mg sand fly protein/min)	31.366 (11.549) [A]	17.947 (6.207) [B]	17.461 (6.553) [B]
BETA (nmol β -naphthol consumed/mg sand fly protein/min)	18.656 (6.235) [A]	11.000 (3.466) [B]	9.507 (3.329) [B]
GST (mmol reaction product/mg sand fly protein/min)	0.409 (0.201) [A]	0.402 (0.227) [A]	0.174 (0.186) [B]
MFO (μ g cytochrome C)	4.047 (1.704) [A]	2.667 (0.606) [B]	2.731 (0.960) [B]
PNPA (Δ absorbance/min)	2.200 (1.695) [A]	1.704 (2.909) [A]	-1.345 (2.278) [B]

Discussion

Target-Site Insensitivity.

Para. Pyrethroid and DDT insecticides target the α -subunits of voltage-gated sodium ion channels proteins, which are encoded by the *para* gene. Pyrethroids and DDT kill insects by causing knockdown. Knockdown is a physiological response where the voltage-gated sodium ion channels' inactivation is blocked, which causes action potential spasms, involuntary movements, and muscle spasms (Martins et al. 2009). TSI in *para* is known as knockdown resistance (*kdr*). *Kdr* decreases channels' sensitivity to insecticides by decreasing ligand affinity and/or altering the kinetics of channels by favoring the closed-state and accelerating deactivation (Bloomquist and Miller 1986, Davies et al. 2007, Burton et al. 2011, Dong et al. 2014). The protein encoded by the *para* gene has four domains, each of which has six transmembrane helices. Parallel convergent evolution of *kdr* in the sixth transmembrane helix of the second domain, especially at

the 1,011th, 1,014th, and 1,016th amino acids, have been discovered in many insects, which was our reasoning for examining these three codons in our permethrin-resistant-selected colonies of *P. papatasi* and *Lu. longipalpis* (Soderlund and Knipple 2003, Dong et al. 2014).

At the 1,011th codon, all insecticide-susceptible sand flies genotyped in this study had an ATT genotype (isoleucine). Insecticide-susceptible *Aedes aegypti* mosquitoes also have an isoleucine (ATA) at this locus that is substituted for a methionine (ATG) or valine (GTA) in *kdr* individuals (Bregues et al 2003, Saavedra-Rodriguez et al. 2007). The most parsimonious route to *kdr* for *Phlebotomus* species or *Lu. longipalpis* at the 1,011th codon requires one step: a transition of the terminal T to become a G (methionine). The isoleucine becoming a valine requires at least two steps: The initial A to become a G, and the terminal T to become an A.

At the 1,016th codon, all insecticide-susceptible sand flies we genotyped in this study had a valine (GTT or GTC (only in *P. longicuspis*)). *Kdr A. aegypti* mosquitoes have either a glycine (GGA) or isoleucine (ATA). For all the sand flies genotyped, at least two steps would be needed to develop *kdr* similar to *A. aegypti* at the 1,016th locus (Bregues et al. 2003, Saavedra-Rodriguez et al. 2007, Rajatileka et al. 2008, Marcombe et al. 2012).

There is a high frequency of *kdr* convergence at the 1,014th codon. The native leucine in many insects of agricultural and public health importance has been substituted for a phenylalanine, serine, histidine, cysteine, or tryptophan in *kdr* individuals (Dong et al. 2014). This demonstrates the biochemical importance of the leucine's interaction with pyrethroids or DDT (Martinez-Torres et al. 1997, Martinez-Torres et al. 1998). All the insecticide-susceptible *Phlebotomus* species and the two *Lu. longipalpis* populations genotyped in this study had a leucine at the 1,014th codon (TTA). In many insecticide-susceptible insect vector species that also have a TTA at the 1,014th codon, *kdr* arises from two routes using single substitutions. First, in insecticide-resistant mosquitoes and triatomines the terminal A is substituted for a T (phenylalanine) (Martinez-Torres et al. 1998, Martinez-Torres et al. 1999, Ranson et al. 2000,

Lüleyap et al. 2002, Enayati et al. 2003, Karunaratne et al. 2007, Liu et al. 2009, Chen et al. 2010, Singh et al. 2010, Xu et al. 2011, Fabro et al. 2012). Second, in some mosquitoes, the middle T is substituted for a C (serine) (Ranson et al. 2000, Lüleyap et al. 2002, Chen et al. 2010, Singh et al. 2010, Verhaeghen et al. 2010). Both *kdr* routes described above require only one base change from the TTA genotype in the insecticide-susceptible sand flies we genotyped to substitute the leucine for a phenylalanine or serine. To substitute a histidine, cysteine, or tryptophan amino acid for the native leucine, two mutations need to occur.

Ace-1. In nematoceran flies, including sand flies, one gene, *ace-1*, encodes the acetylcholinesterase enzyme (Weill et al. 2002), which is targeted by organophosphate and carbamate insecticides. These insecticides inhibit acetylcholinesterase by phosphorylating/carbamylating the catalytic serine hydroxyl group of the enzyme's oxyanion hole (Fukuto 1990). At the 119th codon of *ace-1*, there have been several examples of convergent TSI evolution in several important mosquito vectors (Weill et al. 2003, Weill et al. 2004, Liu et al. 2005, Cui et al. 2006, Djogbénou et al. 2008). At this locus, the native glycine is substituted for a serine (GGC to AGC). The sterically bulkier serine, because it is in the oxyanion hole, turns over insecticides quicker or blocks the insecticide from performing its function (Weill et al. 2004).

To date, there has been no conclusive evidence of TSI in any sand fly species, although Surendran et al. (2005) found insensitive acetylcholinesterase in *P. argentipes* from Delft Island in Sri Lanka, and Hassan et al. (2012) found insensitive acetylcholinesterase in *P. papatasi* in Surogia village in Sudan. Both examples may be attributed to a TSI mutation in *ace-1*, but they were not genotyped. Insecticide-susceptible mosquitoes have a GGC codon at amino acid 119 (glycine), and resistant individuals have substituted the initial G for an A (serine). The *Phlebotomus* species we genotyped had a GGA genotype at amino acid 119. These species need two mutations to occur (terminal A to C and initial G to A) to become resistant following the route of mosquitoes. Both susceptible *Lu. longipalpis* populations have a GGC genotype and

would only need the initial G to become and A to become resistant, identical to the route of mosquitoes (Coutinho-Abreu et al. 2007).

Implications. Despite evidence of TSI in other vector species, there is no evidence of TSI in our four artificially-selected colonies in any of the generations we genotyped. Similarly, Fawaz et al. (2016) found no evidence of *kdr* after 16 generations of permethrin selection in a laboratory population derived from a field population of Egyptian *P. papatasi*. There are several reasons for why TSI have not yet been found in sand fly populations. First, these mutations may exist in field populations, but they have not been discovered. There is a gap in the understanding of the prevalence and mechanisms of resistance in sand fly populations around the world because of challenges in collecting flies and because of a lack of standardized sand fly bioassays (Li et al. 2015). Resistance cannot be detected and understood if the tools to test for it are lacking.

Second, the fitness costs of TSI mutations may be too great. Fitness costs of insecticide resistance are well-documented in vectors (Shi et al. 2004, Berticat et al. 2008). TSI mutations account for large percentages of the resistant phenotype (Saavedra-Rodriguez et al. 2008) but can impose large fitness costs (Berticat et al. 2008). The *para* and *ace-1* proteins are functionally important, and the exonic sequence between sand fly species and other nematoceran vectors is conserved (Coutinho-Abreu et al. 2007). Mosquito TSI mutations in *para* and *ace-1* proteins cause sluggish and hyperactive nervous systems, respectively, which significantly decrease fitness (Berticat et al. 2008).

Conventional synthetic insecticides pose incredible selection pressures for resistance by killing young adult female vectors. Despite the strong negative physiological changes TSI mutations impose by decreasing fitness, the benefits of resistance (survival) outweigh these costs and help resistance spread in a population. However, if the benefits of resistance could be lessened, the increased costs of resistance would make resistance less likely to spread in a population, as is seen in late-life-acting insecticides to control malaria control (Read et al. 2009).

One way that insects reduce costs is by having further genetic changes that alleviate the strong deleterious effects caused by the initial adaptation, and refine it to minimize fitness costs (Kliot and Ghanim 2012). Mosquitoes with simultaneous TSI in both *para* and *ace-1* had fewer fitness costs than mosquitoes with only TSI in one gene because the two TSI mutations were compensatory for each other (Berticat et al. 2008).

TSI mutations reduce the activity of their target proteins. It is logical to propose that the loci of these mutations occur at insecticide-binding sites (Dong et al. 2014), which could be why there are only a few loci where TSI occurs (Ffrench-Constant et al. 1998) and why parallel convergence is common in these genes across many insect orders (Chevin et al. 2010, Dong et al. 2014). This makes identifying TSI sites predictable because they have direct interactions with the insecticide (Stern and Orgogozo 2009, Stern 2013). We hypothesized that TSI resistance in our sand fly colonies, if found, would be convergent at loci where TSI has been found in other vectors (i.e. the 1,011th, 1,014th, and 1,016th codons of *para* and the 119th codon of *ace-1*)? However, TSI loci do not always follow a lock-and-key binding interaction; amino acids can have epistatic interactions with other amino acids that together confer TSI resistance (Ffrench-Constant et al. 1998, Dong et al. 2014). While genotyping the known loci for evidence of TSI, it is prudent to examine the entire gene for novel TSI mutations.

Lastly, it has been observed that substituted amino acids having varying effects on resistance. For example, the mutated leucine at the 1,014th codon of *para* has been shown to substitute for a phenylalanine, serine, histidine, cysteine, or tryptophan. These amino acids all provide varying levels of resistance to different pyrethroid insecticides and have varying fitness costs. Knowledge of the mutated amino acid can be useful for vector control officials when determining insecticide rotations and insecticide intensity (Rinkevich et al. 2006; Burton et al. 2011; Dong et al. 2014).

Metabolic Detoxification. Unlike TSI resistance, there are few examples of MD resistance in the literature. First, in Montes Claros, Brazil, a region that had used pyrethroids to control sand flies since 1986, Alexander et al. (2009) found a population of *Lu. longipalpis* to have elevated esterases and decreased MFO and GST activity. The *Lu. longipalpis* permethrin-resistant-selected colony demonstrated a decreased ALPHA esterase activity and no change in BETA, GST, or MFO activity. The permethrin-resistant-selected *P. papatasi* colony had very similar results to the Montes Claros population: elevated ALPHA esterase activity and decreased MFO and GST activity. Second, a population of *P. argentipes* on Delft Island, Sri Lanka had elevated esterase. Delft Island is known to have been heavily treated with malathion during anti-malarial campaigns (Surendran et al. 2005). Unlike this *P. argentipes* population, the malathion-resistant-selected *P. papatasi* and *Lu. longipalpis* laboratory colonies both had decreased ALPHA and BETA esterase activity. Third, Hassan et al. (2015) found 60% of DDT-resistant *P. argentipes*, collected from three villages in the Bihar state, to have elevated GST activity. The *P. papatasi* permethrin-resistant-selected colony had decreased GST activity, and the *Lu. longipalpis* permethrin-resistant-selected colony had no change in GST activity. Fourth, in Surogia village, Sudan, Hassan et al. (2012) found *P. papatasi* to have elevated esterases and GSTs. This region of Sudan has historically used malathion and propoxur as part of control programs targeting *Anopheles* mosquitoes. Opposite of this field population, our malathion-resistant-selected *P. papatasi* and *Lu. longipalpis* colonies both had decreased esterases and decreased GSTs.

Lastly, in a laboratory population of *P. papatasi* from Egypt under permethrin selection, Fawaz et al. (2016) observed, from the control generation to the F₁₆ generation, no significant differences in GST activity and a decrease in ACE inhibition. Interestingly, MFO and esterase activity increased significantly in the early generations of exposure but returned to approximate control generation levels by the F₁₃ and F₁₆ generations. Our permethrin-resistant selected *P. papatasi* colony also had a significant decrease in ACE inhibition, but it had significant decreases

in GST and MFO activity. Like the Fawaz et al. (2016) *P. papatasi* colony's esterase activity, our *P. papatasi* colony had a very significant increase in ALPHA activity in the F₆ generation and it decreased from the F₆ generation to the F₁₁ generation (although still significantly greater activity than the susceptible population). Our permethrin-resistant selected *Lu. longipalpis* colony had no significant decrease in ACE inhibition, GST activity, and MFO activity. Unlike Fawaz et al. (2016) *P. papatasi* colony's esterase activity, our *Lu. longipalpis* ALPHA and BETA activity had significant decreases in the early generations, but was increasing towards the latter generations, although it was not significant different from the susceptible population.

The disparity between enzyme activities involved in MD documented from sand fly populations around the world can provide important insights about these enzymes. Similar results between populations of which enzymes contribute to MD should not always be expected because it is common for enzyme classes to detoxify multiple insecticide classes. (Hemingway and Karunaratne 1998, Hemingway 2000, Corbel et al. 2007, Perera et al. 2008, Che-Mendoza et al. 2009, David et al. 2013). Additionally, despite strong evidence for MD resistance being conferred by enzyme upregulation, decreased enzyme can also confer resistance. Decreased enzyme activity conferring MD has been best described for MFOs and esterases. MFOs function by first enzymatically activating insecticides, which they later detoxify; resistance can develop by having fewer MFOs that bioactivate fewer insecticides (Scott 1999). Additionally, the "Ali-esterase hypothesis" posits that there is functional redundancy with overlapping substrate specificity between enzymes of the same family (e.g. carboxylesterases). Mutations in some of these enzyme family members makes them unable to hydrolyze typical substrates, which would present as decreased activity of that enzyme family. For example, in organophosphate-resistant house flies and blow flies, esterase activity was found to be low because mutations had caused them to be unable to hydrolyze esterases, but in doing so, some of the esterases had gained enhanced ability to hydrolyze organophosphates (Newcomb et al. 1997, French-Constant et al.

2004). Many of the enzymes that we measured for our four resistant-selected colonies also had reduced activity (Tables 8-11), and the possibility for a similar mechanism as the ali-esterase hypothesis should be investigated further as a mechanism of metabolic detoxification resistance.

The detoxifying enzymes which contribute to the resistance phenotype may depend on fitness costs. Like TSI resistance, the fitness costs of MD resistance may have been too large for all the colonies to upregulate the activity of these enzymes. For the insect vector, resistance has costs correlated with pleiotropic changes in its biology, physiology, and behavior. Resources are allocated to traits affecting metabolic processes at the cost of other traits (Chevillon et al. 1997), and the allocation for resources to possibly produce more detoxifying enzymes was too great. Perhaps the energy resources for survival were allocated elsewhere that had fewer fitness costs. The re-allocation of resources to/from metabolic detoxification enzymes affects a vector's longevity, immune response, and ability to transmit pathogens (Rivero et al. 2010).

For a vector's longevity, MD resistance can require the overproduction of detoxifying enzymes, often at the expense of survival and the ability to combat oxidative stress (Rivero et al. 2010). There is added oxidative stress from normal metabolism and respiration when hemoglobin is digested in the blood-meals of blood-feeding insects (Graça-Souza et al. 2006, (Diaz-Albiter et al. 2012). MFOs and esterases increase oxidative stress by producing reactive oxygen species (ROS) during oxidative respiration, which cause internal damage and decrease a vector's longevity; GSTs protect against oxidative stress by solubilizing and excreting ROSs to increase longevity (McCarroll and Hemingway 2002, Vontas et al. 2002, Enayati 2003, Che-Mendoza et al. 2009, David et al. 2005, Rivero et al. 2010).

MD resistance also alters a vector's immune response. MD-associated enzymes are pleiotropic in their ability to recognize of foreign parasites, to affect the transduction of immune signaling, and to affect the pathogen targeting mechanisms (Rivero et al. 2010). GSTs, because of their ability to neutralize ROSs in response to pathogen invasion, may increase the

susceptibility of a vector to the parasite by limiting the ability of ROSs to activate the apoptotic cycle in the pathogens (Kumar et al. 2003, MacLeod et al. 2007). Esterases and MFOs, in contrast, make vectors more refractory to pathogen infection by increasing parasite melanotic encapsulation through increased ROS production (Kumar et al. 2003). Lastly, overproduced enzymes can favor parasite development by depleting resource pools, which limits the vector's ability to mount an immune response. However, if the resources are directed away from the parasite's ability to consume them, then the parasite's development may be limited (Rivero et al. 2010, Vézilier et al. 2010).

MD resistance can also affect a vector's ability to transmit pathogens by altering the vector microenvironment where parasites develop. When the insect host becomes resistant to insecticides, this environment can be modified that may be advantageous or disadvantageous to the parasite. For example, the physiological changes resulting from MD resistance may make the vector toxic for parasites. McCarroll et al. (2000) and McCarroll and Hemingway (2002) showed that insecticide resistant *Culex quinquefasciatus* mosquitoes that overproduced ESTs were more refractory to *Wuchereria bancrofti* filariae than susceptible *C. quinquefasciatus*. The larval worms died when the redox potential changed in the tissues where they were living because of overproduced ESTs. This connection between ESTs and ROS production could extend to other parasites that are susceptible to oxidative stress. A future aim could be to examine how *Leishmania* survive in sand flies with MD resistance.

Conclusions. In this experiment, the artificial selection for insecticide resistance in laboratory populations of *P. papatasi* and *Lu. longipalpis* failed to demonstrate convergent mechanisms found in other vectors. However, we did observe colony survival in successive generations over the course of several years, which does suggest that resistance is a heritable phenotypic trait with a genetic mechanism, but a mechanism that we did not find in this experiment. In future investigations, we plan to examine the genetic variation of our susceptible

populations of *P. papatasi* and *Lu. longipalpis* to make inferences about other possible mechanisms of insecticide resistance.

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CHAPTER 6

STANDING GENETIC VARIATION IN LABORATORY POPULATIONS OF INSECTICIDE-SUSCEPTIBLE *PHLEBOTOMUS PAPTASI* AND *LUTZOMYIA LONGIPALPIS* (DIPTERA: PSYCHODIDAE: PHLEBOTOMINAE) FOR THE EVOLUTION OF RESISTANCE**Abstract**

Genome-wide associations can locate and characterize alleles that underlie traits that are associated with a phenotype of interest. Alleles allow for adaptation to increased fitness when encountering novel selective pressures. Insecticides are selective pressures that have forced populations of phlebotomine sand flies (Diptera: Psychodidae), vectors of *Leishmania* parasites, to develop resistance around the world. However, there is little information about the genetic mechanisms of their adaptation to insecticide exposure. Using genotype-by-sequencing, we created DNA libraries of insecticide-susceptible *Phlebotomus papatasi* and *Lutzomyia longipalpis* that survived or died from a sub-lethal exposure to either permethrin or malathion. A genome-wide efficient mixed model association was used to examine the standing genetic variation in these populations and to associate alleles with adaptive survival to insecticide. For each treatment, we estimated the proportion of the phenotypic variance explained by the genetic data (i.e. heritability), the proportion of the genetics with measurable effect sizes, and the number of single nucleotide variants with measurable effects. For all treatments, survival to an insecticide exposure is a heritable trait with modest genetic architecture and polygenic mechanisms. Both *P. papatasi* and *L. longipalpis* had alleles for survival that associated with many genes throughout their genomes. The implications of polygenic resistance are discussed for previous work that has not found evidence of monogenic resistance in *P. papatasi* and *L. longipalpis* colonies having been exposed to permethrin and/or malathion for several years. Inferences are made about the

utility of laboratory association studies compared to field observations in terms of insecticide resistance.

The ability to understand, characterize, and analyze the alleles that underlie traits has always interested biologists (Nielsen 2005). These traits give rise to the incredible phenotypic diversity observed in organisms. All organisms are under the control of the fundamental forces of evolution: mutation, selection, recombination, and drift (Feyereisen et al. 2015). Natural selection is the driving force of evolution; it is opportunistic if new variants are available (Nei 2007, Brakefield 2011). Variants from standing genetic variation or from new mutations allow populations to adapt to new selective pressures (Barrett and Schluter 2008). These responses allow populations to achieve fitness optima, and novel selective pressures force adaptation to a new optimum (Orr 2002). Adaptation is necessary for a population when exposed to a new selection pressure in order to increase its fitness.

Synthetic insecticides are a great example of a selection pressure, and they have forced insects to adapt for survival since the 1940s (Hemingway and Ranson 2000, Alexander and Maroli 2003). Insecticides have been extremely useful because they kill vectors to reduce disease transmission, but there are many examples of insecticide resistance in the most important vector populations around the world (Hemingway and Ranson 2000, Rivero et al. 2010). Phlebotomine sand flies (Diptera: Psychodidae) are vectors that transmit *Leishmania* protozoans that cause leishmaniasis to humans, a disfiguring, stigmatizing, and lethal disease causing tens of thousands of deaths each year worldwide (Hotez 2008, World Health Organization (WHO) 2010, Alvar et al. 2012, WHO 2013).

The continued application of insecticides has been a tremendous selective pressure for resistance in sand fly populations. Today, resistant sand fly populations have been documented in the Middle East, southern Asia, and South America (Surendran et al. 2005, Alexander et al. 2009,

Dinesh et al. 2010, Faraj et al. 2012, Hassan et al. 2012, Saeidi et al. 2012, Hassan et al. 2015, Khan et al. 2015). Despite the recent findings of widespread resistance in sand fly populations around the world, there is little information about the genetic and molecular mechanisms of resistance in these populations.

Populations adapt to new selective pressures in two ways: from standing genetic variation or from new mutations, which result in monogenic or polygenic responses, often depending on the strength of selection (Barrett and Schluter 2008). In previous research, we did not find examples of monogenic resistance via target-site insensitivity (TSI) or metabolic detoxification (MD) in *Phlebotomus papatasi* and *Lutzomyia longipalpis* colonies under artificial selection for resistance over the course of several years (Denlinger et al. in review). Resistance is known to be more complicated than TSI or MD; many genes with different mechanisms can collectively contribute to the resistance phenotype (David et al. 2005, Vontas et al. 2005, Vontas et al. 2007). More robust methods are now needed to scan the entire sand fly genome for markers associated with insecticide exposure survival.

Genotype-by-sequencing (GBS) is a new method for exploring thousands of single nucleotide variants (SNVs) throughout a genome to identify associations between loci and the phenotype involved in adaptation as well as the strength of each locus' contribution to adaptive evolution, either directly or through linkage disequilibrium (Hirschhorn and Daly 2005, Romay et al. 2013, Comeault et al. 2014, Comeault et al. 2015). GBS do not describe causation between SNVs and the phenotype, only statistical associations with a degree of uncertainty (Guan and Stephens 2011, Comeault et al. 2014). It is a first step by providing large coverage across the entire genome to identify many candidate SNVs that could be involved in a trait's complex architecture (Comeault et al. 2014).

The goal of this research was to understand if insecticide exposure in laboratory populations of insecticide-susceptible *Phlebotomus papatasi* and *Lutzomyia longipalpis* is pre-

adaptive by using GBS to examine their standing genetic variation, and if it is pre-adaptive, to identify and to map the genetic variants that confer the survival phenotype. That is, we wanted to know if the standing variation in these populations was sufficient for selection to drive insecticide resistance into a population. We hypothesized that there would be little standing genetic variation in our laboratory populations, and therefore, the ability to survive an insecticide exposure would be polygenic and rely upon many genes with small effect sizes that would cumulatively allow for survival. To our knowledge, this is the first experiment to incorporate GBS with genome-wide association mapping to understand the mechanisms of insecticide resistance in any vector species. GBS is potentially a valuable tool for identifying and mapping important genetic variants associated with insecticide resistance adaptation beyond which mechanisms are currently assessed in insecticide resistance studies.

Materials and Methods

Sand Fly Colonies. Laboratory colonies of insecticide-susceptible *P. papatasi* and *L. longipalpis* were maintained at Utah State University (Logan, UT). Both species were derived from 30-year established colonies maintained at the Walter Reed Army Institute of Research (WRAIR) (Silver Spring, MD) that had been originally collected from Jordan and Jacobina, Brazil. All life stages were maintained and reared following Denlinger et al. (2015), Denlinger et al. (2016a), and Denlinger et al. (2016b).

Insecticide exposure. One hundred ninety-two adult *P. papatasi* and *L. longipalpis*, both males and un-blood-fed females, were exposed to a sub-lethal dose of either permethrin or malathion. This was done using lethal concentrations (LC) that caused X% mortality of permethrin or malathion in a modified CDC bottle bioassay protocol (Denlinger et al. 2015). *Phlebotomus papatasi* were exposed to 50 µg/ml permethrin and 25 µg/ml malathion, the LC₅₁ and LC₅₇ respectively. *Lutzomyia longipalpis* were exposed to 25 µg/ml permethrin and 10 µg/ml

malathion served as the LC_{63} and LC_{68} . These LCs were the same LC values used to initiate resistant-selected colonies in Denlinger et al. (in review).

Following insecticide exposure, all sand flies were captured via mechanical aspiration and released into 1-pint cardboard containers with a mesh top onto which a cotton ball saturated with 30% sugar-water was placed and served as an energy/water source. The containers were kept in the same growth chamber as the insecticide-susceptible colonies. Sand flies were held in these containers for 24 h when mortality was scored as a complete cessation of movement (Perea et al. 2009, Denlinger et al. 2015). The insecticide exposure phenotype was scored as binary: survived exposure or died from exposure.

Genotype-by-Sequencing.

DNA Extraction and Library Preparation. Libraries were prepared separately for the permethrin- and malathion-exposed 192 *P. papatasi* or *L. longipalpis* (four libraries in total). For each library, total DNA was extracted from all 192 sand flies individually using Qiagen's DNeasy 96 Blood & Tissue Kit (Qiagen Inc., Valencia, CA, USA). Barcoded restriction-site associated DNA libraries were generated (Nosil et al. 2012, Parchman et al. 2012, Gompert et al. 2014, Comeault et al. 2015). For each library, genomic DNA of each sand fly was digested with the restriction enzymes *EcoRI* and *MseI* (NEB, Inc. Ipswich, MA, USA), and then *EcoRI* and *MseI* adaptor oligonucleotides were ligated onto the digested DNA fragments. All 192 sand flies received the same universal *MseI* adaptor, which contained an Illumina adaptor and complementary *MseI* restriction site nucleotides; unique *EcoRI* adaptors allowed for individual sand fly recognition and contained an Illumina adaptor, a 10-bp individual barcode, and an addition nucleotide to complement the restriction site. Next, DNA from each sand fly was amplified through two rounds of PCR, all 192 individual PCR reactions were pooled together, and the pooled library was size-selected between 200-300bp using the Blue Pippin at the USU Center for Integrated Biosystems. The size-selected DNA libraries were sequenced at the

University of Texas Genomic Sequencing and Analysis Facility (Austin, TX, USA) on lanes of the Illumina HiSeq 2500 or HiSeq 4000 platforms (one library per lane).

Sequence Assembly and Data Analysis. All bioinformatics were conducted through a Linux terminal running the bash shell. Custom Perl scripts were first used to remove the *MseI* and *EcoRI* barcodes (Nosil et al. 2012). A Burrows-Wheeler Transformation was used to index the *P. papatasi* and *L. longipalpis* reference genomes provided in VectorBase.org (Giraldo-Calderón et al. 2015). Single-end reads of each sand fly were aligned to their respective reference genome using Burrows-Wheeler Alignment Tool (Li and Durbin 2009). The following criteria were established for alignment: a maximum of 4-bp differences, a seed length of 20-bp, two mismatches in the seed, a gap between mismatch penalties and their current values, and a quality threshold for read trimming set to 10. Only reads with a single best match were aligned. Alignment files were converted to binary format, which were then sorted and indexed.

SAMtools mpileup was used to implement a Bayesian model that distinguished between variable nucleotides and errors. Here, the probability of the observed sequence data was calculated as a product across all individual reads, given the base quality scores and the assumption that all individuals were homozygous for the reference allele. Based on the standard neutral model given a specified value of $\theta = 4N_e\mu$, the prior probability that the nucleotide was invariant was calculated. By combining the prior probability with the likelihood to produce the posterior probability, a nucleotide position was assumed to be variable when a low probability resulted. Variants were called by combining the treatment groups of each sand fly species because of the supposed inbred homogeneity among the susceptible population from which all of the flies were taken. In bcftools, the standard neutral model was applied for the prior probability distribution with the per nucleotide value of θ set to 0.001, variants at loci where sequence data for no less than 80% of all sand flies were used, and variants where the posterior probability of having an invariant allele given the sequence data and quality scores under a null model was less

than 0.01 (Li 2011). Genotype posterior probabilities were derived, for each population separately, from a product of genotype likelihoods at each locus and a Hardy-Weinberg prior distribution based on the minor allele frequencies (MAF). Posterior probability means were calculated to provide predictive probability of a particular genotype for an individual. For each treatment, the total population MAF combined for the two populations (population that survived insecticide exposure, population that succumbed to insecticide exposure) were calculated using twenty iterations of the Expectation-Maximization algorithm (Li 2011, Gompert et al. 2014), which maximizes the model likelihood with respect to the genotype likelihoods (Gompert et al. 2014).

Genome-wide Association Mapping. Associating the insecticide exposure survival phenotype with genotypes was completed using Bayesian sparse linear mixed models (BSLMMs) with the software Genome-wide Efficient Mixed Model Association (*gemma*) (Zhou et al. 2013). BSLMMs in *gemma* estimate the proportion of phenotypic variation that can be explained by \mathbf{u} , which captures SNVs with infinitesimal effect sizes, or β , which captures SNVs with measurable effect sizes. Such results were derived through Bayesian parameters: the proportion of the phenotypic variance explained (PVE) by the genetic data (β and \mathbf{u}), the proportion of PVE explained by genetic variants (PGE) with measurable effect sizes only (β), and the number of large effect SNVs explaining the phenotypic variance ($n-\gamma$). Thirty independent Markov-chain Monte Carlo (MCMC) chains on a binary BSLMM were run for insecticide susceptibility. MCMC chains had 100,000 burn in steps, chain lengths of 1,000,000, a thinning interval of 10, and all other parameters set to default values. Based on a Gelman-Rubin diagnostic test in the R package (CODA), optimal convergence was established with 30 MCMC chains.

In addition to estimating the three parameters, *gemma* provides posterior inclusion probabilities (PIP) in the BSLMMs that quantify the probability of each SNV contribution to insecticide susceptibility, given the data. Model average point estimates (MAPE) were derived

from PIPs for estimating SNVs that had non-zero measurable effects on insecticide susceptibility variation (Zhou et al. 2013). Posterior inclusion probabilities and model average point estimates for genetic architecture parameters (PVE, PGE, and $n-\gamma$) were summarized based on the posterior median and the 95% highest posterior density interval using the R package (CODA).

In VectorBase, we examined SNVs with aberrantly higher absolute-value MAPE scores using the Variant Effect Predictor (VEP) tool, which describes the genetic consequence of each SNV in relation to the genome (Giraldo-Calderón et al. 2015). If a SNV had a consequence associated with a gene, VEP provides access to the name of the gene and its function, if annotated.

Cross-Validation. A cross-validation was performed using *gemma* to predict phenotypes from a test data set containing missing phenotypic values. Here, the same phenotype and genotype files were used for both fitting BSLMM and obtaining predicted values, whereby individuals in the test data set were labeled with either true phenotype values or as missing (e.g. “NA”). Only predicted values were obtained for individuals with missing phenotypes (20% of the individuals in the test set). Receiver operator characteristic (ROC) curves were constructed to determine the predictive power in correctly classifying surviving and perished sand flies, using the R package (*rocr*). Here, we provide insight into the genetic interpretation of the area under the curve (AUC) when the test classifier is a predictor of insecticide survival.

Results

Insecticide Exposure. In the sub-lethal insecticide exposure tests on the susceptible populations, 128/192 (66.7%) *P. papatasi* survived exposure to permethrin, and 45/192 (23.4%) survived exposure to malathion. From the *L. longipalpis* colony, 130/192 (67.8%) survived exposure to permethrin, and 96/192 (50%) survived exposure to malathion.

Summary Statistics.

Phlebotomus papatasi. One hundred eighty-seven out of 192 permethrin-exposed individuals yielded sufficient reads for analysis. A total of 80,516,505 DNA sequences were processed, and 51,290,933 (64%) successfully aligned to the reference genome. The average number of reads per individual was 430,570, and the average length of a read was 46-bp. All 192 malathion-exposed individuals yielded sufficient reads for analysis. A total of 221,625,299 DNA sequences processed, and 111,682,651 (50.4%) successfully aligned to the reference genome. The average number of reads per individual was 1,154,304, and the average length of each read was 48-bp. Across each individual alignment, there was an average of 6 reads covering each position in the genome, ranging from 0 - 8004 reads with no observable pattern with respect to scaffold number. There was a total of 38,657 variant SNPs called from permethrin- and malathion-exposed *P. papatasi*.

Lutzomyia longipalpis. One hundred eighty-two out of 192 individuals exposed to permethrin yielded sufficient reads for analysis. A total of 207,072,345 DNA sequences were processed, and 78,155,513 (37.7%) successfully aligned to the reference genome. The mean number of reads per individual was 1,134,460, and the average length of each read was 47-bp. One hundred fifty-three out of 192 individuals exposed to malathion yielded sufficient reads for analysis. A total of 75,785,403 DNA sequences were processed, and 34,016,329 reads (45%) successfully aligned to the reference genome. The mean number of reads per individual was 495,329, and the average length of each read was 47-bp. Across individual alignments, the average depth of coverage at each position in the genome was 16 reads with 0 – 4678 reads with no observable pattern in terms of scaffold number. There was a total of 18,856 variant SNPs called from permethrin- and malathion-exposed *L. longipalpis*.

Minor Allele Frequencies. There is a strong positive correlation among the estimated minor allele frequencies between the perished and surviving *P. papatasi* exposed to permethrin

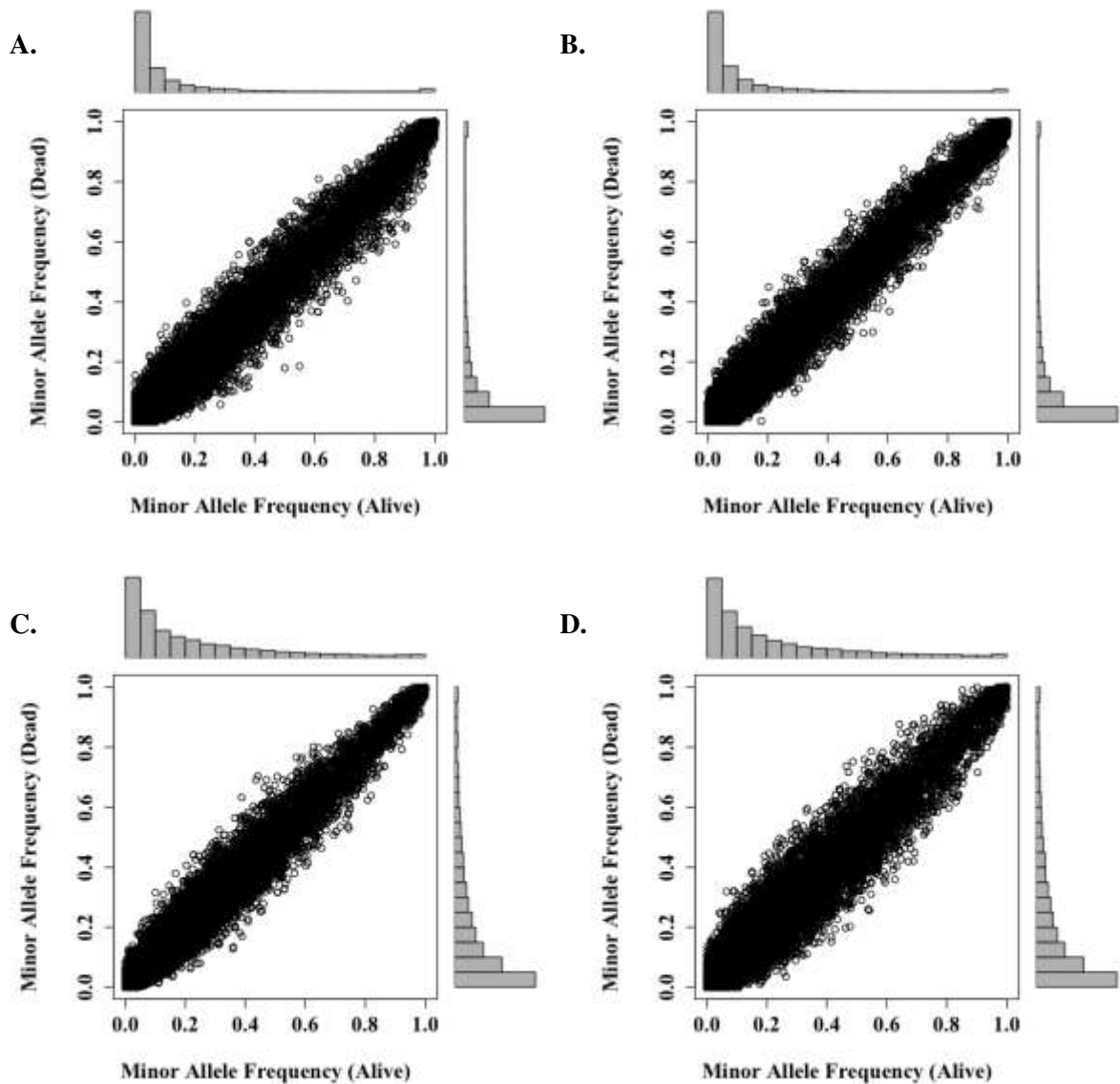


Fig. 6.1. Correlations between minor allele frequencies between the sand flies that survived and perished in the *P. papatasi* permethrin treatment (A), *P. papatasi* malathion treatment (B), *L. longipalpis* permethrin treatment (C), and *L. longipalpis* malathion treatment (D).

exposure ($r = 0.985$, Fig. 6.1A.) and exposed to malathion ($r = 0.987$, Fig. 6.1B.). Similarly, the correlations were strong for the *L. longipalpis* exposed to permethrin ($r = 0.981$, Fig. 6.1C.) and exposed to malathion ($r = 0.968$, Fig. 6.1D.).

Genome-wide Association Mapping.

Phlebotomus papatasi. For survival to permethrin, 62% of the total phenotypic variation is explained by genetic data (CI for PVE = 26.7% - 99.9%). Approximately 69.7% of the genetic data (CI for PGE = 31% - 100%) is due to loci with measurable effects on resistance, including an average of 7 SNVs (CI for $n-\gamma$ = 0 - 60). For survival to malathion, the genotypic data can explain 14.7% of total phenotypic variation (CI for PVE = .0001% - 53.6%). Approximately 36.4% of the genetic data (CI for PGE = 0% - 93%) is based on loci with measurable effects for resistance, including an average of 15 SNVs (CI for $n-\gamma$ = 0 - 217).

Lutzomyia longipalpis. For survival to permethrin, the genotypic data can explain 35.6% of the total phenotypic variation (CI for PVE = .001% - 76.1%). Of this explained variation, 39.5% (CI for PGE = 0% - 93.5%) can be explained by 28 SNVs (CI for $n-\gamma$ = 0 - 243) with measurable effects. For survival to malathion, our genotypic data can explain 90.1% of total phenotypic variation (CI for PVE = 40.7% - 99.9%). Of this explained variation, 29.8% (CI for PGE = 0% - 91.6%) can be explained by 58 SNVs (CI for $n-\gamma$ = 0 - 258) with measurable effects.

We found each *P. papatasi* treatment group to have few SNVs with large MAPE scores (Fig. 6.2A., B.). Across *L. longipalpis* treatment groups, there were relatively more SNVs associated with large MAPE scores (Fig. 6.2C., D.). Top ranking MAPE scores in the permethrin treatment groups across species exhibited higher posterior inclusion probabilities than those in the malathion treatment groups (Table 6.1-4.). For each treatment, at least one SNV was associated directly with a gene, whether with a known function or not, according to the VEP tool in VectorBase. The two highest MAPE score SNVs in the *L. longipalpis* treatment group exposed to malathion had associations with four genes (Table 6.4.). For each species, the genes associated with the highest MAPE score SNVs was different (Table 6.1-4.). Although, for both malathion treatment groups, a SNV associated with zinc fingers was found (Table 6.2., 6.4.).

Table 6.1. Association mapping of SNVs with the largest model-average point estimates from the *Phlebotomus papatasi* exposed to permethrin treatment.

Scaffold:Position	Model-Average Point Estimate	Posterior Inclusion Probability	Genetic Consequence	Gene	Function
1601:26833	2.7265	0.412	Intergenic variant		
3565:10014	1.0922	0.467	Downstream variant	PPAI005735	Unknown
99828:233	0.3957	0.091	Intergenic variant		
67723:636	0.1885	0.177	Intergenic variant		
53775:723	0.1167	0.089	Intergenic variant		

Table 6.2. Association mapping of SNVs with the largest model-average point estimates from the *Phlebotomus papatasi* exposed to malathion treatment.

Scaffold:Position	Model-Average Point Estimate	Posterior Inclusion Probability	Genetic Consequence	Gene	Function
661:31493	0.0228	0.009	Synonymous variant	PPAI009906	Serine protease
2202:3597	0.009	0.012	Intergenic variant		
48932:4971	0.0059	0.005	Downstream variant	PPAI008313	Mitochondrial substrate/solute carrier
5205:7108	0.0055	0.005	Upstream variant	PPAI008803	Zinc finger
29:84808	0.0043	0.006	Intergenic variant		

Table 6.3. Association mapping of SNVs with the largest model-average point estimates from the *Lutzomyia longipalpis* exposed to permethrin treatment.

Scaffold:Position	Model-Average Point Estimate	Posterior Inclusion Probability	Genetic Consequence	Gene	Function
9743:508	0.0195	0.0394	Intergenic variant		
136:192398	0.0167	0.0283	Downstream variant	LLOJ001674	Unknown
2068:4242	0.016	0.0201	Intergenic variant		
35:185239	0.0144	0.0197	Intron variant	LLOJ005493	Orange domain-like
113:53407	0.0142	0.0135	Intron variant	LLOJ000771	Isoprenoid synthase

Table 6.4. Association mapping of SNVs with the largest model-average point estimates from the *Lutzomyia longipalpis* exposed to malathion treatment.

Scaffold:Position	Model-Average Point Estimate	Posterior Inclusion Probability	Genetic Consequence	Gene	Function
31:237972	0.5558	0.0159	1. Downstream variant 2. 5' UTR 3. Downstream variant 4. Upstream variant	1. LLOJ005038 2. LLOJ005039 3. LLOJ005040 4. LLOJ005041	1. Protein disulfide isomerase 2. PDCD5-related 3. Nuclear envelope phosphatase-regulatory-like 4. Microtubule-associated protein RP/EB
8:211637	0.3679	0.0267	1. Synonymous variant 2. 5' UTR 3. Upstream variant 4. Downstream variant	1. LLOJ009054 2. LLOJ009053 3. LLOJ009055 4. LLOJ009056	1. Zinc finger 2. Zinc finger 3. Transmembrane Fragile-X-F-associated protein 4. Intra-flagellar transport protein
25:59200	0.2606	0.0086	Synonymous variant	LLOJ004221	Transcription factor CP2
1643:2211	0.1591	0.0084	Intergenic variant		
1282:13137	0.1256	0.0159	Downstream variant	LLOJ001414	Glycosyltransferase

Cross-Validation. For *P. papatasi*, the area under the ROC curve (AUC = .68) suggests standing genetic variation to be a robust predictor for permethrin resistance (Fig. 6.3A.), whereas standing genetic variation in *P. papatasi* is not a sufficient predictor for malathion resistance (AUC = .36; Fig. 6.3B.). In *L. longipalpis*, standing genetic variation is a variable predictor for permethrin resistance (AUC = .53; Fig. 6.3C.), and malathion resistance (AUC = .59, Fig. 6.3D.).

Discussion

We found evidence that laboratory colonies of insecticide-susceptible *P. papatasi* and *L. longipalpis* had sufficient variation for survival to sub-lethal doses of insecticides. Survival to an insecticide exposure is a heritable trait with a modest genetic architecture. We found some support for this hypothesis. The heritability from the standing genetic variation in our populations for survival to an insecticide exposure is greater than we anticipated. Because of this stronger heritability, the genetic architecture is polygenic, but there is evidence that several SNVs in each treatment are strongly associated with this phenotype. The lack of support for a monogenic mechanism for survival in this experiment supports the lack of convincing evidence of TSI or MD monogenic resistance in previous experiments (Denlinger et al. in review). These

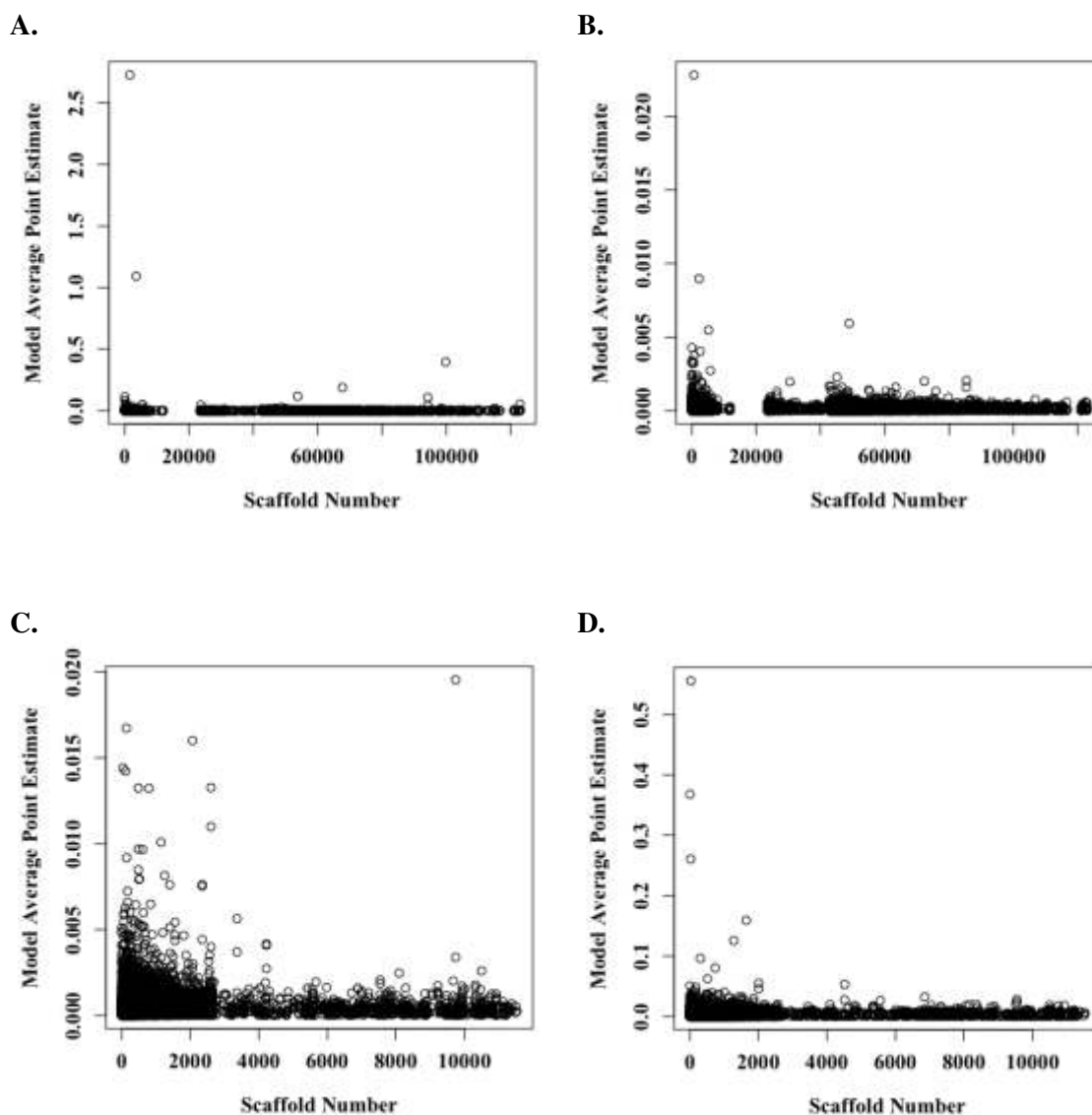


Fig. 6.2. Model-averaged point estimates for the effects of individual SNVs, or the functional variants they are potentially in LD with, across the scaffolds for the *P. papatasi* permethrin treatment (A), *P. papatasi* malathion treatment (B), *L. longipalpis* permethrin treatment (C), and the *L. longipalpis* malathion treatment (D).

loci have not been detected with TSI or MD methods that are used to currently assess insecticide resistance.

Minor Allele Frequencies. For the susceptible *P. papatasi* and *L. longipalpis* treatment groups, there were no major differences in MAFs for most SNVs, but few SNVs display notable discrepancies. As such, there are some SNVs with slightly higher allele frequencies in the

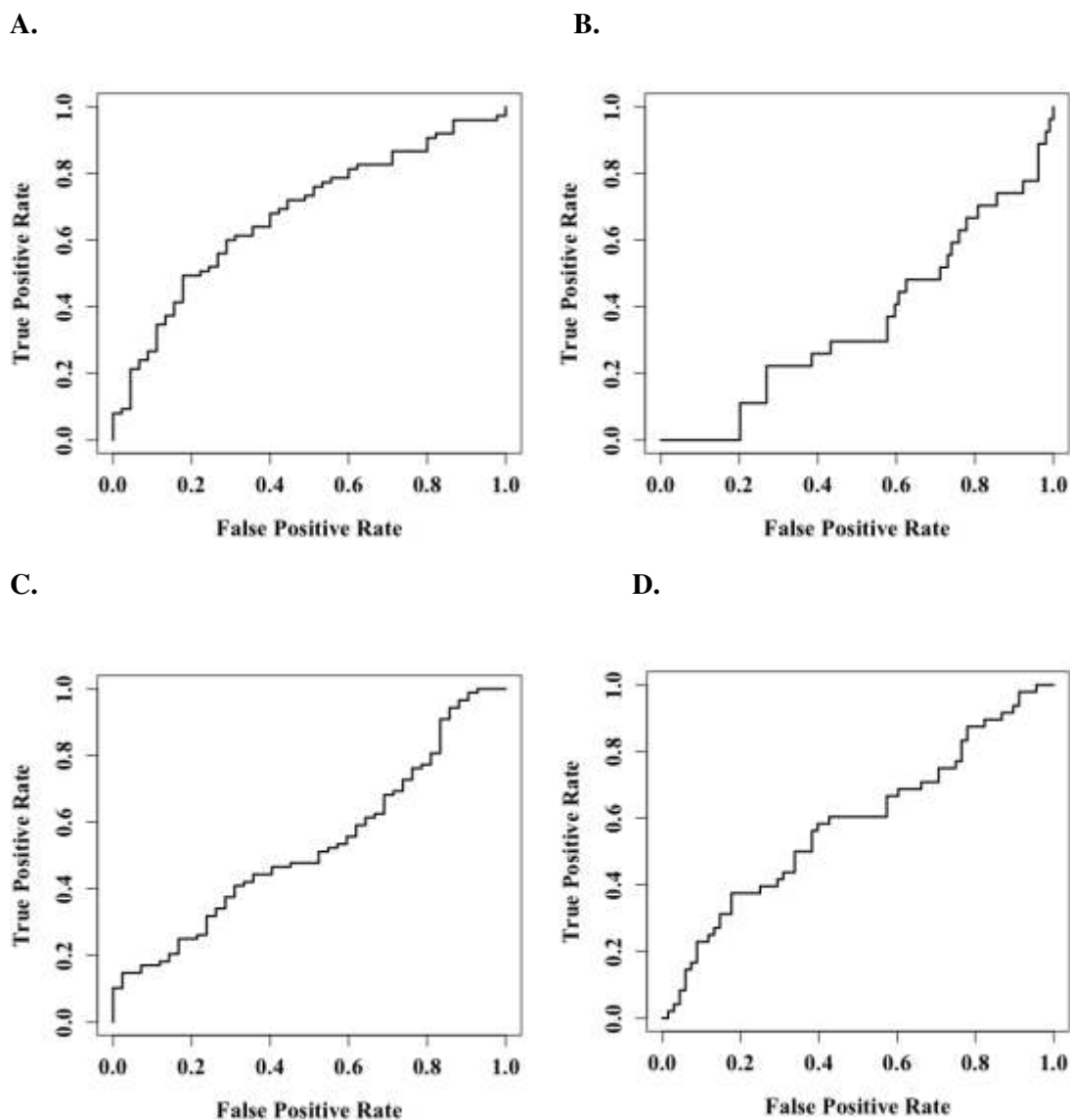


Fig. 6.3. Potential to predict survival phenotype with area under the receiver operating characteristic curve measuring the genomic profile of the *P. papatasi* permethrin treatment (A), the *P. papatasi* malathion treatment (B), the *L. longipalpis* permethrin treatment (C), and the *L. longipalpis* malathion treatment (D).

perished group and lower allele frequencies in the surviving treatment, and vice versa. Outliers between these groups indicate potential SNVs associated with insecticide exposure survival and susceptibility. This suggestion is further supported through genetic architecture analyses.

Genetic Architecture/Predictive Strength. The genetic architecture of survival ability to insecticide exposure was estimated using polygenic Bayesian mapping models across the

genome. Such models entail not only quantitative estimates of PVE, PGE, and $n\text{-}\gamma$, but they also account for uncertainty in the identification of candidate SNVs underpinning the phenotype for insecticide survival (Guan and Stephens 2011). The level of confidence to estimate these parameters varied for each species. The credible intervals spanned a large portion of the range when estimating PVE, PGE, and $n\text{-}\gamma$, which indicates that there is not robust support for inferences. In turn, the interpretations below include considerations for the uncertainty reported.

Phlebotomus papatasi. Survival to a sub-lethal dose of permethrin is heritable, and most of that heritability comes from SNVs that have measurable effects for survival. There is statistical confidence that these SNVs are adaptive for this phenotype given their high model average point estimates that are associated with high posterior inclusion probabilities. This confidence translates to power for predicting whether an insecticide-susceptible *P. papatasi* will survive or die from an exposure to a sub-lethal dose of permethrin given their phenotypes.

Interestingly, survival to a sub-lethal dose of malathion is almost a fifth as heritable as survival to a sub-lethal dose of permethrin. Perhaps the susceptible population of *P. papatasi* did not already have the genetic variation to survive malathion's different mode of action from permethrin. Only one third of the heritability for malathion-exposure can be explained by SNVs with measurable effects, which is about half from the permethrin-exposed treatment. There is less confidence that these SNVs are associated with the survival phenotype, and the association scores for the highest ranking SNVs are much lower than the scores for the highest ranking SNVs in the permethrin-exposed treatment. Not surprisingly, our power to predict survival to exposure to malathion is not as strong as the power to predict survival to permethrin given their phenotypes.

These results follow the percent survival of the resistance selection from Denlinger et al. (in review). The permethrin-resistant-selected population had less variance in survival from generation to generation compared with the malathion-resistant-selected population. The

malathion selected population was never able to maintain consistent, high percentage survival like the permethrin selected population was for several generations. This suggests that a greater level of standing genetic variation and higher levels of heritability can allow for greater survival in response to insecticide exposure.

Lutzomyia longipalpis. Phenotypic variation for survival ability in permethrin-exposed *L. longipalpis* is only moderately heritable. To some extent, the genetic underpinnings for such variation can be explained by a small number of causal variants with measurable effects for survival. Regardless, given the genotypes of insecticide-susceptible *L. longipalpis*, there is little predictive power whether survival or death will result from a sub-lethal exposure to permethrin given their genotypes. This lack of predictive strength is probably due to only moderate levels of heritability for causal variants associated with survival. We thus have little confidence these measurable-effect SNVs contribute to the survival phenotype, given the relatively low model average point estimates that are associated with low posterior inclusion probabilities, across the genome.

Conversely, *L. longipalpis* survival ability when exposed to a sub-lethal dose of malathion is very heritable, but much of the genetic basis is owed to SNVs with infinitesimal effects. Granted there are a few candidate variants with large model average point estimates, but their posterior inclusion probabilities are not supportive for survival ability to mainly be due to measurable effects. This finding is reflected by the relatively low model average point estimates and posterior inclusion probabilities associated with candidate SNVs, as well as the lack of predictive power for the survival phenotype. Given the genotypes of insecticide-susceptible *L. longipalpis*, and despite the significant heritability, there is only moderate predictive power whether survival or death will result from a sub-lethal exposure to malathion. Such predictive strength is may be derived from a low percentage of SNVs with measurable effects.

Gene Associations. Intergenic variants and variants associated with genes were among the top five highest ranking SNVs in all four treatment groups. The variants associated with genes were found in genes or upstream or downstream of them. Some genes do not yet have an annotated function in the sand fly genomes. The genes that are annotated have a diverse range of metabolic and biochemical functions: serine proteases, mitochondrial substrate/solute carriers, zinc fingers, orange domain-like functions, isoprenoid synthase, protein disulfide isomerase, PDCD5-related function, nuclear envelope phosphates regulatory function, microtubule associated protein RP/EB function, transmembrane X-F-associated function, intra-flagellar transport, transcription factor CP2, and glycosyltransferase. We must be cautious in correlating SNVs with resistance. GBS does not identify causal variants; it only statistically associates SNVs with a trait (Comeault et al. 2014). However, some of these genes have been associated with insecticide resistance in other vectors and agricultural pests. Even the intergenic variants could serve important biochemical functions as gene expression regulators (Elshire et al. 2011).

Serine proteases (*P. papatasi* malathion exposure), like acetylcholinesterases, are inhibited by organophosphates, like malathion. They are up- or down-regulated in resistant insects (Chambers and Oppenheimer 2004, Vontas et al. 2007) and are important for synthesis and conformation of detoxifying enzymes in the presence of organophosphates (Ahmed et al. 1998). Zinc fingers (malathion exposure in both sand fly species) are transcriptional repressors (Kasai and Scott 2001). In *Musca domestica*, mixed functional oxidase (MFO) promoters bind transcription repressor genes that contain zinc finger moieties. The MFO promoters in pyrethroid-resistant *M. domestica* bind the repressor genes less than in susceptible individuals because of polymorphisms in the repressor gene, which causes increased transcription of MFOs (Gao and Scott 2006). MFOs detoxify pyrethroids (Perera et al. 2008). But, in our *P. papatasi* and *L. longipalpis* malathion-resistant-selected colonies, we found decreased MFO activity (Denlinger et al. n review). This could be from decreased MFO transcription, the opposite of the

findings in Gao and Scott (2006). MFOs can also detoxify organophosphates (Perera et al. 2008). It is possible that the upstream variant of the zinc finger encoding gene contributes to MFO repression. Decreased MFOs can confer resistance because they first must enzymatically activate insecticide, which they later detoxify. With fewer MFOs, there are fewer bioactivated insecticides (Scott 1999). Perhaps variants near or within zinc fingers contribute to increased or decreased MFO expression, both of which can lead to insecticide resistance.

Several SNVs were found that associated with a protein in the *L. longipalpis* treatment exposed to malathion. A SNV was found associated with a protein containing a disulfide isomerase function. GSTs in insects are known to alter isomerase activity (Sheehan et al. 2001). In the same treatment, microtubule associated protein RP/EB were upregulated found in lambda-cyhalothrin resistant *Aphis glycines*. Microtubule associated proteins interact with postsynaptic proteins in the nervous system. They could help stabilize dendrites to normalize nerve function when malathion disrupts synaptic transmission by inhibiting acetylcholinesterase (Lepicard et al. 2014). Intra-flagellar transport proteins were less abundant in imidacloprid-resistant *Myzus persicae* (Meng et al. 2014). Glycosyltransferases are detoxification enzymes, and overexpression of some uridine diphosphate-glycosyltransferases has been shown to confer resistance in lepidopteran agricultural pests (Li et al. 2016).

Standing Genetic Variation. Variable levels of standing genetic variation have been found in laboratory colonies of sand flies (Mukhopadhyay et al. 1997, Lanzaro et al. 1998, Mukhopadhyay et al. 1998, Mukhopadhyay et al. 2001). Despite more homogenous laboratory populations, insecticide exposure survival is a known heritable trait and can lead to resistance (Feyereisen 1995, Hemingway et al. 2002, Rivero et al. 2010). In theory, alleles for survival will increase in frequency towards fixation with continued selection, disseminate throughout the population, and result in greater population survival over the course of continued exposure (Xu et al. 2012).

In our controlled artificial selection over several years, we did not observe the percent survival increase that we expected, and survival from generation to generation was variable (Denlinger et al. in review). Noticeable, sustained survival in our populations could take many generations over many years, similar to what has been found in other artificial selection experiments (Shepanski et al. 1977, Goldman et al. 1986, Gore and Adamczyk Jr. 2004, Wirth et al. 2004). Adaptation is not always quick, regular, and linear, as expected in Fisher's geometric model; a trajectory towards optimum insecticide exposure survival can be convoluted and undulating because beneficial alleles can have negative pleiotropic effects, and subsequent mutations move towards this optimum by both contributing towards optimal phenotype and ameliorating previous mutation's negative effects (Lande 1983, Otto 2004, Labbé et al. 2007).

The rate of evolution in a population depends on multiple factors, including the initial allele frequency (Roush and McKenzie 1987). The insecticide-susceptible colonies used in this experiment were derived from 30-year inbred populations that were most likely homozygous for many traits, with the emergence of pre-adaptive alleles being removed through purifying selection and/or through stabilizing selection because of fitness costs. Despite evidence of sufficient standing genetic variation for selection to act upon, this variation could have been very little. Therefore, fixation of resistance alleles in these laboratory populations could take a very long time. We aim to re-assess the later generations of our artificial-selected colonies using GBS to examine if the genetic contributors of resistance-selection at later generations are similar as to what we observed this experiment's single generation of exposure.

Polygenic Adaptation. Polygenic insecticide resistance under laboratory conditions has been studied theoretically and empirically (McKenzie et al. 1992, Ffrench-Constant et al. 2004, Ffrench-Constant 2013). Selection for resistance in a laboratory population falls within the phenotypic distribution of the susceptible population, often below the LC_{100} for an insecticide (Roush and McKenzie 1987, Ffrench-Constant et al. 2004, Oakeshott et al. 2013). This selection

process is conducted to allow survivors for subsequent generations. In doing so, existing, common variation is selected for, which produces polygenic resistance. Because of the homogeneity of laboratory populations, very low initial frequency of resistance alleles (as low as 10^{-13}), the high fitness costs of those resistance alleles, and the weakness of the selection process, the evolution of resistance from major-effect alleles is very unlikely (Lande 1983, McKenzie et al. 1992). Even a LC_{90} of an insecticide has the potential to produce polygenic resistance (McKenzie and Batterham 1994). Our lineages were exposed to an approximate LC_{50} of permethrin and malathion, so it is certainly expected to find evidence of polygenic resistance and is possibly a reason why monogenic resistance was not found in Denlinger et al. (in review). Monogenic resistance can be successfully selected for in the laboratory if selection concentration is set above the LC_{100} of an insecticide (McKenzie and Batterham 1998). With diagnostic doses for many insecticides for sand flies recently described (Denlinger et al. 2016a), selection for major-effect alleles is possible in the future.

Monogenic adaptation. Resistance selection in field populations is much greater (above the LC_{100} for an insecticide) and can be outside of the phenotypic range of insecticide tolerance. This can result in the rapid selection of rare, major-effect mutations that can lead to monogenic or oligogenic TSI, MD, or both epistatically (Whitten et al. 1980, McKenzie and Batterham 1998, Ffrench-Constant et al. 2004, Saavedra-Rodriguez et al. 2008, Hardstone et al. 2009, Edi et al. 2014). Here, large population sizes of field populations act as a great source of rare mutations, whereas the small population sizes of inbred individuals in a laboratory population only lead to an accumulation of small effect-size mutations (McKenzie et al. 1992, Ffrench-Constant 2013). It is the heterogeneity of field populations that allows for rare variants to exist (Groeters and Tabashnik 2000). Interestingly, rare variants may precede the selection for resistance. For example, In Australia, mutations for organophosphate resistance in *Lucilia* blow flies predated the use of malathion. Examples of standing genetic variation of resistance alleles in field

populations, prior to insecticide use, demonstrate that these alleles are under balancing selection and do not carry a high enough fitness cost (Ffrench-Constant 2007). Standing alleles in populations are known to quickly increase in frequency from human induced evolution (Messer et al. 2016). This may be why resistance has evolved very rapidly when insecticides are first introduced as a control method (Hemingway and Ranson 2000).

Laboratory strains initiated from field populations with monogenic resistance may not always evolve monogenic resistance because of the factors associated with polygenic resistance selection (Groeters and Tabashnik 2000, Zhu et al. 2013, Kasai et al. 2014). This may be why Fawaz et al. (2016) did not find TSI mutations in their laboratory colony of initiated from Egyptian *P. papatasi*. Even so, resistance in the field may be more polygenic than initially perceived, and this could be due to fitness costs and pleiotropy from major-effect mutations. Microarrays have found many genes with various functions involved in resistance, more than could be found by simply testing for TSI or MD (Pedra et al. 2004, David et al. 2005, Vontas et al. 2005, Vontas et al. 2007, Djouaka et al. 2008). These findings demonstrate that insecticide resistance, in both the field and laboratory, is a complicated phenotype that combines major-effect changes (TSI or MD) and many other alleles that are beginning to be discovered and understood.

Resistance Control Implications. Despite the theoretical work of understanding insecticide resistance in laboratory populations, it behooves insect vector management programs to be cautious about proposing management strategies based only on what has been observed in artificial-selection experiments, as these results do not always empirically verify what is observed in the field (Ffrench-Constant 2013). Even within different laboratory colonies of the same species or population, polygenic resistance can be different (Dapkus and Merrell 1997, Daborn et al. 2002, Ffrench-Constant 2013). Nevertheless, the importance of artificially selecting for

resistance should not be underestimated because of the ability to predict variants of resistance mechanisms for new insecticides to be used in the field (McKenzie and Batterham 1998).

In the 1950's laboratory colonies of DDT-resistant *D. melanogaster* were found to have cross-resistance to other classes of insecticides, which suggested that MD was responsible for resistance. This turned out to be accurate and was important in providing useful information about the mechanisms of resistance in the absence of the underlying genetics (Ffrench-Constant 2013). The utility of artificial selection has also been shown for important agricultural pests for resistance to *Bacillus thuringiensis* (Bt) transgenic crops. Prior to its use, resistance to Bt was theoretically unlikely. Artificial selection for Bt resistance demonstrated that resistance was possible and the modes of action were elucidated. Together, resistant laboratory strains of insects have been useful for estimating levels of resistance, the fitness costs of resistance, and the heritability of resistance in field populations. These findings have helped establish effective resistance management strategies (Devos et al. 2013, Ffrench-Constant 2013).

We found that selecting for and developing insecticide resistant laboratory colonies of sand flies is possible, but challenging. There is sufficient standing genetic variation in our laboratory colonies for polygenic resistance mechanisms, and we probably had low levels of tolerance/resistance in our permethrin-resistant-selected or malathion-resistant-selected colonies. Polygenic resistance is not frequently found in field populations of insects because of greater selection pressure and larger pools of genetic diversity, but it is possible (Raymond and Marquine 1994, Groeters and Tabashnik 2000). Polygenic insecticide resistance is found in nature and is maintained by low mutation rates and minimal migration, both of which are a source of new alleles for monogenic resistance (Raymond and Marquine 1994, Zhu et al. 2013). A question that remains is whether polygenic resistance likely in field populations of sand flies? Sand flies are weak fliers, distribute poorly, and are vagile, which together can lead to small, genetically structured populations (Doha et al. 1991, Morrison et al. 1993, Hamarsheh et al. 2007, Belen et

al. 2011, Khalid et al. 2012, Orshan et al. 2016). The weaker effect of selection in smaller populations, and the stronger effect of drift, could dilute resistant alleles should they arise through mutation (Lanfear et al. 2014). Compound these factors with little gene flow from poor migration, or with gene flow from susceptible sand flies that were unexposed to insecticide due to inadequate insecticide coverage in the environment, and susceptible alleles could remain commonplace in a population.

For our laboratory populations, predictions, not assumptions and conclusions, should be made about the mechanisms of insecticide resistance in field populations (Mukhopadhyay et al. 1997). Our laboratory colonies should serve as a model, not a standard or representative of sand flies in the field. For the results of our experiment to be more heuristic, future laboratory experiments should investigate resistance using much higher doses of insecticide. More research of TSI, MD, and other resistance mechanisms using GBS need to be investigated in natural populations. If so, these predictions from our artificially-selected strains can enable proactive approaches for developing effective integrated vector management programs. Aspects of insecticide use, refuge populations to allow for gene flow especially when insecticide coverage for vectors is uneven, understanding heritability and dominance levels of resistance, understanding fitness costs, and the dynamics of polygenic resistance becoming monogenic resistance can be further studied (Mallet 1989, McKenzie et al. 1992, Tabashnik et al. 2003, Neve et al. 2009).

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CHAPTER 7

SUMMARY & CONCLUSIONS: AN EVOLUTIONARY COMPARISON BETWEEN THE INSECTICIDE- AND ANTIMICROBIAL-RESISTANCE PANDEMICS

Summary

The goal of my dissertation research was to further our collective understanding of insecticide susceptibility and resistance mechanisms in phlebotomine sand flies, an understudied group of vectors that transmit the neglected tropical disease leishmaniasis. My research took many forms and incorporated both applied research and basic research. Public health is about preventing disease and promoting health by making informed decisions that consider medical, economic, anthropologic, and societal inputs. Hopefully, the work presented in this dissertation will positively impact leishmaniasis-focused public health around the world.

I began by comparing known and novel techniques of blood-feeding an important leishmaniasis vector species (Chapter 2). Our ability to move the insecticide resistance and vector biology fields forward requires the ability to raise large-sized colonies for use in research. From there, my research took an applied approach to determine the susceptibility status of two leishmaniasis vectors to ten different insecticides (Chapter 3, 4). I first determined lethal concentrations of these ten insecticides using a combined modified version of two worldwide-used bioassays for determining susceptibility (Chapter 3). This dose-response assay will become more important when time-response assays for assessing insecticide-susceptibility are no longer feasible. Our lethal concentrations from Chapter 3 were critical for artificially selecting for resistance in our laboratory populations (Chapters 5, 6). Working from lethal concentrations, we next determined diagnostic doses and diagnostic times for the same ten insecticides (Chapter 4). These diagnostics represent the largest repository of diagnostics for any sand fly species using the CDC bottle bioassay, an under-utilized approach for assessing insecticide resistance. We have

now provided research with new avenues for assessing resistance that can be used to determine diagnostics for sand fly species in leishmaniasis foci around the world.

I concluded by examining the mechanisms of resistance in laboratory colonies of *Lutzomyia longipalpis* and *Phlebotomus papatasi* under artificial selection (Chapter 5) and retroactively looked at the standing genetic variation of our insecticide-susceptible colonies (Chapter 6). I did not find conclusive examples of convergent resistance mechanisms that are seen in other insect vectors, through target-site insensitivity or metabolic detoxification, but I found moderate levels of genetic variation in my laboratory colonies (Chapters 5). Moderate levels of genetic diversity in these populations implies that there is sufficient variation for resistance selection to act upon throughout the genome to facilitate resistance development in a population. How similar are these results to those for field populations of sand flies? That remains to be determined, and disparities between field populations and laboratory populations can make direct comparisons of genetic diversity and mechanisms of resistance difficult, but these laboratory results should serve as a starting point for understanding the intricacies of resistance and how mechanisms can be exploited for vector control efforts.

An Evolutionary Comparison Between the Insecticide- and Antimicrobial Resistance Pandemics

The paradigm that insecticide resistance undermines the successful control of vector-borne diseases by causing disease resurgences is well-founded (Rivero et al. 2010, Cohen et al. 2012). This may not always be true (McCarroll and Hemingway 2002), but often resurgences are attributed to an increase of pathogens' basic reproductive number (R_0) which results from some intrinsic change in the vectors' or pathogens' biology or phenology (Hemingway and Ranson 2000, Rivero et al. 2010, Thomas and Read 2016). How resistance affects R_0 for each vector in different geographic regions needs to be examined in the epidemiological context of a disease focus for better resistance management (Rivero et al. 2010).

Successful disease eradication, by reducing R_0 , whether vector-borne or other infectious disease, requires a multifaceted approach. Elimination of rinderpest and smallpox, and diseases on the verge of elimination, dracunculiasis and polio which remain endemic in very few countries, relied upon the understanding of a confluence of epidemiological factors (Fenner 1982, Barry 2007, Morens et al. 2011, Cochi et al. 2016). However, for vector-borne diseases, disease control can rely entirely on using insecticides (Hemingway et al. 2016). While a critical component, insecticides should not be the only approach to controlling vectors and therefore disease transmission: the global threat of resistance is real, lethal, and should be a sobering impetus for considering how we use insecticides. Vector resistance to insecticides is not new, but there is a salient, conspicuous idea that needs to be addressed: what happens if the insecticides that we have relied upon fail?

The cognizance of widespread insecticide failure is becoming more pronounced (WHO 2012, Hemingway et al. 2016). Proactive approaches are needed to develop rational strategies into ensure the success of other integrated vector management practices, and these approaches need data, financial support, and resources to train those involved in managing this crisis. Concerning insecticides, there are often few options for insecticide rotations, inconsistent resistance monitoring, an unwillingness to share resistance data, and a lack of infrastructure to manage these data. These responsibilities fall on both the disease-endemic countries and global public health partners (Chanda et al. 2016). Funding is a crucial component of public health programs focusing on vector control, but it has waned because of recent successes due to the “out of sight, out of mind” paradox: past success and reduced disease burden make diseases less “visible,” so the urgency for more financial support lessens. Complacent thinking often leads to new disease outbreaks, such as with recent outbreaks of measles, rubella, pertussis, and diphtheria, that can be more financially expensive than had there been sustained support (Cohen et al. 2012).

I see many parallels between the global threats of insecticide resistance and antimicrobial resistance. Instead of vectors, there are microorganisms; instead of insecticides, there are antimicrobials. The efforts for control, too, can be synonymized for antimicrobial resistance and insecticide resistance. Comparable to worldwide insecticide resistance, worldwide antimicrobial resistance may be the most significant challenge facing the worldwide health care infrastructure this century (Engelhardt and Wright 2016). The CDC's director Tom Frieden has brought much attention to the public, calling these resistant microbes "superbugs" and "nightmare bacteria," which has gained traction in the media (McKenna 2013). The situation has become so dire that President Obama acted by declaring his National Action Plan for Combating Antibiotic-Resistant Bacteria (Nizet 2015).

Like any organism, microbes are under the control of the fundamental forces of evolution: mutation, selection, recombination, and drift (Feyereisen et al. 2015). Unlike insects, microbes have been under these influences for a much longer time: the fossil evidence shows they have existed for at least 3.5 billion years, and they have filled an incredible diversity of niches (Khan and Aziz 2016). These microbes have been in evolutionary combat for billions of years for survival, so it is not surprising that antimicrobial genes are found in natural populations; they already had the genetic variation needed for adaptation prior to human antimicrobial use (Davies and Davies 2010, Pawlowski et al. 2016). Unfortunately, these alleles have shown to have few fitness costs and are maintained in populations, either for resistance or other biochemical functions (Nordmann et al. 2007, Davies and Davies 2010). The speed at which resistance has developed to novel antimicrobials and antimicrobial classes is striking, both in the laboratory and in the field. Laboratory experiments at Roy Kishony's lab at Harvard University have shown that bacteria can evolve resistance to concentrations of antibiotics orders of magnitudes higher than initial exposure concentrations in under two weeks (Baym et al. 2016). Like insects, bacteria must navigate a fitness landscape with their resistance mechanisms to reach a fitness optimum

with while minimizing the effects of negative mutations (Palmer and Kishony 2013).

Antimicrobial resistance was inevitable, and the public health infrastructure was not ready (Davies and Davies 2010).

There was a warning for the threat of antimicrobial resistance. Alexander Fleming, the serendipitous microbiologist, had the evolutionary foresight to be wary of his “magic bullet” penicillin and how the missuse of antimicrobials could hasten the global resistance pandemic we find ourselves entrenched in today.

“The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily under dose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant.” (Barriere 2015)

“But I would like to sound one note of warning.... It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body.” (Khan and Aziz 2016)

Fleming’s dire understandings of antimicrobial resistance have come to fruition today. In the United States alone, the CDC estimates that antimicrobial resistant microbes kill 23,000 people a year and sicken more than 2 million. The healthcare costs of these individuals are staggering: \$20 billion in costs and \$35 billion in lost productivity (Nizet 2015). Many microbes are culpable. *Acinetobacter baumannii* (“Iraqibacter”), *Burkholderia pseudomallei* (melioidosis), *Campylobacter* spp., *Candida albicans* (candidiasis), *Clostridium difficile* (“CDiff”), *Enterobacter* spp., *Enterococcus faecium*, *Escherichia coli*, *Klebsiella pneumoniae*, *Mycobacterium tuberculosis* (MDR and XDR tuberculosis), *Neisseria gonorrhoeae* (gonorrhea),

Plasmodium falciparum (malaria), *Pseudomonas aeruginosa*, *Staphylococcus aureus* (MRSA, VRSA, and LRSA), *Trichomonas vaginalis* (trichomoniasis), and *Vibrio cholerae* (cholera), have been found to be resistant, all of which cause serious human disease (Plough 1945, Dunne et al. 2003, Davies and Davies 2010, Buono et al. 2015, Egan 2015, Sanguinetti et al. 2015).

These resistant microbes are global, and they, like vector-borne diseases, have been opportunistic from antimicrobial use in health care facilities, civil unrest, famine, and natural disasters (Davies and Davies 2010). As a species, we have exacerbated the problem immensely by applying evolutionary selective pressures through antimicrobial use in health care and agriculture settings (Alanis 2005, Barriere 2015). I think the same is true for insecticides. Microbes have surged to cause lethal infections from three areas: nosocomial infections, community-acquired infections, and livestock-acquired infections (Davies and Davies 2010).

What has been done, and how can we forward in our effort to curb the damaging health care and economic repercussions of antimicrobial resistance? It is critical to have a strong global collaboration and funding for industry, academia, and governments (Alanis 2005). First, to apply new selective pressure, there needs to be a robust pipeline for new antimicrobials, which is drying up (Alanis 2005). It is obvious that we are losing our last-resort antimicrobials at an alarming rate, but the pharmaceutical companies we have relied on, are halting their R&D efforts because of economic incentives and repressing regulations (Nizet 2015, Engelhardt and Wright 2016). The Food and Drug Administration (FDA) has been forced to reboot its approach to new antimicrobial development. Janet Woodcock, the FDA's director of the Center for Drug Evaluation and Research, has recognized the need to change clinical trial designs and the importance of opening new pathways for drug development (Shlaes et al. 2013). Antimicrobials have saved our lives; we need to save their lives too. They, like insecticides, should be treated as nonrenewable resources. There needs to be energy and momentum for innovation in the markets. New antimicrobials, like Teixobactin, offer hope, but Ling et al. (2015)'s claims of resistance

development being unlikely should be met with met with hesitancy and watchfulness. Microbes are fickle, and we have seen how quickly resistance can develop to other antimicrobials (Plough 1945). For the antimicrobials that we still have available, there needs to be tremendous stewardship: limiting drug misuse and overuse; requiring accurate prescriptions; not using antibiotics to treat viral infections; continued education for physicians, veterinarians, farmers, and the public; decreasing patients' hospital stays; using narrow-spectrum antimicrobials as much as possible; (Nordmann et al. 2007, Perry and Hall 2009, Davies and Davies 2010, Barriere 2015, Engelhardt and Wright 2016, Khan and Aziz 2016).

Second there needs to be continued vigilance for resistance, especially in hospital settings where antimicrobial resistant infections are devastating, and for new technologies (Alanis 2005, Barriere 2015, Engelhardt and Wright 2016). There needs to be better infection prevention in all environments, especially where people are very susceptible, and if infections arise, diagnostics to quickly distinguish patients with resistance is imperative. Simultaneously, understanding patients' pre-existing conditions and proclivities for acquiring resistant infections will save time and money by developing proactive approaches for managing resistant infections. Initially testing new patients for colonization by antimicrobial-resistant microbes can allow for swift action and isolation to prevent further infection of other health care workers and other patients. New technologies, including advances in genome technologies, could give rise to new antimicrobial adjuvants, prebiotics, probiotics, and bacteriophage products. Lastly, while it may seem obvious, proper hygiene in health care facilities is crucial for preventing antimicrobial-resistant infections (Trick et al. 2007).

Third, there should be a motivation for non-traditional therapies that are not always initially considered. For gastrointestinal infections, such as *Cdiff*, the use of fecal transplants is gaining favor despite the offensive perception that it carries. Fecal transplants work by taking the gut microbiota, in the form of stool, from a healthy individual and transplanting that material into

a Cdiff-infected person in the hopes that the transplanted microbiota will be a form of biological control to fight against the Cdiff bacteria. This approach is more cost-effective than antibiotics, and it is approaching a 90% cure rate (Simonson 2016). For cutaneous antimicrobial-resistant infections, medicinal maggot debridement therapy is making a comeback. First introduced to Western medicine by Dr. William Baer, who observed the healing effects of maggots in wounded soldiers during WWI (Baer 1931), the saliva of blow fly maggots has been shown to be effective against resistant microbes; like fecal transplant, maggots should remain a viable option when traditional pharmaceuticals fail (Sherman et al. 2000). Lastly, the rise in cases of infections with antimicrobial resistant pathogens may, of course, be linked to increasing resistance, but perhaps it could also be linked to the health of our immune systems? Are we, as a people, becoming too clean? Dr. Martin Blaser has been at the forefront of this thinking: how do changes in our own microbiome have lasting effects on our health as we age (Blaser 2014). The systemic health of our bodies intrinsically relies on the health of our microbiota, and people need to take better care of the microbes that call our bodies home. Our microbiome provides us physical protection, helps regulate our metabolism, and aids in the development of our immune system. Blaser's team argues that the overuse of antibiotics, along with Cesarean delivery, at young ages lead to microbiota immaturity, which present, later in life, as malnutrition, obesity, diabetes, inflammatory bowel disease, asthma, allergies, attention deficit hyperactivity disorder, celiac disease (Blaser 2014, Blaser 2016, Bokulich et al. 2016). Let alone selecting for resistance, too many antibiotics and obsessive cleanliness deplete our own microbiomes, which makes us more susceptible to infection by resistant microbes, and it requires us to use more of the same antibiotics. The biologic costs of using antibiotics on people from pregnancy to post natal ages needs continued research to see the effects on a person's ability to innately fight infection.

Is there hope in the antimicrobial resistance and insecticide resistance fields? I certainly think so. We are currently battling the same evolutionary selection pressures in the forms of

resistant microbes and resistant insects. Resistance in both fields is dynamic and fluid. We must be the same to overcome these challenges. I fear, with antimicrobials and insecticides, that we are competing against the Red Queen: we develop an antimicrobial/insecticide, the microbe/insect evolves resistance, and then we develop a new antimicrobial/insecticide and the microbes/insects keep pace by evolving new mechanisms of resistance, and so on (Robson 2005). Therefore, it will take our collective intellect, ingenuity, creativity, and observation to make strides in overcoming these evolutionary challenges. Could we develop antimicrobials/insecticides that are evolutionary proof? (Read et al. 2009). Might we be able to use CRISPR-Cas9 to make gene drive systems to permanently disrupt microbe or insect vector populations? (Hammond et al. 2016). We need an integrated, multifaceted, and interdisciplinary approach to combat these challenges, and it requires the global effort from individuals, communities, government, businesses, academia, the media, NGOs, and people everywhere (WHO 2012).

As a personal conclusion, we are in a unique transition era that is seeing the inchoate impact that science communication is having in both the insecticide resistance and antimicrobial resistance fields (or any field). Hollywood has, probably for good, propagated awareness of infectious diseases through movies such as *Outbreak* and *Contagion*, and newspapers, magazines, books, and the internet inundate people with stories. But, social media has reinforced and educated people like never before (Brossard 2013, Jarreau and Porter 2017). Millennials, including the next generation of scientists, are proficient at using social media to communicate and learn. Social media to communicate science should be embraced, not ignored, because of its potential to be utilized by so many more people around the world than any other form of communication. I have found bloggers and Twitter users to be very informative, and I believe that science writers, such as Maryn McKenna, Carl Zimmer, Ed Young, and so many other “scicomm” people, can publicize these issues much better and efficiently than scientists do. Personally, while writing scientifically about science is important and needed to communicate

with other scientists, I think it can often be dry and unmotivating. Many non-scientists may not care about what is written in a science journal article, but the ability to precisely articulate main points in a blog, Tweet, post, hashtag, is very effective, motivating, and sincerely needed. Flashy and simple headlines, descriptive writing, and attention-grabbing photos are attractive. It is crucial to have people who can move between hard science and social media; they need to be a hybrid, both knowledgeable of the scientific process but also creative enough to communicate it to the non-scientist. Social media is beginning to be embraced by scientists, big science organizations, journals, and societies because I think everyone is beginning to understand the effectiveness and utility of social media for the future. I think there will be a shift in the science field in the coming years to use social media as the main form of communication. Hopefully scicomm can be very effective in reaching everyone around the world about the perils that we face with insecticide resistance, antimicrobial resistance, and the resurgence of diseases once thought to be defeated. As a graduate student in the public health field, our research has the end goal of tangibly helping people, and no matter what field of science we work in, we owe it to communicate with other scientists and the public. Frankly, the public pays for most of our research, and we should make our results visible to give them a return on their investment. Communication needs to be embraced, and we as scientists should be excited to share science with others so that we can motivate the next generation by fostering a love and curiosity for the world we share.

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APPENDICES

Appendix A: Coauthor Permission Letters

November 4, 2016

Mr. David S. Denlinger
Department of Biology
Utah State University
Logan, Utah, USA
84322-5305

Dear David,

As a coauthor on the following manuscript, I grant you my permission to use this work in your dissertation:

Denlinger, D. S., A. Y. Li, S. L. Durham, P. G. Lawyer, J. L. Anderson, and S. A. Bernhardt.
2016. Comparison of in vivo and in vitro methods for blood feeding of *Phlebotomus papatasi* (Diptera: Psychodidae) in the laboratory. *J. Med. Entomol.* doi:
<http://dx.doi.org/10.1093/jme/tjw074>.

Sincerely,

A handwritten signature in blue ink that reads "Andrew Li". The signature is written in a cursive style.

Andrew Y. Li
Researcher, USDA ARS
Invasive Insect Biocontrol and Behavior Laboratory
Beltsville, MD

October 25, 2016

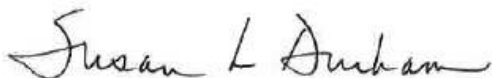
Mr. David S. Denlinger
Department of Biology
Utah State University
Logan, Utah, USA
84322-5305

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<http://dx.doi.org/10.1093/jme/tjw074>.

Sincerely,

A handwritten signature in cursive script that reads "Susan L. Durham".

Susan L. Durham
Statistician
Ecology Center
Utah State University

November, 24 2016

Mr. David S. Denlinger
Department of Biology
Utah State University
Logan, Utah, USA
84322-5305

Dear David,

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<http://dx.doi.org/10.1093/jme/tjw074>.

In my opinion, you were the primary contributor to this work, which constituted independent and creative effort in design, implementation, and manuscript preparation.

Sincerely,



Phillip G. Lawyer
Research Entomologist
Laboratory of Parasitic Diseases
Intracellular Parasite Biology Section
National Institutes of Health

October 27th, 2016

Mr. David S. Denlinger
Department of Biology
Utah State University
Logan, Utah, USA
84322-5305

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Denlinger, D. S., A. Y. Li, S. L. Durham, P. G. Lawyer, J. L. Anderson, and S. A. Bernhardt.
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<http://dx.doi.org/10.1093/jme/tjw074>.

Sincerely,



Joseph L. Anderson
Undergraduate Researcher
Department of Biology
Utah State University

November 15, 2016

Mr. David S. Denlinger
Department of Biology
Utah State University
Logan, Utah, USA
84322-5305

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2016. Comparison of in vivo and in vitro methods for blood feeding of *Phlebotomus*
papatasi (Diptera: Psychodidae) in the laboratory. *J. Med. Entomol.* doi:
<http://dx.doi.org/10.1093/jme/tjw074>.

Sincerely,



Scott A. Bernhardt
Assistant Professor
Department of Biology
Utah State University

October 27, 2016

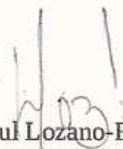
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Department of Biology
Utah State University
Logan, Utah, USA
84322-5305

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Sincerely,



Saul Lozano-Fuentes, PhD
CEO Mosquito Scientists, LLC

November, 16 2016

Mr. David S. Denlinger
Department of Biology
Utah State University
Logan, Utah, USA
84322-5305

Dear David,

As a coauthor on the following manuscript, I grant you my permission to use this work in your dissertation:

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In my opinion, you were the primary contributor to this work, which constituted independent and creative effort in design, implementation, and manuscript preparation.

Sincerely,



Phillip G. Lawyer
Research Entomologist
Laboratory of Parasitic Diseases
Intracellular Parasite Biology Section
National Institutes of Health

October 24, 2016

Mr. David S. Denlinger
Department of Biology
Utah State University
Logan, Utah, USA
84322-5305

Dear David,

As a coauthor on the following manuscript, I grant you my permission to use this work in your dissertation:

Denlinger, D. S., S. Lozano-Fuentes, P. G. Lawyer, W. C. Black IV, and S. A. Bernhardt. 2015. Assessing insecticide susceptibility of laboratory *Lutzomyia longipalpis* and *Phlebotomus papatasi* sand flies (Diptera: Psychodidae: Phlebotominae). *J. Med. Entomol.* 52: 1003-1012.

Sincerely,

A handwritten signature in blue ink that reads "William C. Black IV". The signature is written in a cursive, flowing style.

William C. Black IV
Professor
Department of Microbiology, Immunology and Pathology
Colorado State University

November 15, 2016

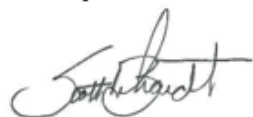
Mr. David S. Denlinger
Department of Biology
Utah State University
Logan, Utah, USA
84322-5305

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Denlinger, D. S., S. Lozano-Fuentes, P. G. Lawyer, W. C. Black IV, and S. A. Bernhardt. 2015. Assessing insecticide susceptibility of laboratory *Lutzomyia longipalpis* and *Phlebotomus papatasi* sand flies (Diptera: Psychodidae: Phlebotominae). *J. Med. Entomol.* 52: 1003-1012.

Sincerely,



Scott A. Bernhardt
Assistant Professor
Department of Biology
Utah State University

October, 29 2016

Mr. David S. Denlinger
Department of Biology
Utah State University
Logan, Utah, USA
84322-5305

Dear David,

As a coauthor on the following manuscript, I grant you my permission to use this work in your dissertation:

Denlinger DS, Creswell JA, Anderson JL, Reese CK, Bernhardt SA. Diagnostic doses and times for *Phlebotomus papatasi* and *Lutzomyia longipalpis* sand flies (Diptera: Phlebotomidae: Phlebotominae) using the CDC bottle bioassay to assess insecticide resistance. *Parasit Vectors*. 2016; 9: 212.

Sincerely,



Joseph A. Creswell
Undergraduate Researcher
Department of Biology
Utah State University

October 27th, 2016

Mr. David S. Denlinger
Department of Biology
Utah State University
Logan, Utah, USA
84322-5305

Dear David,

As a coauthor on the following manuscript, I grant you my permission to use this work in your dissertation:

Denlinger DS, Creswell JA, Anderson JL, Reese CK, Bernhardt SA. Diagnostic doses and times for *Phlebotomus papatasi* and *Lutzomyia longipalpis* sand flies (Diptera: Psychodidae: Phlebotominae) using the CDC bottle bioassay to assess insecticide resistance. *Parasit Vectors*. 2016; 9: 212.

Sincerely,



Joseph L. Anderson
Undergraduate Researcher
Department of Biology
Utah State University

October 25, 2016

Mr. David S. Denlinger
Department of Biology
Utah State University
Logan, Utah, USA
84322-5305

Dear David,

As a coauthor on the following manuscript, I grant you my permission to use this work in your dissertation:

Denlinger DS, Creswell JA, Anderson JL, Reese CK, Bernhardt SA. Diagnostic doses and times for *Phlebotomus papatasi* and *Lutzomyia longipalpis* sand flies (Diptera: Psychodidae: Phlebotominae) using the CDC bottle bioassay to assess insecticide resistance. *Parasit Vectors*. 2016; 9: 212.

Sincerely,



Conor K. Reese
Undergraduate Researcher
Department of Biology
Utah State University

November 15, 2016

Mr. David S. Denlinger
Department of Biology
Utah State University
Logan, Utah, USA
84322-5305

Dear David,

As a coauthor on the following manuscript, I grant you my permission to use this work in your dissertation:

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Sincerely,



Scott A. Bernhardt
Assistant Professor
Department of Biology
Utah State University

Appendix B: Publisher Permission Letters


JOURNALS PERMISSIONS

to me ▾

Oct 17



Dear David Denlinger,

RE. David S. Denlinger et al. Comparison on In Vivo and In Vitro Methods for Blood Feeding of *Phlebotomus papatasi* (Diptera: Psychodidae) in the Laboratory. *Journal of Medical Entomology* (2016) 53 (5): 1112-1116

David S. Denlinger et al. Assessing Insecticide Susceptibility of Laboratory *Lutzomyia longipalpis* and *Phlebotomus papatasi* Sand Flies (Diptera: Psychodidae: Phlebotominae). *Journal of Medical Entomology* (2015) 52 (5): 1003-1012

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 Louise

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From: David Denlinger [mailto:david.denlinger@aggiemail.usu.edu]
Sent: 11 October 2016 21:40
To: JOURNALS PERMISSIONS
Subject: Publishing rights for dissertation

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Best wishes,

Ricardo Sison Jr.

Customer Services Executive

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Ricardo.SisonJr@springernature.com

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CURRICULUM VITAE

David S. Denlinger, B.S.

Ph.D. Candidate
 Utah State University
 5305 Old Main Hill
 Logan, UT 84322-5310
 ddenlinger89@gmail.com
 732-996-5376

Career Objectives

I am looking to further my career in medical entomology by seeking an employment position in the military, public health, vector biology, or epidemiology fields.

Education

Ph.D. Candidate

Utah State University (USU), Logan, UT
 2011-Present

Dissertation: Sand flies and soldiers: understanding the mechanisms of insecticide resistance in *Phlebotomus papatasi* and *Lutzomyia longipalpis* (Diptera: Psychodidae: Phlebotominae)

Adviser: Scott Bernhardt, Ph.D., MPH

Bachelor of Science

University of Delaware, Newark, DE
 2007-2011

Majors: Biology and Entomology, *magna cum laude*

Thesis: Does Genetically Engineered Corn Affect Pheromone Production or Larval Development Time in the European Corn Borer *Ostrinia nubilalis* (Hübner)?

Adviser: Charles Mason, Ph.D.

Publications

1. Denlinger, D. S., A. Y. Li, S. L. Durham, P. G. Lawyer, J. L. Anderson, and S. A. Bernhardt. 2016. Comparison of *in vivo* and *in vitro* methods for blood feeding of *Phlebotomus papatasi* (Diptera: Psychodidae) in the laboratory. *J. Med. Entomol.* 53: 1112-1116.

2. Denlinger DS, Creswell JA, Anderson JL, Reese CK, Bernhardt SA. Diagnostic doses and times for *Phlebotomus papatasi* and *Lutzomyia longipalpis* sand flies (Diptera: Psychodidae: Phlebotominae) using the CDC bottle bioassay to assess insecticide resistance. *Parasit. Vectors.* 2016; 9:212.

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Conference Presentations

Denlinger, D. S., Y. M. Linton, Z. Gompert, and S. A. Bernhardt. “Worldwide collections of sand flies (Diptera: Psychodidae: Phlebotominae) to assess insecticide resistance. International Congress of Entomology, Orlando, FL. 2016; Invited oral presentation: 12 minutes.

Denlinger, D. S., P. G. Lawyer, K. Saavedra-Rodriguez, and S. A. Bernhardt. “Understanding the molecular mechanisms of insecticide resistance in *Phlebotomus papatasi* and *Lutzomyia longipalpis* sand flies (Diptera: Psychodidae).” Pacific Branch of the Entomological Society of America Tucson, AZ. 2014; Poster presentation

Denlinger, D. S., P. G. Lawyer, W. C. Black IV, and S. A. Bernhardt. “Molecular characterization of insecticide resistance in *Phlebotomus papatasi* and *Lutzomyia longipalpis* sand flies.” The American Society of Tropical Medicine and Hygiene, Washington, D.C. 2013; Poster Presentation

Denlinger, D. S., P. G. Lawyer, W. C. Black IV, and S. A. Bernhardt. “Molecular characterization of insecticide resistance in *Phlebotomus papatasi* and *Lutzomyia longipalpis* sand flies. The International Congress of the Society of Vector Ecology, La Quinta, CA. 2013. Invited oral presentation: 10 minutes

Teaching Assistant Experience

Utah State University, Logan, UT, Fall 2011-Spring 2017

- *Graduate Teaching Assistant*: Introductory Biology I (BIOL 1610/1615)
-Laboratory instructor covering basic microscopy, enzymes, genetics, molecular biology techniques, and photosynthesis
- *Graduate Teaching Assistant*: Introductory Biology II (BIOL 1620/1625)
-Laboratory instructor covering the scientific method and hypothesis testing, phylogenetics, population genetics, and animal growth
- *Graduate Teaching Assistant*: Honors Introductory Biology I and II (BIOL 1610, 1620)
-Introductory Biology I and II with added student-led presentations on scientific journal articles
- *Graduate Teaching Assistant*: Preparatory TA for Biology I (BIOL 1610)
-Responsible for laboratory preparation
- *Graduate Teaching Assistant*: Special Problems (BIOL 2988)
-Develop curriculum, program itinerary, organize social activities, and coordinate with labs participating in program for 23 USU Eastern Blanding students during summer STEM program

Teaching Experience

Utah State University, Logan, UT, Spring 2016

- BIOL 3500: Plagues, Pests, and People (undergraduate)

Campus Involvement, Achievements, and Accomplishments

USU Department of Biology

1. Native American STEM Mentorship Program Co-Graduate Facilitator

Utah State University, Department of Biology, Logan, UT

February 2015- Present

Description: Supervise visiting USU Eastern Blanding students during summer work at USU

Logan.

Duties: Develop program itinerary, organize social activities, coordinate with labs participating in the program, and serve as a primary contact with students.

Achievements: Nearly a quarter of those students are now enrolled as full-time, matriculated students here at the Logan campus and are pursuing Bachelor's degrees.

2. USU Biology Graduate Student Association Graduate Program Committee

Representative

Utah State University, Department of Biology, Logan, UT

August 2015-October 2016

Description: Graduate student representative of the Biology Department's Graduate Program Committee

Duties: Review and recommends aspects of graduate students' Program of Study, review new graduate student applications.

3. Co-Host Dr. David Denlinger, The Ohio State University, USU Ecology Seminar Series

Utah State University, Department of Biology and Ecology Center, Logan, UT

October 2015

Description: Graduate student host and facilitator of Dr. Denlinger's visit and seminar presentation

Duties: Assist with developing seminar itinerary, helped transport to the airport and to different locations on campus, gave a campus tour, and introduce Dr. Denlinger before one of his seminar presentations

4. Entomology Club (Treasurer 2015-Present; Vice President 2014-2015)

Utah State University, Department of Biology, Logan, UT

Fall 2011-Present

Description: USU campus-wide club consisting of undergraduate and graduate students that focuses on understanding about and teaching others about insects

Duties: Led outreach events for Valentine's Day and Halloween to educate USU students and the public about insects. Volunteered at Science Unwrapped lectures and participated in outreach events to local elementary schools (K-12) throughout Cache Valley. Helped to create an educational display with the Merrill-Cazier Library titled "Small Wonders: The Expansive World of Insects"

5. Department of Biology Graduate Student Association

Utah State University, Department of Biology, Logan, UT

Fall 2011-Present

Description: USU club consisting of all graduate students in the Department of Biology

Duties: Helped raise funds via plant sales and silent auctions to support BGSA for travel awards and emergency funds. Did biology outreach at Science Unwrapped lectures and Pow-Wows.

Technical Skills

Programming: Cygwin, SAS, R

Sequencing: SeqMan Pro, EditSeq

Survival Curve Analysis: IRMA QCal

Other: Microsoft Office Suite

Current Professional Memberships

Peer Reviewer- Journal of Insect Science (2017-Present)
Peer Reviewer- Journal of Medical Entomology (2016-Present)
Peer Reviewer- Journal of Economic Entomology (2016-Present)
Entomological Society of America
Society of Vector Ecology

Grants/Scholarships/Awards

The USU Graduate Enhancement Award, May 2016 (\$4000)
The USU College of Science Claude E. Zobell Graduate Scholarship, April 2016 (\$1000)
The USU Department of Biology Joseph K.- K. Li Graduate Student Research Award, April 2016 (\$1000)
The USU Research and Projects Grant, March 2015 (\$974.40)

Press

July 9, 2016. The Herald Journal. < http://news.hjnews.com/allaccess/outsmarting-bugs-usu-professor-studies-dna-of-mosquitoes-to-advance/article_927d56e6-4378-5311-8ffb-e37ccfca94fc.html?utm_medium=social&utm_source=twitter&utm_campaign=user-share>.