DISSERTATION

DDT AND PYRETHROID RESISTANCE IN *XENOPSYLLA CHEOPIS* (ROTHSCHILD), THE ORIENTAL RAT FLEA IN NORTHERN UGANDA

Submitted by

Abbe D. Ames

Department of Microbiology, Immunology, and Pathology

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall 2011

Doctoral Committee:

Advisor: William C. Black IV

Kenneth L. Gage
Janet C. McAllister
Boris C. Kondratieff
Patricia A. Cole

ABSTRACT

DDT AND PYRETHROID RESISTANCE IN *XENOPSYLLA CHEOPIS* (ROTHSCHILD), THE ORIENTAL RAT FLEA IN NORTHERN UGANDA

Development of insecticide resistance by vectors of disease is a well-recognized and continuous concern for public health officials. Monitoring insects for development of resistance to the chosen toxicants is part of effective management philosophy.

Several programs to control mosquito vectors of malaria utilize insecticides with similar modes of action targeting the insect. Fleas can vector plague and in many areas inhabit the same environment that is the focus of mosquito management. Non-target insect development of resistance is a phenomenon most commonly associated with agriculture, but can also apply to insect vectors that threaten public health. Rapid and effective methods of monitoring for the possible development of insecticide resistance in fleas are important measures taken to prevent or suppress a plague outbreak.

This study describes the development and application of a new field assay for evaluating phenotypic demonstration of insecticide resistance in fleas, results of biochemical analyses performed to evaluate possible development of metabolic detoxification pathways, and the subsequent elucidation of the *para* voltage gated sodium channel gene in *Xenopsylla cheopis* (Rothschild) with concurrent analyses of the prevalence and effects of knockdown (*kdr)* mutations in the gene.

The field assay used a glass Petri dish coated with a dose of a chosen insecticide and a time mortality assay that was performed for 60 minutes. Discriminating concentrations, established on colony reared fleas, was tested on field collected fleas in northern Uganda. Fleas from villages with a history of indoor residual spraying (IRS) of DDT and /or pyrethroid were tested with those insecticides and significant increased survival was demonstrated. Phenotypic resistance to DDT was demonstrated with an 81.8% survivorship. Lambda-cyhalothrin tested fleas from three villages demonstrated phenotypic resistance of levels of 57.7%, 60.5%, and 58% survivorship.

Enzyme profiles indicated increased levels of expression of α-esterase and β-esterase in field caught fleas compared to colony-reared fleas. Fleas potentially exposed to DDT and/or pyrethroids had higher levels than did unexposed fleas. An increase in insensitive acetylcholinesterase was found in fleas from villages with no known history of IRS. No increase in glutathione S-transferase was noted in any population.

The *para* voltage gated sodium channel gene for *X. cheopis* was amplified and sequences for colony and Ugandan fleas were analyzed with emphasis on knockdown resistance (*kdr)* evolution in the fleas. Extensive evidences of selective pressures influencing genetic profiles of *kdr* development faster than expected for random mutation or recombination were found. The phenylalanine allele, associated with *kdr*, was found at an average of 95.1% frequency in villages with an IRS history. Field caught fleas with no known insecticide exposure had an allele frequency of 13.3%

All three studies clearly indicate resistance is developing quickly in Ugandan flea populations and should be addressed with surveillance and management.

ACKNOWLEDGEMENTS

I would like to acknowledge the thought-provoking guidance all my committee members provided to me. I have discovered that not only are they subject matter experts in their respective fields of science, they are the products of many years of experience in regulatory and political arenas and have taken the time to make sure I emerge as a freshly minted PhD with less dogma and more forward thinking. I am eternally grateful for the sincere efforts Bill, Ken, Janet, Boris and Pat put forth to guide my intellectual growth.

If I started to mention by name all the individuals who contributed to the success of my projects, I would be frightened I would accidentally forget someone and hurt them because the list is extensive! Nearly the entire 4th floor of the Centers for Disease Control and Prevention in Fort Collins either voluntarily helped me or was buttonholed for any helpful information I thought I might glean. Janae Stovall was probably just as happy as I was when sequencing was a fait accompli because that meant she finally had a break from me pestering her for ideas. Old friends like Jenn Holmes were there for me and new ones like Sarah Billeter and Leah Colton helped whenever I asked. Cassie Scott helped me transition from small animal clinics to a laboratory. In actuality, the entire CDC Fort Collins campus from security to the Director's office always had enthusiastic words of encouragement for me whenever we saw each other. Karla and Karen in Bill Black's lab were so understanding and helpful too.

My entire family and friends around the world continued to support and encourage me, especially when the 3-year deadline loomed and I had a seemingly insurmountable amount of writing to do. My wonderful, long suffering son, Talon, probably could have written the dissertation himself from hearing me ramble about

problems and new concepts I wrestled with so much. I am forever indebted to my friend Christine Lorenz, who's unswerving support and belief in my purpose got me here.

Thanks to the CDC for funding the research and the trip to Uganda to collect the fleas. The incredible trapping crew, headed by Joseph Tendo, were funny, skilled, and taught me all sorts of tricks to trapping rats. The Ugandan Virology Research Institute in Kampala and Arua were very generous with their assistance and were instrumental in my success in Uganda.

Thanks to the United States Army Veterinary Corps for funding my PhD and for the opportunity to master new skills.

DEDICATION

To my parents, Dr. Sherman and Mrs. Elizabeth Ames, and my son, Talon. They are my generational bookends that have supported me unconditionally throughout all my endeavors, especially this one. To Christine Lorenz, an exceptional person and an incredible friend.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. LITERATURE REVIEW

A. INTRODUCTION

Many of the emerging diseases threatening public health today are transmitted by arthropod vectors. The risk posed by these diseases can be dramatically reduced by the use of insecticides. Unfortunately, the introduction of these insecticides has caused many species to develop mechanisms of resistance to most classes of insecticides. Early detection, characterization of resistance mechanisms, and understanding genetic responses to selective pressures are some keys to successful vector management systems that can reduce both public health risks and integration of long-term genetic resistance adaptations to insecticidal selective pressures. This dissertation describes a novel, inexpensive, field-expedient insecticide resistance bioassay, characterizes metabolic detoxification responses and describes voltage-gated sodium channel *para* gene knockdown resistance mutations in populations of *Xenopsylla cheopis* (Rothschild) the flea vector of *Yersinia pestis* (Yersin) collected in northern Uganda.

1. Global resurgence of diseases leading to more intense chemical control measures

Emerging and remerging vector borne diseases have a significant deleterious impact on global economies and public health (Binder et al, 1999; Morens et al, 2004; Smolinski et al, 2003). The reasons surrounding these changes are multifactoral and complex. Increasing human population densities and the associated environmental changes have created or exacerbated conditions conducive to the proliferation of certain arthropod vectors (Service, 1991). Climate change has certainly contributed to the prevalence of emerging arboviruses (Gould & Higgs, 2009).

The World Health Organization (WHO) and many scientific publications report that the use of insecticides can dramatically reduce the risk of arthropod-borne diseases,

as demonstrated in the intense DDT usage in the 1940s for typhus, and 1950s-1960s to control malaria. Krogstad (1996) reported that insecticide resistance was one of two major reasons for the failure of the malaria eradication program and was expected to impact the reemergence of vector-borne diseases. Many editorial discussions, like Dash et al. (2007), have analyzed the issues surrounding DDT and its ban and importance in vector control. The WHO still recognizes the rationale for indoor residual spraying (IRS) of DDT in malaria control but only until a more effective, affordable and safe alternative tool is available (Raghavendra et al, 2011). And although mosquitoes remain the central focus of most vector control programs, other public health pests including fleas, ticks, cockroaches, bedbugs and houseflies have also developed resistance (Davies et al, 2007; Gratz, 1977; Soderlund & Knipple, 2003).

2. Arthropod responses to insecticidal selective pressures

Resistance to insecticides as defined by the Insecticide Resistance Action Committee (http://www.irac-online.org). is '*a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species*'. Georghiou and Taylor (1986) considered it to be the genetic development by a target population of the ability to tolerate a dose of a toxicant that would prove lethal to the majority of individuals in a normal population of the same species. Others have simply stated it that it is a genetic change in response to selection by toxicants that may impair control in the field (Sawicki, 1987). Selective pressures over 40 years of intensive use of organic insecticides has led to pesticide resistance in more than 504 species of insects and mites (Roberts & Andre, 1994). Repeated applications of the same insecticides select for

individuals that can survive exposure to the compounds due to genetic difference(s). With time, the resistant phenotype becomes dominant in the population. This resistance is seen as an extremely serious threat to vector control (and crop protection) and many entities, including the WHO, industry, regulatory bodies and the public, consider it an issue that needs a proactive approach (Nauen, 2007; WHOPES, 2006). Resistance to one compound may confer resistance to other compounds in the same chemical class (Brogdon & McAllister, 1998a; Davies et al, 2007; Davies et al, 2008; Hemingway & Ranson, 2000; Nauen, 2007; Soderlund & Knipple, 1999) and is commonly referred to as *cross-resistance*. The first cases of this phenomenon were documented in houseflies in the mid-1970s (Keiding, 1975) and many insects currently found to be resistant to DDT are also resistant to pyrethroids (Davies et al, 2008), as both insecticides target the voltage-gated sodium channel genes in the insect nervous system.

B. INSECTICIDES

Although there are hundreds of insecticides used to control arthropods, there are four main chemical classification schemes and miscellaneous categories of insect growth regulators. A large number of vector control insecticides exhibit primarily two modes of action.

1. Classification

a. Organochlorines (OCs)

Organochlorines are comprised of two groups: DDT and chlorinated cyclic compounds.

DDT (dichlorodiphenyltrichloroethane) was the first of the modern insecticides (Davies et al, 2008; Raghavendra et al, 2011). Technical grade DDT has mainly five isomers. The *p,p′* isomer accounts for about 70% by weight and is the toxic form of DDT. It is a hydrophobic, colorless crystalline solid with good solubility in most organic solvents, fats and oils (Plimmer, 2003). The insecticide DDT is very environmentally persistent due to its low water solubility, low vapor pressure, and moderate stability in sunlight. Persistence in soil is related to temperature and moisture; therefore, the half-life of DDT is about 5 years in tropical areas and about 20-30 years in temperate regions (ATSDR, 2000). This persistence also leads to bioaccumulation. Compared to other insecticides, DDT has a low mammalian toxicity (Plimmer, 2003).

Chlorinated cyclic compounds are represented by lindane and the cyclodienes. Lindane is a hexachlorocyclohexane purified isomer (Yu, 2008). It is odorless, has a higher vapor pressure and is more water soluble than DDT. The cyclodienes are cyclic hydrocarbons and include aldrin, dieldrin, chlordane, heptachlor, endrin, endosulfan, chlordecone, mirex and toxaphene. They are very stable, lipophilic and have a low vapor pressure. This persistence makes them useful for control of termites and soil insects, but has also led to severe usage restrictions in many countries (Radcliffe et al, 2009). For example, the half-life of endrin is 12 years (Plimmer, 2003).

b. Organophosphates (OPs)

This large class of insecticides are highly toxic and are a by-product of chemical warfare research in the development of nerve gases in Germany in World War II (Yu, 2008). Organophosphates degrade rapidly by hydrolysis upon exposure to sunlight, air and soil. Organophosphate insecticides can be grouped according to their toxic action on

insects. Malathion has an action similar to chlorinated hydrocarbons and acts as a contact poison (http://michigan.gov/dnr). Others, like dimefox, are selective systemic insecticides which are absorbed into the plant sap and as such, are not toxic to plant pest predators. Relative to dose and formulation, they, however, have greater acute toxicity than organochlorines (Plimmer, 2003). Organophosphates commonly used to control vectors are malathion, dichlorvos, temephos, naled, fenitrothion, and pirimiphos-methyl.

c. Carbamates

These insecticides are esters of carbamic acid. They are very similar to organophosphates but feature a carbamate ester functional group. Carbamates have their origin in the alkaloid, physostigmine, which is a toxic component of the bean of the plant *Physostigma venosum* (Balf.) and are highly biodegradable (Plimmer, 2003). Familiar insecticides are bendiocarb and propoxur.

d. Pyrethrins

There are six pyrethrin esters known collectively as pyrethrum extracted from *Chrysanthemum cinerariaefolium* Vis.. Pyrethrins occur in high concentrations in the flowerhead. In contrast, pyrethroids are synthetic modifications of the pyrethrin molecule. There are two major classes; type I and type II pyrethroids**.** Their classification is based both on their effects on the cockroach sensory neurons and the presence or absence of an α cyano group. Type II pyrethroids have an α-cyano group in the molecular structure and type I pyrethroids do not. While type I pyrethroids knockdown insects quickly with subsequent recovery, type II pyrethroids cause higher insect mortality. Permethrin is an example of a type I and deltamethrin is a type II. Pyrethrins have shorter environmental persistence than pyrethroids because their chemical structure

is more susceptible to UV light and changes in pH. Another important difference between pyrethrins and pyrethroids is the behavioral effect they have on insects. Pyrethrins induce excitation behavior characterized by erratic and increased movement. This is sometimes referred to as 'flushing' action. This flushing action induced by pyrethrins is highly desirable; because of the increase in movement, it often results in increased insect exposure to pyrethrins. In vector control, most pyrethrin products commonly have synergists added to the formulation by the end user before they are applied because insects can recover from pyrethoids alone (Plimmer, 2003). Insecticides for vector control are alpha-cypermethrin, bifenthrin, cyfluthrin, deltamethrin, etofenprox and lambda-cyhalothrin.

e. Insect growth regulators

Insect growth regulators (IGRs) disrupt the growth and development of the arthropod, eventually leading to death (Hemingway & Ranson, 2005). The five types are juvenile hormone mimics, benzoylphenylureas, diacylhydrazines, triazines, and thiadiazines. Methoprene, fenoxycarb, and pyriproxifen are juvenile hormone mimics that affect morphological maturation and reproductive processes (Mullen & Durden, 2009). Lufenuron is a benzoylphenylurea that inhibits molting and is given orally to pets in tablet form. Diacylhydrazines are all derivatives of hydrazine and are agonists of ecdysteroid receptors. Tebufenozide and methoxyfenozide are diacylhydrazines. Cyromazine is the only triazine insecticide and acts by disrupting molting. Buprofezin is the only currently utilized thiadiazine and acts to inhibit chitin biosynthesis.

f. Synergists

Piperonyl butoxide (PBO) and S,S,S,-tributyl phosphorotrithioate (DEF) are examples of synergists. When a combination of compounds is more toxic than the insecticide alone, synergism is said to occur, with the relatively non-toxic compound acting as the synergist (Yu, 2008). Piperonyl butoxide acts by inhibiting cytochrome P450 monooxygenases, which metabolize insecticides, resulting in increasing toxicity of insecticides. The synergist DEF inhibits esterases.

g. Miscellaneous

Selamectin is in the avermectin class of the macrocyclic lactones. Fipronil is a phenylpyrazole and can be a spray or topical "pour-on" insecticide on pets for flea control. Both classes of insecticides act on the GABA gated chloride channels. Spinosad is derived from the soil actinomycete *Saccharopolyspora spinosa* Mertz and Yao and is administered orally to pets for flea control and used as a larvacide in mosquito control**.** Imidicloprid is a neonicotinoid and binds to the nicotinic acetylcholine receptor and is applied as topical oil on pets.

2. Primary modes of action of the four major groups

a. Voltage-gated sodium channels

DDT and pyrethroids bind to the voltage gated sodium channel transmembrane proteins in nerve cells. This binding causes a delay in closing of the channel during nerve impulse conduction. This prolonged inactivation causes very slow recovery to the resting stage. There are repeated discharges of axonal action potentials (even with a single stimulus) and the resulting neuroexcitation results in hyperactivity, tremors, rigid paralysis and eventually death (Davies et al, 2007).

b. Inhibition of acetylcholinesterase

Organophosphates are generally regarded as irreversibly inhibiting acetylcholinesterase because the rate of dephosphorylation is very slow (days to weeks) (Yu, 2008). The OPs phosphorylate amino acids in the active site of acetylcholinesterase (AChE). The phosphorylated AChE cannot hydrolyze acetylcholine (ACh) which leads to increased concentrations of ACh in the postsynaptic receptor. This leads to excess neuroexcitation in the insect. Exposure to carbamates is reversible because decarbamylation of AChE is rapid, within minutes. (Yu, 2008).

c. GABA-gated chloride channels

Gamma-aminobutyric acid (GABA) functions in the insect nervous system as an inhibitory neuro-muscular transmitter. An action potential triggers the release of GABA from the presynaptic terminal of the neuron which then binds to postsynaptic receptors containing chloride channels in the muscle. This opens the channel and chloride ions flow into the postsynaptic neuron. The increase in anions in the cell causes an inhibitory postsynaptic potential that maintains the membrane at its resting value so that its excitability is decreased (Casida, 2009). Without this inhibition, muscles become overstimulated and rigid. Insecticides including cyclodienes, lindane,and phenylpyrazoles (fipronil and ethiprole) bind to the chloride channel and block its activation by GABA.

3. Uses/Formulations

The first synthetic insecticide was invented in 1873 by Othmar Zeidler and its insecticide activity was discovered in 1939 by Dr. Paul Müller and became known as DDT (Yu, 2008). Prior to this, insect control dates back at least to the classical period in Greece and Rome. Pyrethrum (Persian insect powder) was used in the eighteenth

century, followed by other conventional insecticides for example, mercuric chloride (1860), Paris green (1865), phenols and cresols (1867), naphthalene (1882), Bordeaux mixture (1883), rosin-fish oil soap (1886), calcium arsenate (1907), and nicotine sulfate (1909) (Raghavendra et al, 2011). DDT was extensively used during the Second World War among Allied troops and civilian populations to control louse vectors of *Rickettsia prowazekii* da Rocha-Lima (louse borne typhus) and mosquito vectors of malaria, and was then used as an agricultural insecticide after 1945. DDT was banned for use in Sweden in 1970 and in the United States in 1972 (ATSDR 1994). It was historically the main insecticide used by the WHO to control malaria and remains part of the IRS program until an affordable, effective and safe alternative is found. (Raghavendra et al, 2011).

In the early 1960s, first generation pyrethroids were developed, but these had limited persistence due to instability in sunlight. Second generation pyrethroids introduced in 1974 by Rothamsted Research (Hertfordshire, England) demonstrated increased persistence and mammalian toxicity. The pyrethroids are currently used extensively throughout the world for controlling public health pests. Formulations used for both insecticides in IRS are wettable powders and capsule suspension (Hemingway & Ranson, 2005). Bednets impregnated with pyrethroids are widely distributed to reduce the incidence of malaria. Pyrethroids for flea control are formulated as dusts, shampoos, dips and sprays. Examples would be Long Acting KnockOut Spray™ and Pyrethrin Dip™ by Virbac Animal Health (Fort Worth,TX) and Proticall™ by Intervet Merck Anamal Health, Summit, NJ).

The organophosphates dichlorvos and naled are incorporated into pet flea collars in a microencapsulation time-release design, and naled is also used to control adult mosquitoes and fly populations. Temephos controls mosquito and midge larvae. Malathion, fenetrothion and pirimiphos-methyl are on the WHO list of insecticides for indoor residual spraying (IRS) control of malaria vectors (Raghavendra et al, 2011). Formulations are wettable powders and emulsifiable concentrates. Chlorpyriphos, diazinon, malathion, fenetrothion and pirimiphos-methyl are on the list of dustable formulas WHO recommends for rodent flea control (WHOPES, 2006).

The carbamates bendiocarb and propoxur are also on the WHO recommended IRS list. They are formulated as wettable powders. Carbaryl and the aforementioned two are also used in dust formulations to control rodent fleas (WHOPES 2006).

C. INSECTICIDE RESISTANCE

There is natural variation in any insect's ability to detoxify an insecticide, which inherently makes a designation of an insect as "resistant" a bit subjective. Nevertheless, the WHO has adopted as a definition for a resistant population as one that requires 10 times the amount of pesticide to kill 50% of target insects as compared to a reference population (WHO, 1970).

1. Mechanisms of resistance

Although numerous chemicals are used to control vectors and there are hundreds of examples of resistance, there are very few resistance mechanisms.

a. Behavioral

Behavioral resistance is primarily stimulus dependent and is a result of hypersensitivity or hyperirritability (Coleman & Hemingway, 2007). The affected insect can demonstrate a reduced affinity for entering sprayed houses, or a reduced resting time on sprayed surfaces. These insects may have receptors that are more sensitive to insecticides (Yu, 2008). This mechanism, although present in some species, is more of an avoidance behavior, and as such, is considered significant primarily in malaria vectors (Brogdon & McAllister, 1998a; Roberts & Andre, 1994)

b. Physiological

Physiological resistance involves three factors: reduced penetration, enhanced detoxification and target site insensitivity.

b(i). Reduced penetration

Reduced penetration is a result of the insecticide penetrating the insect cuticle at a lower rate. This mechanism is common but by itself only provides slight resistance and works best in conjunction with other resistance factors, (Hemingway & Ranson, 2005; Yu, 2008). Decreasing the amount or delaying the rate a toxin reaches the insect's target site enables other mechanisms to more effectively detoxify the insecticide as it travels through the insect (Hemingway & Ranson, 2005). The decreased rate of insecticidal uptake could be due to more protein and lipid in the cuticle and increased sclerotization, as seen for example in a DDT-resistant strain of tobacco budworm (Vinson & Law, 1971), or higher total lipids, monoglycerides, diglycerides, fatty acids, sterols, and phospholipids in resistant houseflies' cuticle (Patil & Guthrie, 1979). A study by Ahmad et al. (2006) found that delayed cuticular penetration was a significant aspect of

deltamethrin resistance in Chinese and Parkinson strains of the cotton bollworm (*Helicoverpa armigera* (Hübner)).

b(ii) Enhanced detoxification

According to Hemingway and Ranson (2000) metabolic detoxification is the most common mode of insecticide resistance in insects. Enhanced detoxification resistance involves groups or multigene families of enzymes that have the abilities to interact with xenobiotics and reduce or eliminate their potentially lethal effects. The main enzyme families involved are the cytochrome P-450 dependent monooxygenases (p450s), the esterases and the glutathione S-transferases (GSTs). The monooxygenases are a large family of enzymes and resistance is due to enhanced oxidative metabolism of the insecticides by these enzymes. The P450s catalyze a wide variety of reactions in which a hydroxyl group is added to the diphenyl ether moiety of the insecticide and the product is more easily conjugated with a carbohydrate for enhanced excretion (Plimmer, 2003). The P450s and mixed function oxidases confer resistance to pyrethroids and carbamates, but can include organophosphates and chlorinated hydrocarbons (except cyclodienes) (Yu, 2008).

Esterases target ester bonds in insecticides and subsequently hydrolyze or cleave ester bonds. Some esterases are known to have increased activity through gene duplications leading to increased amounts of transcripts and eventually protein. Resistance to organophosphates, and to a lesser extent carbamates and pyrethroids, has been associated with esterases.

Glutathione S-transferases (GSTs) act by conjugation and dehydrochlorination of the insecticide. The GSTs are associated with resistance to DDT, organophosphates and pyrethroids.

b(iii). Target site insensitivity

There are three main insect central nervous system target sites – the voltage-gated sodium channel, acetylcholinesterase (AChE) in the cholinergic synapses and gamma aminobutyric acid (GABA) sites in chloride channels at neuromuscular synapses. Resistance to DDT and pyrethroids is conferred by a mutation in the voltage-gated sodium channel usually by a substitution of the amino acid leucine with phenylalanine at the same or proximate codon position in domain IIS6 of the protein. These substitutions confer knockdown resistance or, *kdr*. Soderlund and Knipple (2003) provide a thorough review of knockdown resistance. Davies et al. (2007) summarizes the importance of domain II S4-S6 mutations. The genes that confer *kdr* are generally recessive; resistance is only noted in insects homozygous for the mutation (Davies et al, 2008; Hemingway & Ranson, 2000).

Resistance to organophosphates and carbamates is associated with insensitive AChE. This is achieved by structural modifications of the pesticide binding sites in AChE that allow at least partial binding to acetylcholine. This binding allows the AChE to break down the buildup of acetylcholine in the synapse and the synapse can function normally.

Cyclodienes, lindane and fipronil act by binding to chloride channels in the nerve muscle synapses and thereby block its activation by the inhibitory neurotransmitter GABA (Casida, 2009). This lack of inhibition at the synapse leads to hyperexcitation and

paralysis of the central nervous system. Selamectin, on the other hand, opens the chloride channels by binding to the GABA receptor site and acts as a partial agonist. This irreversible flow of chloride ions into the synapse results in loss of sensitivity and paralysis. Resistance is conferred by single nucleotide changes within the same codon of a gene for a GABA receptor involving an amino acid substitution from an alanine to a serine(Hemingway & Ranson, 2005)

2. Methods of detection

Expedient and accurate identification of resistance in target populations is one of the most important factors in successful vector resistance management (Brogdon & McAllister, 1998a; Nauen, 2007; Roberts & Andre, 1994; Soderlund & Knipple, 2003). Generally, resistance detection methods fall into three broad categories: bioassay, biochemical or molecular genetic.

a. Bioassay

Bioassays of agricultural pests generally use topical application of the toxin directly on the insect's body and mortality is assessed using a predetermined LC_{50} dose. Field bioassays to test for phenotypic expression of resistance also include the standard World Health Organization adult susceptibility test (WHO, 1970) originally developed in 1963 or the CDC bottle bioassay (Brogdon & McAllister, 1998b). Although a mainstay, the WHO bioassay has many shortcomings: it is expensive, test strips are not available for many commonly used insecticides, available dosages do not apply to all species, actions of synergists cannot be integrated, results are difficult to correlate with biochemical resistance test results, mosquitoes must maintain contact with pyrethroid strips (Brogdon and McAllister 1998b), only one insecticide can be tested per insect, it is

necessary to have large numbers of field caught insects to establish discriminating doses, the insecticide degrades on the shelf leading to false resistance results, and using discriminating dosages prevents estimation of levels of resistance (Roberts and Andre 1994). The CDC bottle bioassay is a great improvement with better speed, identifying mechanisms affecting susceptibility changes, and increased versatility and sensitivity. Both assays focus on flying insects (mosquitoes) by physical design. The WHO test simulates a mosquito resting on the wall of an insecticide-treated house, then leaving (or falling on the floor) (Brogdon and McAllister 1998b). Bottle assay assumes dosing by tarsal contact on a wall, net, curtain, etc. This can also be a drawback. Crawling arthropods, for example fleas and ticks, also need a field-expedient assay.

b.Biochemical assays

Biochemical assays detect the generic activity of insecticide metabolic enzymes. Originally, a single insect yielded one assay. During the 1980s and 1990s improvement was seen in the format by going from a single test tube assay to the microplate format, allowing multiple enzyme assays on a single insect. Others were developed using a microplate-spectrophotometric analysis (Brogdon, 1984; Brogdon, 1988; Brogdon & Barber, 1990), high-pressure liquid chromatography (Brogdon & Dickinson, 1983), dot-blot testing on single insects (Dary et al, 1991), and assorted variations on the central theme. Biochemical tests are rapid, multiple assays that can be performed on single insects to detect generic activity potentially correlated with metabolic resistance. Disadvantages of these tests are that the reagents are not readily available, resistance enzymes vary naturally with age, developmental stage and sex, and global use would require standardized kit production, and field conditions (temperature, humidity) could

affect results (Roberts & Andre, 1994). Detailed comparisons between bioassay and biochemical procedures have been made (Brogdon, 1989; Brogdon et al, 1992; Brown & Brogdon, 1987).

c. Molecular genetic methods

Molecular genetic detection methods include DNA isolation, the polymerase chain reaction (PCR) (and several variations thereof), sequencing, and cloning. A newer *in vitro* method uses microarray technology. An example is the detoxification chip of the malaria vector *Anopheles gambiae* Giles used to monitor detoxifying gene expression levels (David et al, 2005). Researchers constructed the chip to assay 230 potential detoxification transcripts in *A. gambiae* corresponding to the cytochrome P450s (103 transcripts), mixed function oxidases (41MFOs), the glutathione-S transferases (35GSTs), and the carboxylesterases (31COEs). Another novel development involves the use of microarrays containing expressed sequence tags (ESTs) to identify genes conferring or associated with insecticide resistance (Nauen 2007). They can also be used to measure changes in expression levels, detect single nucleotide polymorphisms (SNPs), or to genotype or resequence mutant genomes (David et al, 2005). Husseneder et al. (2010) used the technique for genetic analyses and population assignment in the cat flea. Microarray while very expensive is both sensitive and specific. However, gene expression does vary by developmental stage, age, and sex. Furthermore, microarrays are species specific and are currently limited to use in a well equipped research laboratory.

3. Prevalence/Importance

There are currently no effective drugs or vaccines for diseases such as dengue, dengue hemorrhagic fevers and Chagas disease. Prevention relies completely upon

prevention of transmission of the disease by the insect vector and the most important element of vector control strategies is the use of insecticides (IRAC 2011). Most insecticides were developed for agriculture (Yu 2008). Almost all insecticides used in public health are also used in agriculture. When vector species breed close to, or in, crop areas they may be exposed to the same insecticides used for agricultural pest control (Shyamal et al, 2008).

The degree to which most insect control programs have relied on DDT was brought to light by the Stockholm Convention on Persistent Organic Pollutants (POPs) when they recommended restrictions on the use of DDT (Raghavendra et al, 2011). Mosquito and sandfly control currently rely more on pyrethroid treated nets (ITNs) and materials (ITMs).

Over 500 pest insect species have evolved resistance to at least one insecticide during the last 40 years and the increase in the number in those 40 years has been exponential (AFPMB, 2011). The burden that vector-borne diseases put on the public health system is accounted for in more than human suffering. It is estimated that malaria alone has accounted for more than a 20% reduction in the gross national product of Africa over the past 15 years (IRAC, 2011). The plethora of government, university, industry and private watchdog entities focused on managing insecticide resistance and online resistance search engines and databases is testament alone to the magnitude of the problem (Sawicki et al, 1978).

D. RESISTANCE IN THE ORIENTAL RAT FLEA, *XENOPSYLLA CHEOPIS* (ROTHSCHILD)

1. History of plague

Plague is, arguably, one of the world's most "famous" (or infamous) diseases and has been called "the ultimate scourge of mankind" (Kelly, 2005). Until recently it was an epidemic disease subject to International Health Regulations and as such was a notifiable disease to the WHO (1983). The disease is thought to have originated in Central Asia with the first recorded epidemic possibly being described in the Bible as an outbreak among the Philistines in 1320 BC (Tikhomirov, 1999). The first recorded pandemic, called Justinian's plague occurred between 542 and 546AD. The second, now known as the "Black Death" is believed to have originated in the city of Caffa (now called Theodosia) on the Crimean shore of the Black Sea. Tartars fleeing the infected city spread it to Asia. It was spread to Europe by Italian merchants (Kelly, 2005). From 1347 to 1350 plague spread throughout Asia, Africa and Europe and wreaked devastation on an apocalyptic scale. An estimated 20 million people died in Europe alone (Cantor, 2001). It is thought to have killed an estimated 50 million people in total (Tikhomirov 1999).

The third major pandemic started in Canton and Hong Kong in 1894 and in 10 years killed an estimated 10 million people (USACHPPM, 1995). In its spread to surrounding countries from 1896 to 1948, 12 million deaths occurred in India alone (Mullen & Durden, 2009). Manchuria in 1910–1911 witnessed about 60,000 deaths due to pneumonic plague and the same number again in 1920–1921. Foci of this pandemic still exist. In 1965-1971, during the Vietnam War, 25,000 cases of plague were reported.

Epidemics still happen even in modern times, most often in developing countries where plague is endemic and living conditions are unsanitary and rat-infested (Gage & Kosoy, 2005). A minor outbreak occurred as recently as the summer of 1994 in Surat,

India following an earthquake in September 1993. In the United States, 61 cases were identified between 1994 and 2003 (Gage and Kosoy 2005). Statistics received by WHO from 1954 to 1997 indicated that plague affected 38 countries, with 80,613 cases and 6,587 deaths. The highest number of cases during those years (6,004) occurred in 1967 and the minimum in 1981 was 200 (Tikhomirov 1999). The annual number of cases in 1967-1971 was over 4,000 and the total for that period was 22,335 cases and 975 deaths. Asia reported the largest proportion of cases with 58.4%, Africa had 27.8% and the Americas reported 13.8%. Subsequent mortalities were 54.6%, 34.4% and 11.0% respectively.

The Ugandan Ministry of Health and the CDC reported 127 clinical cases of plague in the Arua and Nebbi Districts in northwestern Uganda in November 2006. Twenty eight patients died (Prevention, 2009) In November 2008 another outbreak occurred (Pathology, 2008). Four cases and two deaths occurred in the Arua District and five cases and one death were reported in Nebbi District. Other interesting historical accounts of plague can be found in publications by Duplaix (1988) and Mee (1990).

2. Pathology

Plague is an infectious disease of animals (primarily rodents) and humans caused by the bacterial species *Yersinia pestis.* It is a gram-negative, nonmotile, non-spore-forming coccobacillus (Salyers, 2002). When stained with Wright, Giemsa or Wayson stain, the bacterium shows bi-polar staining and when viewed through a microscope, resembles a safety pin (Aiello, 2010). It is carried by a number of animal hosts, primarily rodents, and is spread by the bite of a subsequently infected rodent flea or direct contact with sick or dead animals infected with *Y. pestis.* Infection can also occur by inhalation

of respiratory droplets containing the bacterium or from aerosols generated through laboratory activities or other means. Besides wild and domestic rodents, plague sometimes can affect humans and cats as well as rabbits and hares. A comprehensive list of species associated with plague can be found in Pollitzer and Meyer (1961). Domestic dogs, coyotes, raccoons, badgers, skunks and black bears are considered highly resistant although individual animals might be highly susceptible (AVMA, 2001). Rare cases have developed in goats, sheep, mule deer, pronghorn antelope, nonhuman primates and camels (Aiello, 2010).

There are three clinical manifestations of the disease: bubonic, septicemic, and pneumonic. Bubonic is the classical form of the disease and is characterized by the bacteria's invasion of the lymphoid system through skin inoculation. The infected regional lymph nodes swell and become necrotic. This swollen node is called a bubo. Buboes can occur at any regional lymph node site, including deeper internal ones. The incubation period is normally 2-6 days (Poland & Dennis, 1999). Symptoms can include sudden high fever, chills, headache, tachycardia, malaise, and painful swelling in regional lymph nodes (buboes). The disease progresses rapidly, but with treatment, usually tetracycline and doxycycline, the clinical symptoms will resolve in 3 to 5 days. The untreated human case fatality rate is 50-60% (AVMA, 2001). Symptoms in cats are typically abscesses in the mouth, face or throat regions, buboes especially under the jaw and in the throat/neck area, fever and lethargy. A secondary pneumonia may also develop (AVMA 2001). A clinical study by Eidson (1991) found that 53% of the cats in New Mexico affected by plague demonstrated the bubonic form. Humans can acquire the disease from infected cats, either from the bite of an infected flea they transport or

secondarily by inhaling infected respiratory droplets expelled by the coughing cat. Gage et al. (2000) in a study from 1977 to 1998 found that 23 cases of cat-associated plague in humans occurred in 8 western states which accounted for 7.7% of the 297 cases reported in that period.

Septicemic plague occurs when the pathogen invades the bloodstream. This may occur without a bubo (primary septicemic plague) or septicemia may be secondary to bubonic plague. Gangrene (ergo the name "black death"), blood clotting disorders and multiple organ failure may result (AVMA, 2001). Untreated cases are almost always fatal, but early treatment can result in recovery. Cats with primary septicemic plague will have no obviously enlarged lymph nodes, but will present with fever, lethargy and anorexia. This can progress to overt signs of sepsis which can include vomiting, diarrhes, tachycardia, prolonged capillary refill time, cold extremities, pale mucous membranes disseminated vascular coagulopathy, multi-organ failure and acute respiratory distress syndrome.

Primary pneumonic plague is caused by the inhalation of *Y. pestis* organisms, and although rare, has been reported after handling cats with pneumonic plague (Aiello, 2010). Typically symptoms manifest within 24 to 48 hours post exposure (Poland & Dennis, 1999). Symptoms besides respiratory distress include nausea, abdominal pain, vomiting and diarrhea. Buboes are rarely present. Secondary pneumonic plague is a rare (less than 5%) sequelae to bubonic or primary septicemic plague (Poland & Dennis, 1999). Bacteria spread to the lungs and corresponding symptoms are cough, chest pain, bronchopneumonia, labored breathing and blood in the sputum. Untreated pneumonic plague usually results in death, but early treatment can lead to recovery (AVMA, 2001).

3. Ecology and dynamics of plague

The ecological cycle of plague is typically divided into urban (domestic) and sylvatic (wild-rodent) (Gage & Kosoy, 2006; Gage & Kosoy, 2005; Mullen & Durden, 2009; Pollitzer & Meyer, 1961; Tikhomirov, 1999; USACHPPM, 1995; Wimsatt & Biggins, 2009). Urban plague is associated with commensal rats, and their fleas, both of which live in close proximity to human and their animals. Sylvatic plague is maintained in mammalian species, primarily rodents, and their fleas that typically live in rural areas away from human populations. Sylvatic plague manifests itself in two forms: enzootic and epizootic (Gage & Kosoy, 2006; Gage & Kosoy, 2005; Gage et al, 1995; Mullen & Durden, 2009; Pollitzer & Meyer, 1961; Tikhomirov, 1999; USACHPPM, 1995; Wimsatt & Biggins, 2009). During enzootic cycles, sylvatic plague is typically thought to quietly persist in discrete, moderately resistant or maintenance rodent populations accompanied by low-level flea or host disease (Gage & Kosoy, 2005; Wimsatt & Biggins, 2009). Sustainability of flea populations depends on factors like key nutrients needed by the flea and the *Y. pestis* organism for growth and reproduction, which are provided by continuous hosts (Gage & Kosoy, 2006; Gage & Kosoy, 2005; Pollitzer & Meyer, 1961). Epizootics occur when the disease spills over into more susceptible hosts and is characterized by a progressive and rapid mortality of large numbers of these hosts. A decline in the number of available "immunologically naive" hosts can lead to the spread of *Y. pestis* from one population to another. This poses an increased risk of the spread of plague to humans and other mammals as the fleas seek out new hosts (Gage & Kosoy, 2006).

Although the sylvatic cycle of plague maintains the disease, almost all significant outbreaks of human plague are associated with epizootics in commensal rats (*Rattus rattus* (L) and *R. norvegicus* Berkenhout) and their fleas, especially *X. cheopis* (Gage & Kosoy, 2005; Piesman & Gage, 2000; Pollitzer & Meyer, 1961; Wimsatt & Biggins, 2009). The factors that lead to epizootics are complex and many studies have focused on a variety of them, to include abundance of susceptible hosts, rodent density, and climatic factors (trophic cascade hypothesis), including increased precipitation, and threshold temperatures (Gage & Kosoy, 2006; Gage & Kosoy, 2005).

Understanding the enzootic phase is also key to understanding the persistence of plague. Factors that have been investigated by numerous researchers are: heterogeneity in *Y. pestis* strains, diversity and density of rodent populations, immune status of the host, genetic structure of hosts, physiologic condition of the hosts or vectors, species of flea vector, transmission mechanisms, mutagenic effects on *Y. pestis* of phagocytic cell activity, bacteriophage activity, and interactions between *Y. pestis* and other bacteria (Gage & Kosoy, 2005 and references therein).

Gage and Kosoy (2006) developed four major hypotheses for the persistence of plague based evidence from published research. One is that the bacterium maintains itself through a continuous cycle of enzootic and epizootic periods by depending on a consistent supply of naïve hosts and fleas. Alternatively the carrier-host hypothesis states that carrier hosts maintain the organism during quiescent disease cycles. The telluric hypothesis postulates that *Y. pestis* can survive for long intervals in burrow soil or another substrate. The fourth hypothesis states that fleas act as reservoirs of plague. A

review by Wimsatt and Biggins (2009) and references therein concur with the feasibility of the reservoir hypothesis.

4. Transmission of plague by *Xenopsylla cheopis*

Xenopsylla cheopis, the Oriental rat flea, order Siphonaptera, family Pulicidae, is one of the most efficient, and therefore important, vectors of *Y. pestis* (Chanteau et al, 1998; Eisen et al, 2007b; Gage, 2005; Gage & Kosoy, 2006; Gage & Kosoy, 2005; Gratz, 1999; Lorange et al, 2005; Mullen & Durden, 2009; Piesman & Gage, 2000; Renapurkar, 1990; Shyamal et al, 2008). Although more than 150 species of fleas have been reported to be infected naturally with *Y. pestis* (Pollitzer & Meyer, 1961), most of the taxa are relatively inefficient vectors (Piesman & Gage, 2000). Factors governing the significance of a species' ability to vector *Y. pestis* involve the rodent species used as a host, flea population numbers during peak transmission seasons, geographic distribution of the flea species, the fleas capacity to live off-host for extended periods of time, host-specificity of the flea, and the ability of the flea to become blocked after feeding on an infected host (Eisen et al, 2007b; Gage & Kosoy, 2005; Piesman & Gage, 2000).

Xenopsylla cheopis will readily feed on humans when its ordinary rodent host is not available (Bacot & Martin, 1914; Eisen et al, 2007b; Gage, 2005). The blocking phenomenon is critical for efficient transmission of plague (Bacot & Martin, 1914) and *X. cheopis* is renowned for its ability to become blocked (Gage & Kosoy, 2006; Gage & Kosoy, 2005). Midgut blockage occurs when plague bacteria ingested in a bloodmeal from an infected host quickly multiply within the spine-covered proventriculus at the proximal end of the foregut. The colonies of bacteria that form in a few days multiply enough to block the flea's proventriculus which then also blocks any ingested blood from

passing through to the midgut. The blocked and now starving flea makes repeated attempts to feed and in the process regurgitates *Y. pestis* into the wound created at the bite site on the host (Eisen et al, 2007b; Gage, 2005; Gage & Kosoy, 2006; Gage & Kosoy, 2005; Lorange et al, 2005; Mullen & Durden, 2009; Piesman & Gage, 2000; Wimsatt & Biggins, 2009). Ongoing research is focused on teasing out the complex variables that influence blockage (Eisen et al, 2006; Eisen et al, 2008; Eisen et al, 2009; Eisen et al, 2007a; Eisen et al, 2007b; Wilder et al, 2008) and even challenging the blockage paradigm (Eisen et al, 2009; Webb et al, 2006).

Transmission by mechanical means (i.e. contaminated mouthparts) has also been considered a significant contributing factor (Eisen et al, 2009; Gage, 2005; Gage & Kosoy, 2006; Gage & Kosoy, 2005; Piesman & Gage, 2000).

5. Current status of insecticide resistance in fleas

Reports of flea resistance to insecticides have been extant for 62 years with the first case observed in 1949 involving resistance in the cat flea (*Ctenocephalides felis)* (Bouché) to DDT dusting (Gratz, 1977). The first case of field resistance actually reported in *C. felis* was in 1952 in the southeastern United States (Bossard et al, 1998).

Resistance in *X. cheopis* was reported in a laboratory study wherein resistance was seen up to the F_3 generation (Kilpatrick & Fay, 1952) . The first report of confirmed DDT resistance in *X. cheopis* in the field was in the Poona District of India in 1960 (Patel et al). With the development of the WHO susceptibility/resistance assay in 1960, reports of resistance (mostly to DDT) in fleas increased in developing countries, as a result of IRS programs targeting malaria control (Gratz, 1977). Excellent in-depth reviews of the

history of the development of resistance in fleas can be found in Gratz (1977) and Bossard, Hinkle et al. (1998).

Currently listed on the Arthropod Pesticide Resistance Database maintained by Michigan State University (www.pesticideresistance.org) are 8 fleas in the family Pulicidae that have demonstrated field resistance to insecticides. The flea and the number of reported cases are: *Ctenocephalides canis* (Curtis) (dog flea) 12 cases, *C. felis* (cat flea) 28 cases, *Pulex irritans* (L) (human flea) 13 cases, *Stivalius cognatus* Jordan and Rothschild 1 case, *Synopsyllus fonquerniei* Wagner and Roubaud (Malagasy endemic rural flea) 3 cases, *Xenopsylla astia* Rotschild (Indian rat flea) 4 cases, *X. brasiliensis* (Baker) (African rat flea) 1 case, and, *X. cheopis* (Oriental rat flea) 26 cases. *Ctenocephalides felis* has shown resistance to 13 insecticides in 11 countries and *X. cheopis* has demonstrated resistance to 7 insecticides in 14 countries. Resistance to DDT, other chlorinated hydrocarbons, and pyrethroids in *X. cheopis*, after the report by Patel (1960) in India was next reported in Vietnam (Chow, 1965), Taiwan and Thailand (WHO, 1966; WHO, 1966a), Egypt, Philippines, Burma, and Israel (WHO, 1976), and Madagascar, China and Brazil (Sustriayu et al, 1980; WHO, 1980).

Resistance to fenitrothion and fenthion (organophosphates) by *X. cheopis* appeared in Madagascar in 1986, as did resistance to malathion in India, Tanzania, and Madagascar (WHO, 1986). A report by Renapurkar (1990) indicated that, in India, *X. cheopis* continued to show resistance to DDT, dieldrin, malathion and even fenthion (never used in the area assessed). Plague did occur in the Maharashtra State in 1994 and the human infections from that state were thought to be the sources of infection for the later human cases in Surat where 52 deaths occurred (Dutt et al, 2006). Shyamal et al.

(2008) found, in the Nilgiris District in India that *X. cheopis* was resistant to DDT, malathion, and deltamethrin, but tolerant to permethrin. Tolerance is the natural ability of a population to withstand the toxic effect of an insecticide which can be developed in one generation and subsequently lost when the insects are no longer exposed to the toxicant (Yu, 2008). They concluded the selective pressures on the fleas were not from IRS programs, but from irregular anti-flea practices (i.e. rodent burrow insufflation) and the extensive use of insecticides in agricultural crops where the domestic/peri-domestic rodent interface occurs.

Krishnamurthy and Joshi (1962) observed that the flea indices were higher in towns that had insecticide resistant populations than those where the fleas were still susceptible. Flea indices are useful means for calculating flea densities, which in turn, can have a predictive value for plague risk (Bahmanyar & Cavanaugh, 1976; Mears et al, 2002; Pham et al, 2009; Singchai et al, 2003). The specific flea index is calculated by dividing the number of fleas of a species by the total number of rodents of a species, and a specific index of over 1 for *X. cheopis* indicates a dangerous situation (AFPMB, 2002; Bahmanyar & Cavanaugh, 1976). The risk of plague to humans is associated with an increased index and increased rodent density (Pham et al, 2009; Pollitzer, 1954).

E. RATIONALE AND OUTLINE OF DISSERTATION

In this dissertation a bioassay for detection of phenotypic insecticide resistance in fleas is described. A microplate generic biochemical assay was used to test for the metabolic mechanisms of resistance. I also tested for genetic changes in the *para* gene in *X. cheopis*, a major vector of *Y. pestis*.

My first study used Petri dishes in the development of a novel modification of the CDC bottle bioassay to ascertain the levels of phenotypic expression of resistance to DDT and lambda-cyhalothrin in field collected populations of *X. cheopis* in Uganda (Chapter 2). The second study used microplate enzyme assays performed on field collected Ugandan *X. cheopis* to assess the levels of potential metabolic detoxification resistance responses (Chapter 3). Laboratory colony fleas reared at the CDC in Fort Collins were used as baseline determinants. The last study used molecular techniques such as DNA extraction, PCR and sequencing to ascertain the domain II region of the rat flea *para* sodium channel gene sequence using both colony fleas and field collected Ugandan *X. cheopis* (Chapter 4). Subsequent analyses of the sequences investigated the frequency and distribution of knockdown resistance (kdr) allelic mutations and segregating sites present in each of the populations tested. Phylogenetic analyses also were performed. Single Nucleotide Polymorphisms (SNPs) in *para* were also analyzed for linkage disequilibrium, quantitative trait nucleotides, as well as nucleotide diversity and association with survival following DDT and lambda-cyhalothrin exposure. The results of these studies will provide important new tools and information leading to a more effective control plan for *X. cheopis*, a major vector of *Y. pestis*.

II. A NEW INSECTICIDAL BIOASSAY TECHNIQUE FOR FLEAS

A. INTRODUCTION

Emerging and reemerging vector borne diseases have a significant deleterious impact on global economies and public health (Binder et al, 1999; 2004; Smolinski et al, 2003). The World Health Organization (WHO) and many scientific publications report that use of insecticides can dramatically reduce the risk of insect-borne diseases, as demonstrated in the intense DDT usage in the 1940s for typhus, and 1950s-1960s to control malaria. Krogstad (1996) reported that insecticide resistance was one of two major reasons for the failure of the malaria control program and was expected to impact reemergence of vector-borne diseases. Many editorial discussions, like Dash et al. (2007), have analyzed the issues surrounding DDT and its ban in some countries, and importance in vector control. The WHO recognizes indoor residual spraying (IRS) of DDT in malaria control until a more effective, affordable and safe alternative tool is available (Raghavendra et al, 2011) Although mosquitoes remain a central focus of most vector control programs, other public health pests including fleas, ticks, cockroaches, bedbugs, and houseflies have also developed insecticide resistance (Davies et al, 2007; Gratz, 1977; Soderlund & Knipple, 2003).

Non-target resistance is most commonly associated with agriculture (Fitt et al, 1994; Radcliffe et al, 2009), but it can also occur within other insecticide uses (Tabashnik, 1989). When writing about multiple pesticide use for resistance management strategy, Tabashnik (1989) warns "Even when conditions are appropriate for using a "multiple attack" strategy to delay resistance in one pest species, this approach may speed resistance development in other pests." Multiple insecticide use has occurred more often with medical and veterinary pests than agriculture pests (Tabashnik, 1989). Although fleas are not direct targets in IRS programs, they can have contact with

insecticides either on the rodent host's fur as it moves through treated areas, or possibly rodent (and therefore, flea) nests in roof thatch of treated dwellings

Resistance to one compound may confer *cross resistance* to other compounds in the same chemical class (Brogdon & McAllister, 1998a; Davies et al, 2007; Davies et al, 2008; Hemingway & Ranson, 2000; Nauen, 2007; Soderlund & Knipple, 1999). The first cases of this phenomenon were documented in houseflies in the mid-1970s (Keiding, 1975) and many insects currently found to be resistant to DDT are also resistant to pyrethroids (Davies et al, 2008), because the insecticides share similar targets in the insect nervous system.

Over 500 pest insect species have evolved resistance to at least one insecticide during the last 40 years and the increase has been exponential (AFPMB, 2011). The burden that vector-borne diseases put on public health systems is accounted for in more than human suffering. It is estimated that malaria alone has accounted for more than a 20% reduction in the gross national product of Africa over the past 15 years (IRAC, 2011).

One of the most important factors in successful vector resistance control is expedient and accurate measures of the possible development of resistance in the populations in question (Brogdon & McAllister, 1998a; Nauen, 2007; Roberts & Andre, 1994; Soderlund & Knipple, 2003). Generally, resistance detection methodology falls into three broad categories: bioassay, biochemical and molecular genetic.

Field assays to test phenotypic expression of resistance in malaria programs are usually confined to the standard World Health Organization adult susceptibility test (WHO, 1970) originally developed in 1963 and the CDC bottle bioassay (Brogdon &

McAllister, 1998b). The WHO assay has shortcomings: it is expensive, test strips are not available for many commonly used insecticides, available dosages do not apply to all species, actions of synergists cannot be integrated, results are difficult to correlate with biochemical resistance test results, test chambers have untreated surfaces, and care must be taken to ensure mosquitoes maintain contact with treated strips (Brogdon and McAllister 1998b), it is necessary to have large numbers of field caught insects to provide sufficient sample sizes to perform logistic regressions, the insecticide in the kit degrades leading to false resistance results, and use of discriminating dosages prevents estimation of the levels of resistance (Roberts and Andre 1994). The CDC bottle bioassay is an improvement with better speed, ability to identify mechanisms affecting susceptibility changes, and increased versatility and sensitivity. Crawling arthropods, for example fleas and ticks, may not present the quantity of public health hazards as do mosquitoes, but are nevertheless significant disease vectors and insecticide resistance in these species also requires a field-deployable and field expedient assay.

Plague is, arguably, one of the world's most well-known diseases and has been called the ultimate scourge of mankind (Kelly, 2005). It remains as an epidemic diseases subject to International Health Regulations and as such is notifiable to the WHO (1983). Plague is an infectious disease of animals and humans caused by the bacterium *Yersinia pestis* (Yersin). It is mainly spread by the bite of an infected flea, and if left untreated can be fatal. Foci of plague persist throughout parts of the world overlap exists with some malaria control regions in India and Africa (Tikhomirov, 1999).

In areas of overlap fleas can be exposed to the same insecticides sprayed in IRS programs due to the fact that some of their hosts, commensal rodents, live in or travel

through sprayed homes. Attempts to control fleas in plague outbreaks may fail due to the possibility that the fleas have developed insecticide resistance as a result of this exposure.

Xenopsylla cheopis (Rothschild) the Oriental rat flea, is one of the most efficient, and therefore important, vectors of *Y. pestis* (Chanteau et al, 1998; Eisen et al, 2007b; Gage, 2005; Gage & Kosoy, 2006; Gage & Kosoy, 2005; Gratz, 1999; Lorange et al, 2005; Mullen & Durden, 2009; Piesman & Gage, 2000; Renapurkar, 1990; Shyamal et al, 2008). According to the Arthropod Pesticide Resistance Database maintained at Michigan State University, (www.pesticideresistance.org), *X. cheopis* has demonstrated resistance to 7 insecticides in 14 countries to date.

Currently, no inexpensive, field-expedient assays exist for detecting phenotypic expression of insecticide resistance in fleas. Control and management of a plague epizootic or epidemic could prove ineffective if insecticides are used that target resistance mechanisms that are widespread in local vector populations. To this end, this study undertook the development and testing of a rapid, simple and inexpensive field bioassay to assess phenotypic expression of insecticide resistance in *X. cheopis* fleas in northern Uganda proximal to a plague endemic area.

B. MATERIALS AND METHODS

1. Source of fleas.

Fleas used for baseline data were from laboratory colony *X. cheopis* reared at the Centers for Disease Control (CDC) and Prevention, Fort Collins, Colorado. The colony was started in the mid-1980s at the University of Maryland from fleas taken from rats trapped in the Baltimore, area. The colony was later shipped to the Rocky Mountain Laboratories in Hamilton, Montana. In 1992 fleas from that colony were transferred to the CDC in Fort Collins. Field collected *X. cheopis* fleas were combed off sedated rodents live-trapped from various locations in northern Uganda, Africa (Figure 2.1).

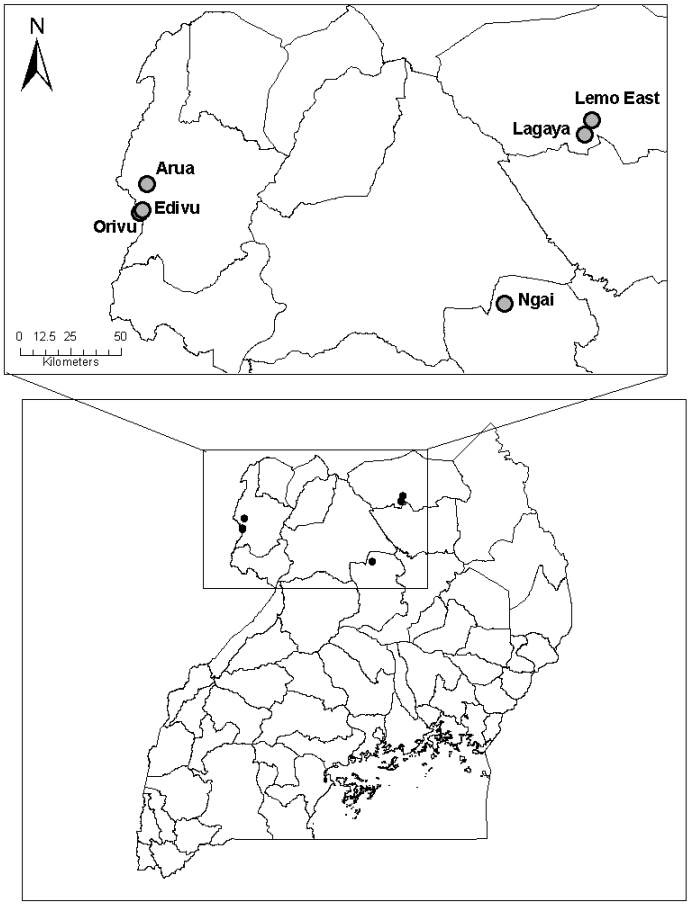

Figure 2.1. Map of Uganda, Africa indicating *Xenopsylla cheopis* field collection sites.

2. Description of dish assay

Round glass Petri dishes and covers, 100mm in diameter and 20mm deep and/or 100mm in diameter and 10mm deep, acetone, and the insecticides of interest were used. Insecticides used were technical grade DDT, permethrin, lambda-cyhalothrin, malathion, and deltamethrin (Chem Service, West Chester, Pennsylvania).

Clean Petri dishes were labeled as to the concentration of insecticide. For the control dishes, 1mL acetone was pipetted into the dish, gently rocked back and forth to ensure even coverage, and allowed to air dry. For insecticide treated dishes, the desired concentration of insecticide dissolved in acetone was added to1 mL acetone that had already been pipetted into the dish. The dish was then rocked evenly back and forth, until no visible liquid was observed, and air dried. All dishes were allowed to 'breathe' in a dark drawer with the lid slightly removed for at least 30 minutes to ensure complete evaporation of the acetone had occurred. Dishes could remain covered in a drawer overnight without loss of toxicity. Dishes were hand washed three times with E-Z™ Foam antibacterial soap (Kutol, Ohio) and rinsed with distilled de-ionized water after each bioassay.

Baseline toxic concentrations for insecticides were estimated under laboratory conditions using fleas collected by mechanical aspiration from colony jars. Approximately 30 live *X. cheopis* fleas were aspirated into 50ml Corning® plastic conical centrifuge tubes. Tube lids were perforated with a 24 gauge needle for air exchange. Small strips of filter paper were inserted in each tube to increase the survival of the fleas. Colony fleas had not been blood fed for at least 24 hours before the assays. The fleas were either then used immediately or placed in an incubator at 28°C and 80%

humidity for up to 1 week, a time period over which significant mortality is not expected. Colony fleas were immobilized prior to transfer to Petri dishes by plunging the conical tubes into ice.

Field assays used *X. cheopis* fleas obtained by first combing them off of live-trapped rodents collected from mud huts in northern Uganda and then anesthetizing them using halothane (CDC-approved Institutional Animal Care and Use Committee (IACUC) protocol #09-022). As described for assays using colony-reared fleas, fleas were placed in each 50ml conical centrifuge tube. The fleas were used for analysis within two hours of collection.

For colony-reared and field collected fleas, a tube of fleas was emptied into each Petri dish and a timer was started as soon as the control group fleas recovered and started moving (approximately 5 min). At 15 minute intervals ending at 60 minutes, all plates were assessed and annotated for flea mortality. Fleas were classified as "dead" if they were laterally recumbent and unable to right themselves and jump when stimulated with a gentle puff of breath under the lid of the plate, and/or tapping on the lid. After the completion of the tests, all fleas were immersed in 75-90% ethanol (EtOH) for genetic testing in Colorado.

3. Discriminating concentration determination

Colony fleas were assumed to be susceptible and used for developing data establishing discriminating concentrations. Lambda-cyhalothrin, DDT, malathion, permethrin and deltamethrin were chosen based on current or proposed indoor residual spraying (IRS) programs in Uganda. Starting points for appropriate concentrations were based on prior data established for lethality in mosquitoes (Janet McAllister, personal

communication). Concentrations were adjusted until the lowest concentration of the insecticide that resulted in 100% mortality in CDC colony fleas within 1 hour was determined.

4. Field assays in Uganda

Fleas collected at the Lemo East and Lagaya sites were processed at the UVRI lab in Arua. Many fleas did not survive the long, hot journey back to the lab and were immersed in 75% EtOH for later genetic analyses in Colorado.

Reagent grade insecticides were suspended in 2ml acetone. Petri dishes were prepared by adding either 65ul of 1mg/ml DDT or 20ul of 0.1 mg/ml lambda-cyhalothrin to 1 ml of acetone in each dish.

Flea immobilization for placement on the dishes was the most difficult hurdle. Commercial cold packs, refrigeration and an electric cold plate were tried. Cold packs were very slow and did not immobilize all the fleas in the tube. Refrigeration timing was subjective and high relative humidity caused condensation inside the centrifuge tubes leading to mortality. Similarly the electric cold table and high relative humidity caused extensive moisture condensation in the tubes and on the plates themselves. This created many droplets of water on the bottom of the Petri dish, creating possible areas of higher, or lower, than normal concentrations of chemical. The fleas stuck and drowned in the "pools".

A field-expedient method of immobilizing fleas was needed to perform the bioassays in Ngai. After collecting about 30 fleas in the 50 ml centrifuge tubes and allowing them time to recover from the combing collection, a strip of filter paper approximately 3.5in X 0.5in was saturated with liquid halothane. Fleas were tapped to

the bottom of the tube. The strip of halothane paper was inserted ¾ of its length into the tube and secured at the top of the tube with the lid leaving a small 'tab' of paper sticking out of the tube. The lids of the tubes had been previously perforated with a 26 gauge needle. Flea sedation was assessed as complete as soon as the fleas ceased moving at the bottom of the tube. The halothane strip could be removed and re-impregnated if necessary. Fleas were then tapped out of the tube onto a treated and labeled Petri dish and the cover was replaced. Most fleas became active within a minute after removal from halothane. At the end of the assay time period, resistant (still alive and jumping) fleas were recovered by opening the Petri dish that had been then placed in the bottom of a ½ gallon plastic zip top bag. Resistant fleas jumped out of the dish. They were removed with forceps and placed in labeled plastic 1.5ml tubes of 75% EtOH for later sequencing. Fleas labeled as susceptible were removed from the dishes and placed in labeled 1.5ml tubes of 75% EtOH.

5. Statistical analysis.

Calibration of the discriminating concentrations were performed using analysis logistic regression program Probit Logit (SAS Institute Inc., Cary, NC). Each concentration of the respective insecticides and the resultant mortality of colony fleas observed at 15 minute increments were entered into a data base. The discriminating dose (LC_{95}) of each insecticide was estimated.

Field tests used a different statistical model because the concentration was fixed and the time was variable. Time is a cumulative variable (i.e. cumulative number dead at successive time points). An underlying assumption in using logistic regression (Probit) is that variables are independent. When using a cumulative variable, such as time, it is

dependent and therefore a different statistical analysis is required (e.g. Weibull or Kaplan-Meyer). We used Weibull as a more robust test when using discrete time points versus continuous scoring of mortality (i.e. recording exact times of death).

The Model

The survival times are assumed to follow a Weibull distribution

$$f\ t\ = \lambda\gamma\ \ \lambda t\ ^{\gamma-1} e^{-\ \lambda t\ ^{\gamma}},$$

where λ determines the scale and γ determines the shape. Its survivorship function and hazard function are

$$S\ t\ = e^{-\ \lambda t\ ^{\gamma}}$$
$$h\ t\ = \lambda\gamma\ \ \lambda t\ ^{\gamma-1},$$

respectively.

We wanted to use a parametric analysis as opposed to a non-parametric analysis since a parametric model generally allows for more precise estimation. Preliminary tests on flea mortality data suggested that the Weibull distribution is flexible enough to fit various data sets with very different mortality patterns.

Data for four dishes run at the same time are used. Dishes within each data set are considered random effects. These random effects are incorporated into the model by adjusting the scale parameter. The proportion of fleas surviving at time *t* in dish *i* is

$$S\ t\ = e^{-\ \lambda_i^{*} t\ ^{\gamma}},$$

where $\lambda_i^{*} = \lambda \exp\ z_i$ and $z_i \sim N(0,\sigma^2)$. This allows for different survival curves for each dish. The survival curves for each dish should be the same, as each dish is a replicate of the same experiment. We test for this equality, the actual test being whether $\sigma^2 > 0$.

6. Comparisons to colony

A survival curve, using the Weibull model described above, was fit to colony data to establish the baseline reaction of susceptible fleas to different insecticides. Using this survival curve, the time required to kill all of the fleas was estimated. However, with a continuous distribution like the Weibull, the probability of survival, $S(t)$, never reaches 0 nor 1. So, we used the time at which the probability of survival reaches 0.5% as an estimate of the time it takes for an insecticide to kill all colony fleas tested. Call this time, t_0. The primary indicator of insecticide resistance is the proportion of fleas in the sample surviving past time t_0. From each sample, a survival curve is estimated. Additionally, based on the variability in the sample, a 95% upper bound for the proportion of fleas alive at t_0 is computed. This upper bound indicates how much the proportion of fleas alive at time t_0 in the sample could vary from 0.5% without suspicion that that the proportion of fleas alive at time t_0 in the population this sample represents differs from 0.5%. If the estimated survival curve crosses t_0 below this upper bound, then we conclude there is no evidence of resistance.

7. Comparisons of two samples

A survival curve, using the Weibull model described above, is fit to both samples. The time at which the probability of survival is 0.5% is computed from the estimated survival curves. Call these time points t_{10} and t_{20}. A 95% confidence bound for the difference in survival probabilities is computed at t_{10} and t_{20}. If both of these intervals contain 0, then the times it takes for the fleas to die off in the two samples are statistically the same.

C. RESULTS

1. Discriminating concentration

Insecticide discriminating concentrations that caused ≥ 95% mortality at 60 minutes of exposure for susceptible laboratory colony *X. cheopis* fleas were: DDT 65.0 µg (95% fiducial limits (95FL) = 63.5 – 355.3 µg) , lambda-cyhalothrin 2.0 µg (97 FL: 0.97 – 2.2 µg), malathion 400.0 µg (95FL: 212.8 – 419.3 µg), permethrin 2.5 µg (95FL: 1.6 - 2.7 µg), and deltamethrin 1.0 µg (95FL: 0.6 - 0.9 µg).

2. Field bioassay

Field immobilization of the fleas with halothane-impregnated strips was rapid, effective and no mortality was observed. Fleas recovered in 1-2 min after removal from the halothane.

Susceptible fleas exposed to 2µg of lambda-cyhalothrin were expected to experience 100% mortality at 46.5 minutes as per colony data. In the Lemo East population, 57.7% of the fleas were still alive at that point (Table 2.1), the Lagaya fleas had a 51.3% survival on the first plate assay (Table 2.1, Figure 2.2) and 69.8% on the second (Table 2.1). For the Ngai populations exposed to 2µg of lambda-cyhalothrin**,** the first assay had 39.4% survival (Table 2.1) and the second had 44.38% survival (Table 2.1). Ngai fleas exposed to 65µg of DDT should have been dead at 45.2 minutes based on assays done with susceptible colony fleas. The first assay indicated that 73.27% survived (Table 2.1) and the second assay showed 90.3% survived (Table 2.1).

Table 2.1. Comparison of LT_{50}, LT_{95}, and mortality of *Xenopsylla cheopis* between logistic regression and Weibull analysis. LT= lethal time in minutes, RR=resistance ratio

Icon		Logistic regression				Weibull			
Strain	Final Mortality	LT50	LT95	RR50	RR95	LT50	LT95	RR50	RR95
Colony	1.00	24	47			25	46		
Lagaya	0.90	9	68	0.4	1.45	58	138	2.32	3.00
Lemo	0.75	17	214	0.7	4.55	55	204	2.20	4.43
Ngai 1	0.85	20	142	0.8	3.02	39	99	1.56	2.15
Ngai 2	0.83	16	201	0.7	4.28	43	98	1.72	2.13
DDT		Logistic regression				Weibull			
Strain	Final Mortality	LT50	LT95	RR50	RR95	LT50	LT95	RR50	RR95
Colony	1.00	32	60			21	38		
Ngai 1	0.53	40	1852	1.3	30.87	98	367	4.67	9.66
Ngai 2	0.52	45	1239	1.4	20.65	106	400	5.05	10.53

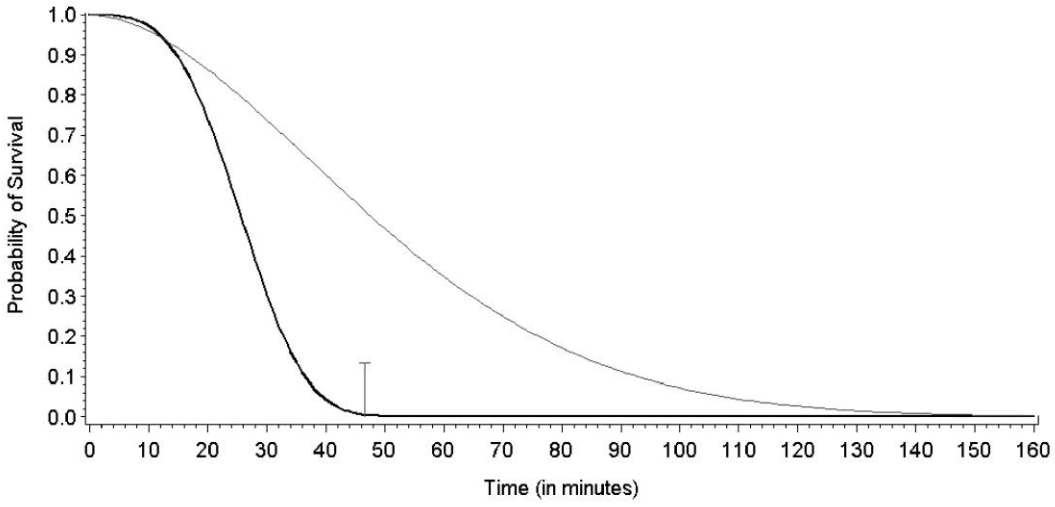

Figure 2.2. Weibull regression survival curve for the Lagaya population of *Xenopsylla cheopis* exposed to lambda-cyhalothrin.

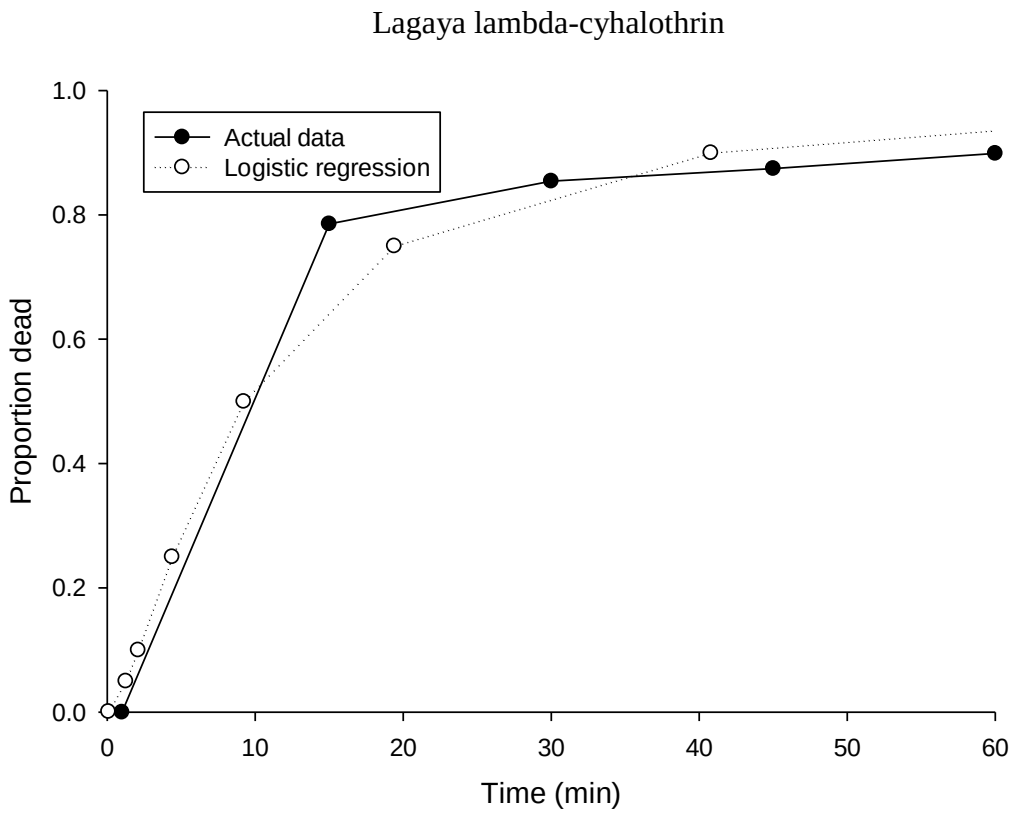

Figure 2.3. Logistic regression survival curve for Lagaya population exposed to lambda-cyhalothrin.

D. DISCUSSION

The inception phase of the bioassay depends on establishment of discriminating concentrations of toxicants resulting in mortality of the insects in a pre-determined period of time. The statistical program of logistic regression used to determine the discriminating dose fit the parameters of the test in that dose and assement time are a variable value, and these variables are independent of each other. Weibull analysis used in the field bioassay is applicable because time for mortality functions as a dependent variable. Readings are taken at predefined time points rather than a continuous recording of exact times of death. Mortality may never occur in some insects and therefore a logrithmic extrapolation of time to death would not apply.

An integral part of any vector control plan is regular surveillance for the development of insecticide resistance in the field (Nauen, 2007; Soderlund & Knipple, 2003). Outbreaks of plague could be considerably more difficult to suppress if vector fleas have developed resistance through exposure to the insecticides being used in IRS programs targeting malaria vectors or other insecticide use. Prior to this study, assesments of insecticide resistance in *X. cheopis* used the standard WHO assay (Gratz, 1977) although several modified methodologies have been utilized for similar purposes in analyzing *C.felis* (Bossard et al, 1998).

The Petri dish assay described in this paper as a modification of the bottle bioassay (Brogdon & McAllister, 1998b) makes field surveillance for insecticide resistance in fleas more rapid and feasible, especially in developing countries. By design, the flat surface of the dish ensures constant contact with the toxicant. Initial investment expenditures are relatively small, requiring only Petri dishes, acetone, and reagent grade insecticides. The dishes can be cleaned and reused making them an economical testing medium. Users need to make their own determination of discriminating doses as susceptibility can vary with environmental and flea-related factors (Bossard et al, 1998). Indeed, glass does not mimic the field environment of rat fleas and may therefore be inaccurate but the phenotypic expression in the local flea population will remain relative (Bossard, 1997).

The village of Lemo East had a history of IRS spraying lambda-cyhalothrin in 2008 and alpha-cypermethrin in 2009. Nearly 50% of the fleas tested from Lemo East demonstrated resistance to lambda-cyhalothrin. Lagaya had only been sprayed with alpha-cypermethrin in 2009 and the plate assays with lambda-cyhalothrin averaged

60.6% resistance in the fleas. The fleas in Ngai had the potential to be exposed to lambda-cyhalothrin and DDT in 2008. Spraying with alpha-cypermethrin was taking place as this study was conducted. An average of 41.9% fleas were resistant to lambda-cyhalothrin and 81.8% were resistant to DDT. The increased expression of resistance to DDT as compared to lambda-cyhalothrin could be the result of a number of factors: the increased persistance of DDT means more consistent contact with the toxicant and therefore more selective pressure on the flea. DDT was used in Uganda from 1950s to 1970s to control cotton pests, and in 1959-60 a WHO sponsored DDT IRS pilot program to control malaria in western Uganda was launched (Zaramba, 2005). The program was terminated in 1963 for unclear reasons, but lack of resources and the end of the WHO malaria reductin campaign were thought to be contributory factors (Zaramba, 2005). The chemical structures of DDT and pyrethroids suggests possible differences in toxicant uptake and metabolic mechanisms in the fleas despite the similar target site for the insecticides (Brogdon & McAllister, 1998a). Further work will need to be done to elucidate this phenomenon and determine if cross resistance or multiple resistance are occurring.

The levels of insecticide resistance that *X. cheopis* fleas expressed in northern Uganda ranged from 51.3% to 90.3% clearly indicating the development of resistance to insecticides that target the voltage-gated sodium channel (DDT and pyrethroids), at levels greater than the 50% resistance level assumed to represent a reasonable threshold for a compound to be considered ineffective (Curtis, 1985). With a spraying history in Lagaya village that only occurred in 2008, it would seem that the fleas have developed knockdown resistance but increased expression of metabolic detoxification enzymes

could also exist or, the reverse scenario. More investigations are needed to identify the possible resistance mechanisms that have evolved. In the meantime, the Petri dish assay can be used for field assessments of resistance in fleas in plague endemic areas and can be a useful tool in planning a vector control program.

.

III. BIOCHEMICAL DETECTION OF INSECTICIDE RESISTANCE MECHANISMS EXPRESSED IN THE ORIENTAL RAT FLEA, *XENOPSYLLA CHEOPIS (*ROTHSCHILD) IN NORTHERN UGANDA, AFRICA

A. INTRODUCTION

Evolution of insecticide resistance by arthropod disease vectors is a well-recognized possible sequelae to vector control programs worldwide, and fleas are no exception (Gratz, 1977). Information from the Arthropod Pesticide Resistance Database maintained at Michigan state University (www.pesticideresistance.org) indicates that *Ctenocephalides felis* (Bouché)(the cat flea) has evolved resistance to 13 insecticides in 11 countries and *Xenopsylla cheopis* (Rothschild) (the Oriental rat flea) has resistance reported to 7 insecticides in 14 countries. While *C. felis* is primarily characterized as a biting nuisance and a pest of domestic animals, *X. cheopis* is one of the most efficient, and therefore important, vectors of *Yersinia pestis* (Yersin), the causative organism of plague (Chanteau et al, 1998; Eisen et al, 2007b; Gage, 2005; Gage & Kosoy, 2006; Gage & Kosoy, 2005; Gratz, 1999; Lorange et al, 2005; Mullen & Durden, 2009; Piesman & Gage, 2000; Renapurkar, 1990; Shyamal et al, 2008).

Metabolic resistance is one of the main mechanisms insects utilize to circumvent the toxic effects of insecticides. According to Hemingway and Ranson (2000) metabolic detoxification is the most common mode of insecticide resistance in insects. Enhanced detoxification resistance involves groups or multigene families of enzymes that have the abilities to interact with xenobiotics and reduce or eliminate their potentially lethal effects. The main enzyme families involved are the cytochrome P-450 dependent monooxygenases (P450s), the esterases, and the glutathione S-transferases (GSTs) (Hemingway & Ranson, 2005). The monooxygenases are a large family of enzymes and resistance is due to enhanced oxidative metabolism of the insecticide (Plimmer, 2003). The P450s catalyze a wide variety of reactions in which a hydroxyl group is added to the

diphenyl ether moiety of insecticides and the product is more easily conjugated with a carbohydrate for enhanced excretion (Plimmer, 2003). Due to their genetic diversity, broad substrate specificity and catalytic versatility, P450s and associated reductases are the only metabolic mechanisms conferring resistance to all classes of insecticides although they primarily confer resistance to pyrethroids and carbamates (Li et al, 2007; Yu, 2008).

Esterases act by targeting ester bonds in the insecticides and subsequently hydrolyze, or cleave, the ester bonds. Some esterases are known to increase activity or sequestration through gene duplication leading to increased amounts of transcripts and eventually protein. Resistance to organophosphates, and to a lesser extent carbamates and pyrethroids, has been associated with this mechanism (Hemingway & Ranson, 2005).

Glutathione S-transferases (GSTs) act by conjugations and dehydrochlorination of the insecticides. The GSTs are associated with resistance to organophosphates, DDT, and pyrethroids (Hemingway & Ranson, 2005).

An enzyme involved in enabling resistance to organophosphates and carbamates is insensitive acetylcholinesterase (AChE), the mechanism is not metabolic, but rather through target site alteration. Acetylcholinesterase is found in the nerve synapses where it is targeted by organophosphate and carbamate insecticides. Organophosphates irreversibly phosphorylate amino acids in the active site of AChE. The phosphorylated AChE cannot hydrolyze acetylcholine (ACh) which leads to increased concentrations of ACh in the postsynaptic receptor (Plimmer, 2003). This leads to excess neuroexcitation in the insect. The reaction in the insect exposed to carbamates is reversible because decarbamylation of AChE is rapid (Yu, 2008). Resistance to organophosphates and

carbamates is associated with insensitive AChE. This is achieved by structural alterations in the target site of the AChE in the insect causing the enzyme to be less sensitive to inhibition by organophosphates and carbamates (Hemingway & Ranson, 2005).

Biochemical resistance assays detect the generic activity of insecticide metabolic enzymes. Many have been developed using a microplate-based spectrophotometric analysis (Brogdon, 1984; Brogdon, 1988; Brogdon & Barber, 1990), high-pressure liquid chromatography (Brogdon & Dickinson, 1983), dot-blot testing on single insects (Dary et al, 1991), and assorted variations on the central theme. Biochemical tests are rapid. Multiple assays can be performed on single insects to detect generic activity that may be correlated with bioassays.

A number of investigations have been performed on resistance mechanisms in *C. felis* (Bass et al, 2006; Bass et al, 2004a; Bass et al, 2004b; Hinkle et al, 1995; Ilg et al, 2010; Kambhampati et al, 1997) but very little has been done with *X. cheopis.* The current study was performed using microplate-based spectrophotometric analysis on laboratory raised and field caught *X. cheopis* fleas with a possible history of exposure to DDT and pyrethroids, primarily as a result of these insecticides being sprayed in dwellings as part of IRS programs targeting malaria vectors. These analyses may elucidate metabolic resistance mechanisms in non-malaria vector species of arthropods, which in turn aid in developing effective management policies for vectorborne diseases.

B. MATERIALS AND METHODS

1. Source of fleas and study areas.

Fleas used for baseline data were from a *X. cheopis* colony reared at the Centers for Disease Control and Prevention (CDC), Fort Collins, Colorado. The colony was started in the mid-1980s at the University of Maryland from fleas taken from rats trapped in the Baltimore area. The colony was later shipped to the Rocky Mountain Laboratories in Hamilton, Montana. In 1992 some of that colony was shipped to the CDC in Fort Collins.

Wild-caught *X. cheopis* were combed off of halothane-sedated *Rattus rattus* (L) (roof rat) that had been live-trapped in Tomahawk™ traps (Tomahawk, WI) from mud dwellings in various locations in northern Uganda (Fig.3.1). Study areas were chosen based on the history of recent insecticide use in IRS programs for malaria vector control. Orivu and Edivu, villages (Fig.3.1) had no reported history of spraying, and were used for collection of baseline wild-type flea data. Lemo East had been sprayed with the pyrethroid insecticides lambda-cyhalothrin in August – October 2008, and alpha cypermethrin in August- October 2009. Lagaya was sprayed with alpha-cypermethrin in October- November 2009. All collections were performed in March and April 2010. Colony fleas were immobilized on ice prior to use. The field collected fleas were frozen at -70°C, and maintained in a frozen state until laboratory analysis at CDC, Fort Collins.

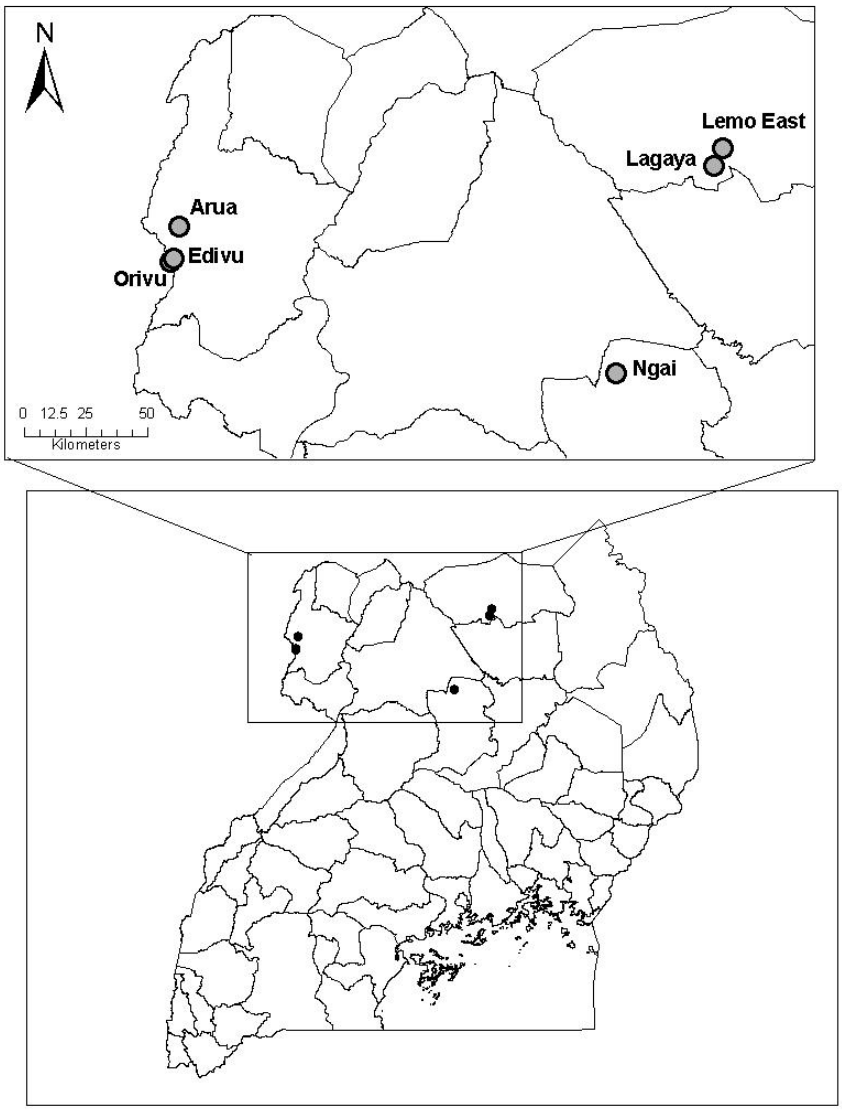

Figure 3.1. Map of Uganda, Africa indicating *Xenopsylla cheopis* collection sites.

2. Microplate assay.

Assays were run to detect non-specific α-esterase, non-specific β-esterase, mixed function oxidases (MFOs), glutathione-S-transferase (GST), and total protein. In addition, insensitive acetylcholinesterase (iAChE) was run to detect the AChE altered target site. Total protein is correlated with body mass was and used as a correction factor (Fonseca-Gonzalez et al, 2009b; Zayed et al, 2006). Protocols published previously by

Brogden (1984; 1988), Brogden and Barber (1990) and Brogden, McAllister et al.(1997) using mosquitoes were adapted for use on fleas by reducing the amount of sample and reagents. Spectrophotometric analysis was performed using a Spectramax M2® (Molecular Devices, Sunnyvale, CA).

Colony fleas were starved for at least 24 hours before the assays. Briefly, whole fleas were ground up with plastic pestles in 100µl of 1M KPO_4 buffer in 1.5ml plastic tubes and then diluted to 1ml with 900µl of additional buffer. Fifty µl of flea homogenate was used per well and tests were performed in triplicate using only half of the published amounts of reagents. The non-specific β-esterase assay uses β-napthyl acetate 5.6 mg/ml (β-napthyl acetate, acetone and KPO_4 buffer) as a substrate and dianisidine 1.0 mg/ml (tetrazotized O-dianisidine and water) in a well with a processed flea. This is incubated for 10 minutes and read at 540nm. Non-specific α-esterase levels are measured using α-napthyl acetate as a substrate (α-napthyl acetate, acetone and KPO_4 buffer) and dianisidine which have an identical incubation and wavelength filter as the β-esterase assay.

Mixed function oxidase reactions use TMBZ 5.0 mg/ml (tetramethyl-benzidine dihydrochloride), methanol, and 0.25M sodium acetate buffer with 3% hydrogen peroxide in the well with the flea homogenate. The reaction is incubated for 5 minutes and read with a 620nm filter.

The Glutathione S-Transferase assay uses reduced glutathione 6.1 mg/ml in KPO_4 buffer and cDNB 2.0 mg/ml (in acetone, and KPO_4 buffer) in the well with the flea homogenate. A reading is made immediately (T_0) and another after 5 minutes (T_5), both

with a 340nm filter. The analysis is based on subtracting the T_0 reading from the T_5 reading.

The insensitive acetylcholinesterase assay determines if an altered acetylcholine site is present, using a competitive assay. This is performed by mixing ATCH 7.5 mg/ml (acetylthiocholine iodide), propoxur 2.1 mg/ml (carbamate), acetone, KPO_4 buffer, and DTNB 1.3 mg/ml (dithio-bis-2-nitrobenzioc acid) in a well with the flea homogenate. If the propoxur binds to the AChE, no reaction with the ATCH can take place (i.e. normal AChE) and the colorometric result is of low intensity. Insensitive AChE will not allow the toxin to bind and therefore a reaction and resulting colorometric change will occur. One reading is made immediately (T_0) and another after 10 minutes (T_{10}) at 414nm. The statistical analysis is based on subtracting the T_0 reading from the T_{10} reading.

The protein assay measures the total amount of protein present using the Bradford method. This procedure uses a protein dye reagent (Bio-Rad Protein Assay, Bio Rad Inc., Hercules CA) and KPO_4 in the well with the flea and is read immediately.

3. Data analysis.

Fleas used in the assay were from 4 villages and 6 colony samples each containing 30 fleas. Forty-four fleas were analyzed from Edivu, 17 fleas from Lagaya, 8 from Lemo East, and 67 from Orivu.

All analyses were performed using Spotfire S+, version 8.1 (TIBCO Software Inc., Palo Alto, CA). To test whether or not the difference in mean enzyme levels was statistically different in fleas from exposed versus unexposed villages, data from the colony fleas was excluded. A generalized linear mixed-effects (GLME) model assuming a gamma distribution (all data values are positive) with an inverse link function was fit to

the wild flea data. Enzyme, exposure, and their interaction were included as fixed effects and flea and village were included as random effects. Diagnostics suggested that there was still some heteroscedasticity in variances among each enzyme and village groups combination, and the model was adjusted to account for this.

Enzyme measurements for the exposed fleas were adjusted by subtracting the terms associated with exposure from the linear component of the generalized linear mixed-effects model and taking the reciprocal. Next data from all the fleas, both wild type and colony bred, were fit to a second GLME, again assuming a gamma distribution and inverse link function. The response vector of enzyme expression consisted of observed enzyme expression for colony and unexposed, wild type fleas while we used predicted enzyme expression for exposed, wild type fleas. In predicting enzyme expression for the exposed fleas, a new source of uncertainty was introduced into the data. This was accounted for in the new model by incorporating a new variance component based on the predicted covariance matrix obtained from the first GLME. Multiple comparisons using Scheffe's method with an overall probability of a Type I error of 0.05 were used to test differences among main effects.

We were concerned that flea sedation by halothane might artificially stimulate increased mixed function oxidase production and therefore upwardly bias our results. The field process of using halothane to anesthetize fleas was reproduced on laboratory colony fleas and subsequent biochemical assays were run. There was no difference in the prior baseline data established (data not shown).

C. RESULTS

1. Microplate assays

Increased transcription and translation as a result of prior exposure to insecticide was considered *a priori* to be a factor in increasing enzyme activity in field collected fleas. The colony fleas had no previous exposure to insecticide. It is not certain whether the wild fleas had been previously exposed to insecticides, however, it was suspected that those trapped in Lagaya and in Lemo had been, as they were removed from rodents that had been trapped in dwellings that had been sprayed with pyrethroids as part of an IRS program to control malaria vectors.

Figure 3.2 illustrates the levels of α-esterase activity expressed by each of the five tested populations of *X. cheopis*. The colony, Edivu and Orivu fleas have similar profiles with low levels of activity. Lemo and Lagaya, villages with an IRS history, have significantly increased levels of expression. Similar profiles for expression of β-esterase are shown in Figure 3.3, with Lemo and Lagaya demonstrating increased levels of activity.

Figure 3.4 represents the levels of MFO activity expressed by the five populations. There is no clear pattern of activity that differentiates any population from another.

Figure 3.5 shows the iAChE absorbance differences in the same populations. The populations from the villages with no known IRS history, Orivu and Edivu, demonstrate an increased absorbance difference indicating an elevated level of iAChE. In comparison to colony and Lemo, the Lagaya fleas show a slight increase in iAChE.

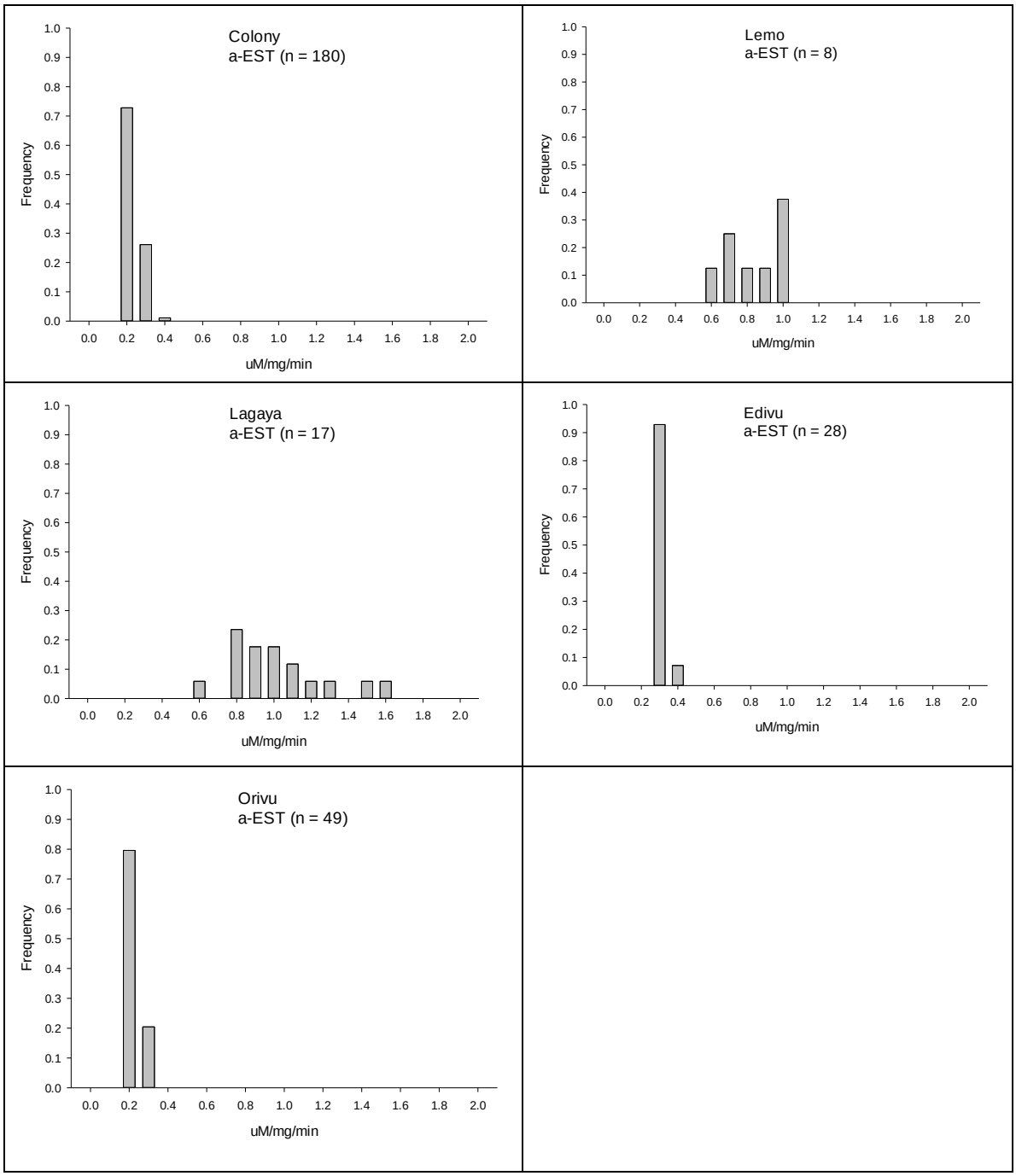

Figure 3.2. Distribution of α-esterase activity in five collections of *Xenopsylla cheopis.*

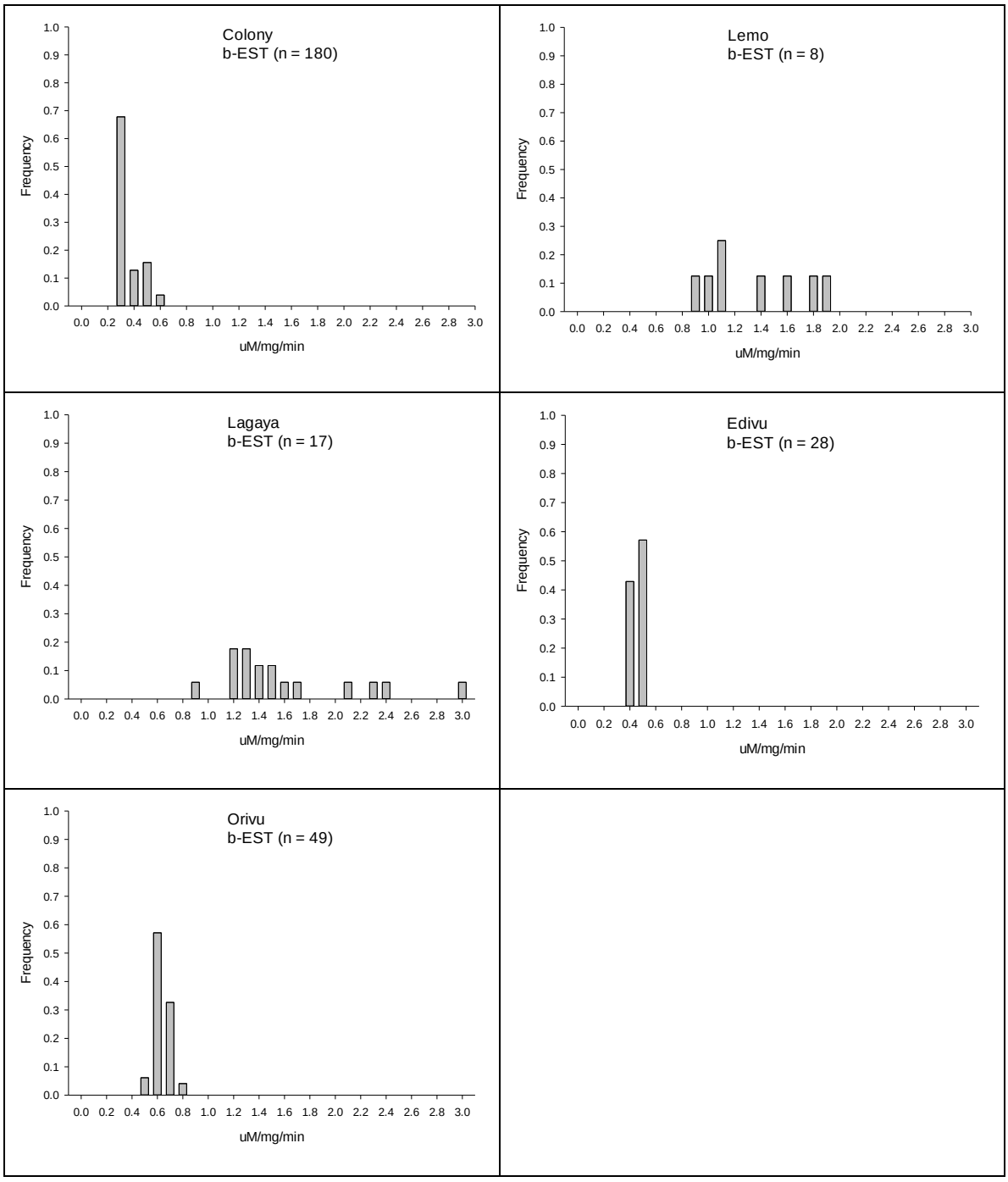

Figure 3.3. Distribution of β-esterase activity in five collections of *Xenopsylla cheopis.*

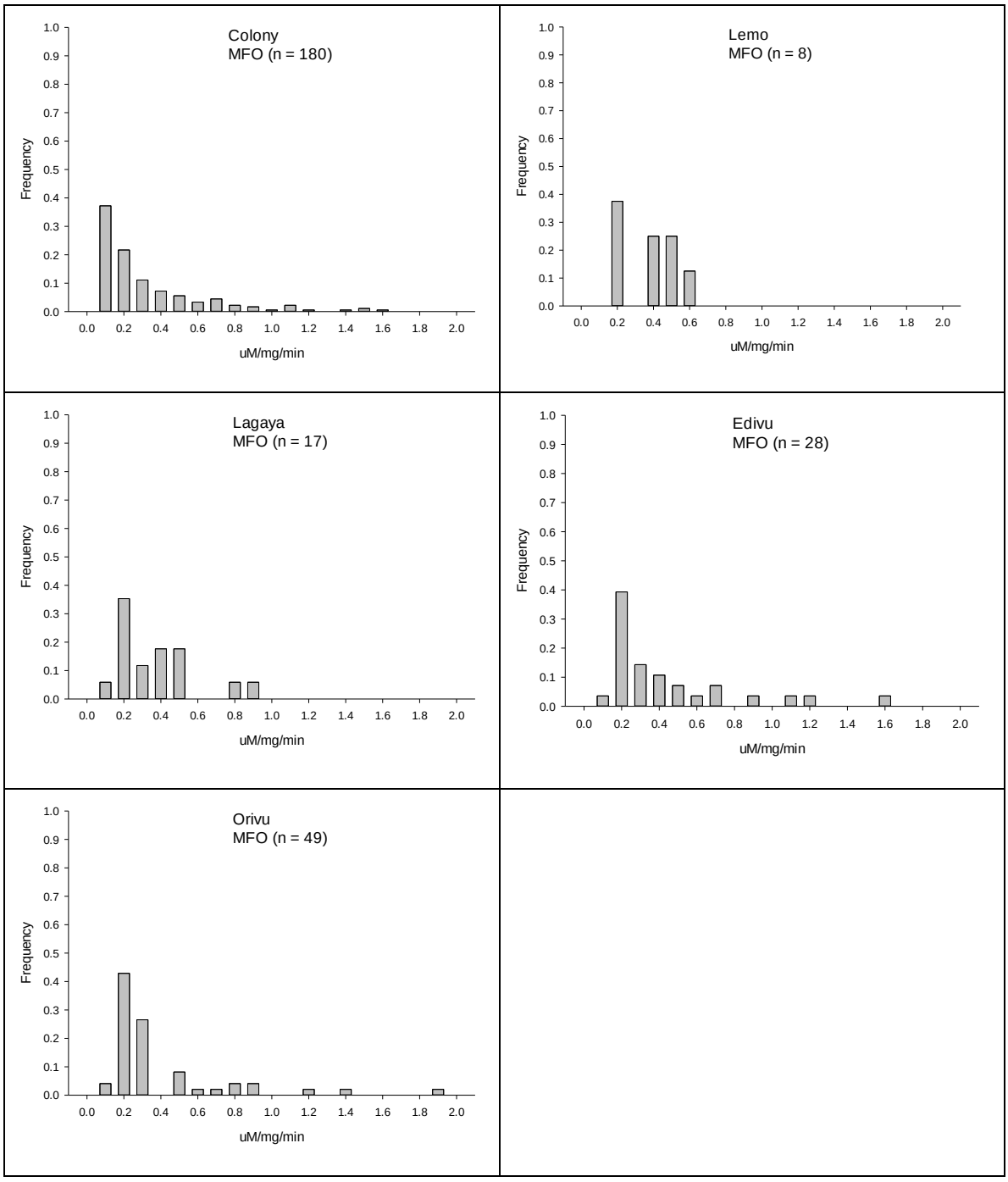

Figure 3.4. Distribution of MFO activity in five collections of *Xenopsylla cheopis*.

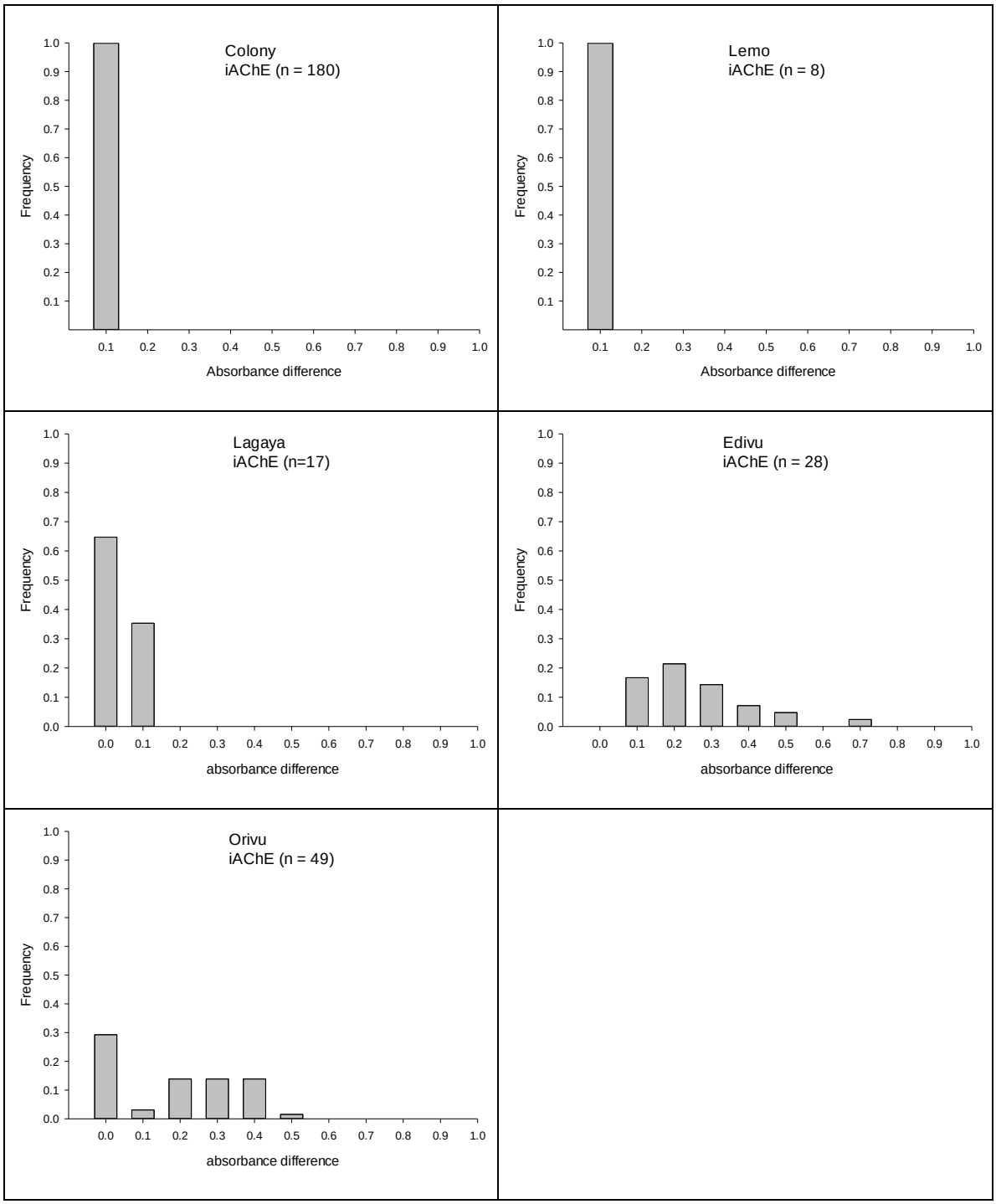

Figure 3.5. Distribution of iAChE absorbance differences in five collections of *Xenopsylla cheopis.*

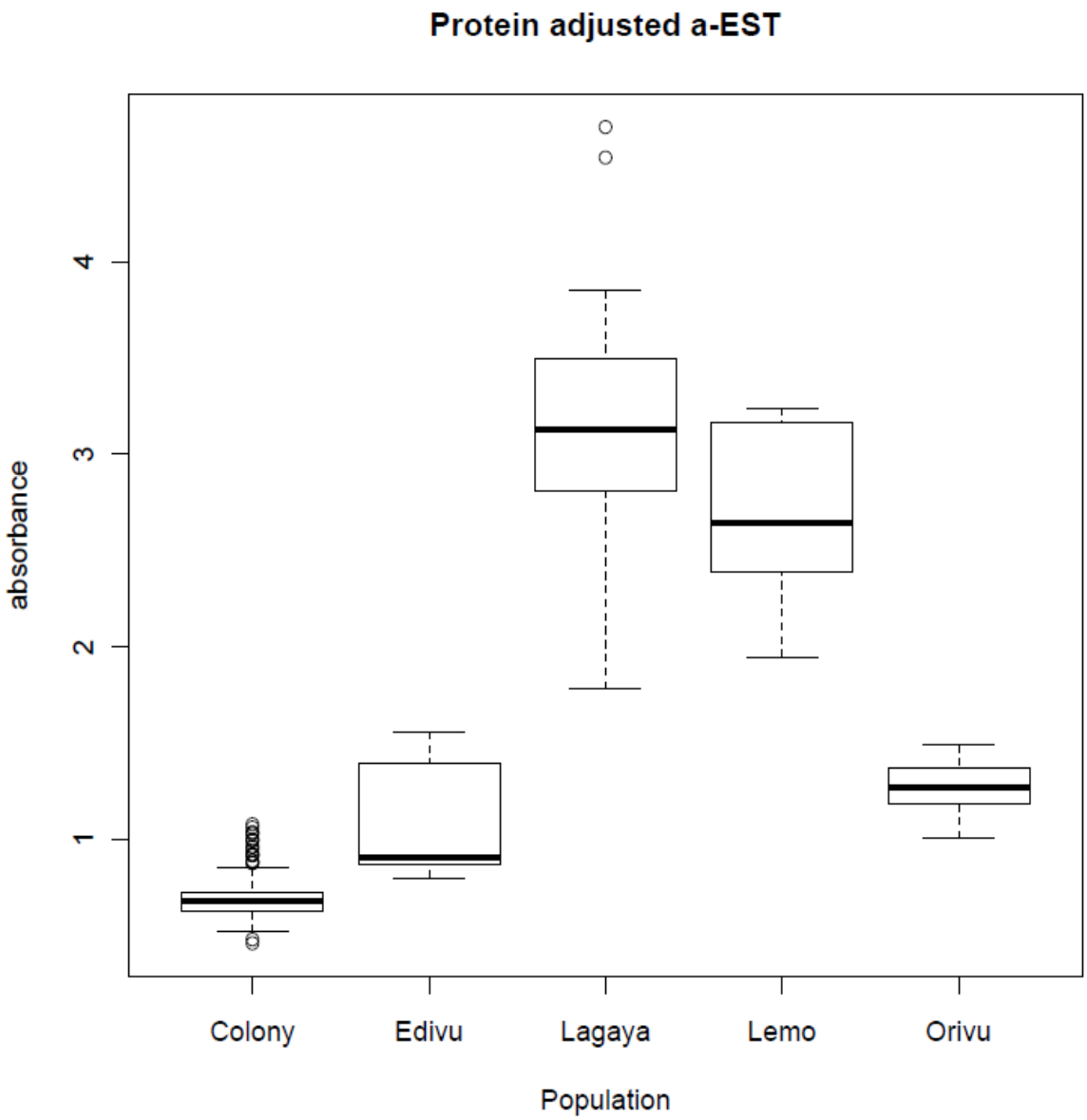

Figure 3.6. Box-and-whisker plot of protein adjusted α-esterase expression by five populations of *Xenopsylla cheopis.* Each single flea test is performed in triplicate, averaged, and divided by the protein value to adjust for body size variations. Open circles are values outside the 95% confidence interval (CI). The top and bottom bars are the maximum and minimum of the CI. Bar in the middle of the box is the 50th percentile (median) and the bottom and top of the box represent the 25th and 75th percentiles, respectively. The dashed vertical line is the whisker and is roughly 2 standard deviations above and below the mean.

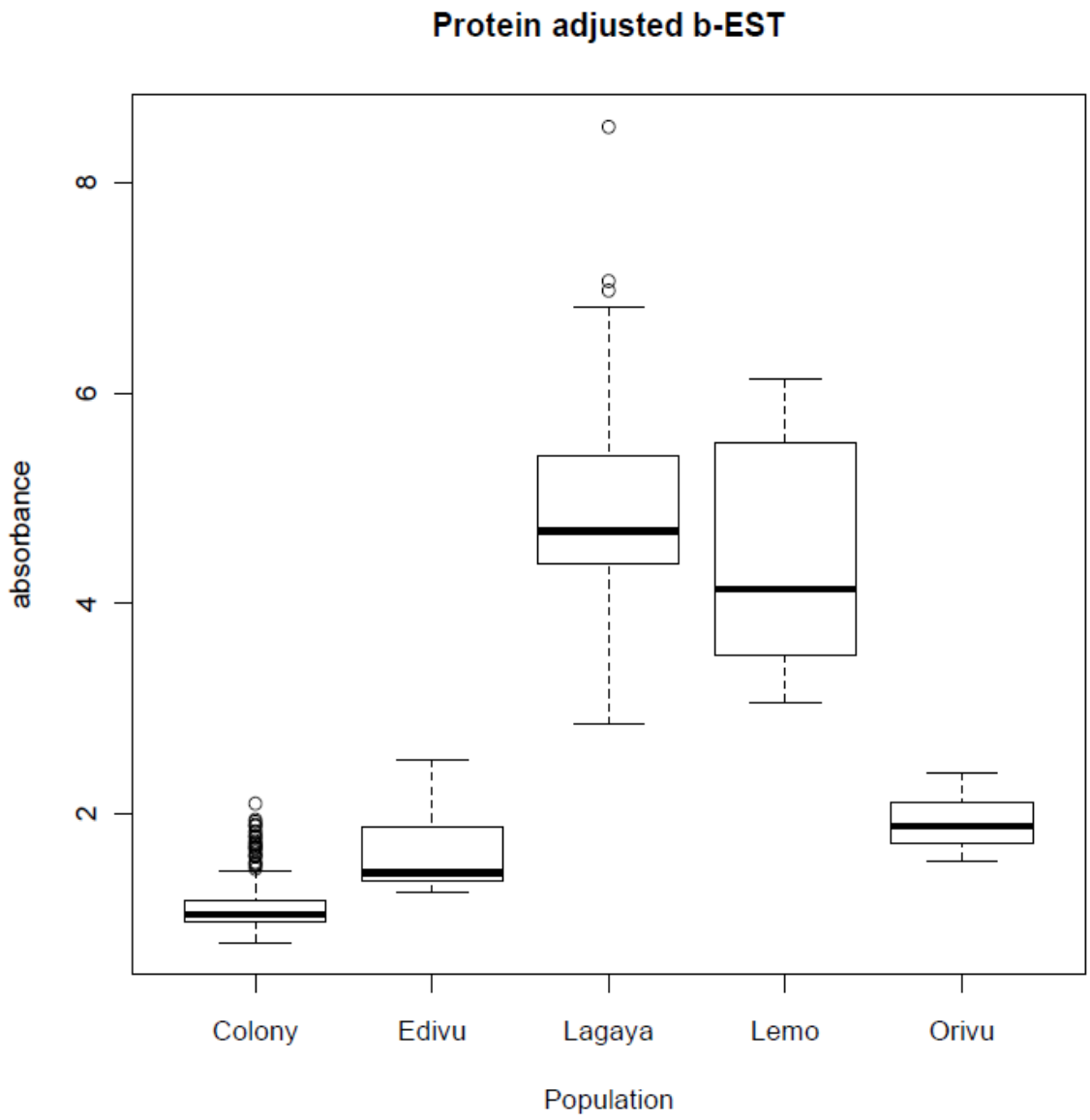

Figure 3.7. Box-and-whisker plot of protein adjusted β-esterase expression by five populations of *Xenopsylla cheopis.* Each single flea test is performed in triplicate, averaged, and divided by the protein value to adjust for body size variations. Open circles are values outside the 95% confidence interval (CI). The top and bottom bars are the maximum and minimum of the CI. Bar in the middle of the box is the 50th percentile (median) and the bottom and top of the box represent the 25th and 75th percentiles, respectively. The dashed vertical line is the whisker and is roughly 2 standard deviations above and below the mean.

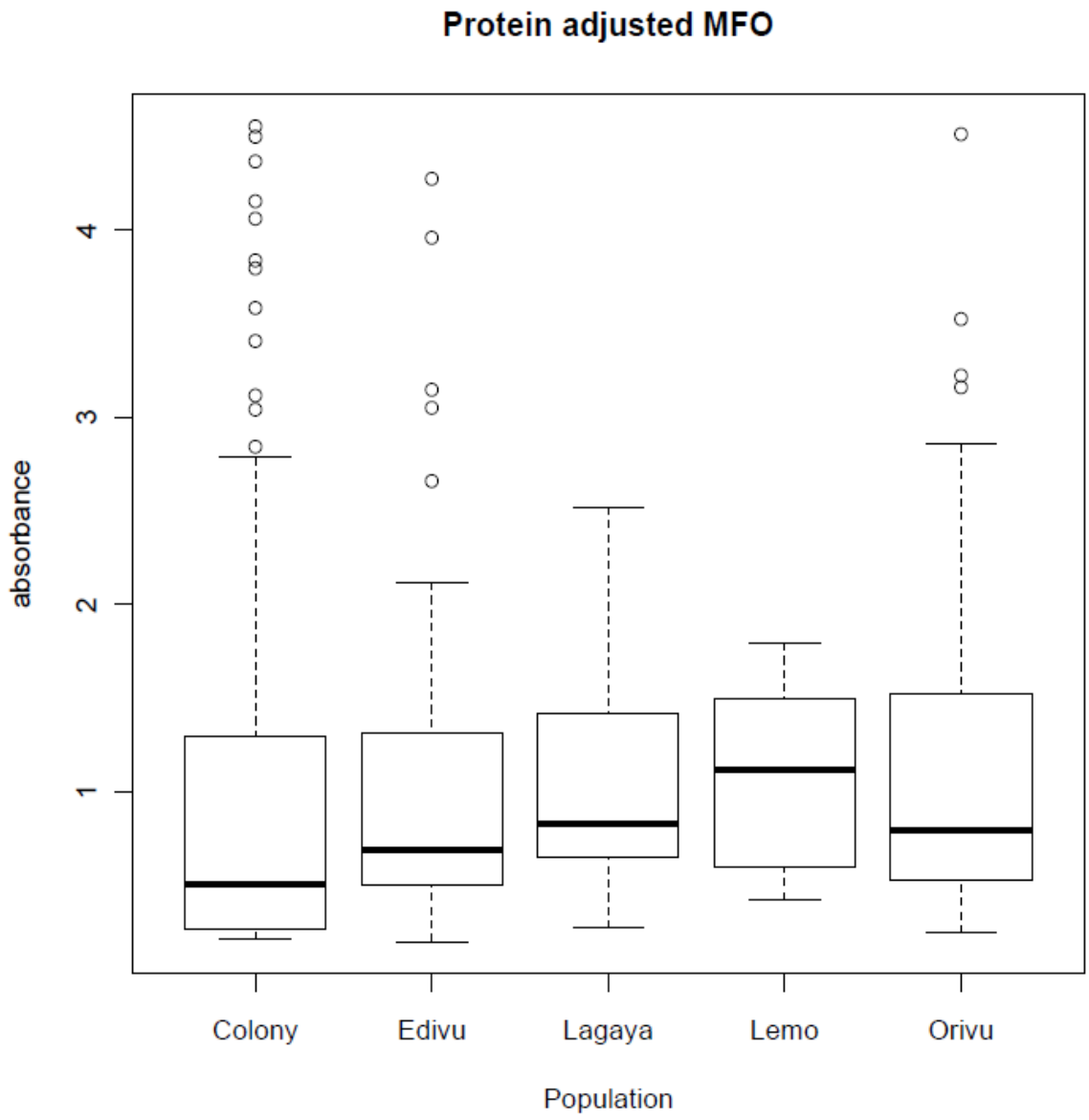

Figure 3.8. Box-and-whisker plot of protein adjusted mixed function oxidase (MFO) expression by five populations of *Xenopsylla cheopis.* Each single flea test is performed in triplicate, averaged, and divided by the protein value to adjust for body size variations. Open circles are values outside the 95% confidence interval (CI). The top and bottom bars are the maximum and minimum of the CI. Bar in the middle of the box is the 50th percentile (median) and the bottom and top of the box represent the 25th and 75th percentiles, respectively. The dashed vertical line is the whisker and is roughly 2 standard deviations above and below the mean.

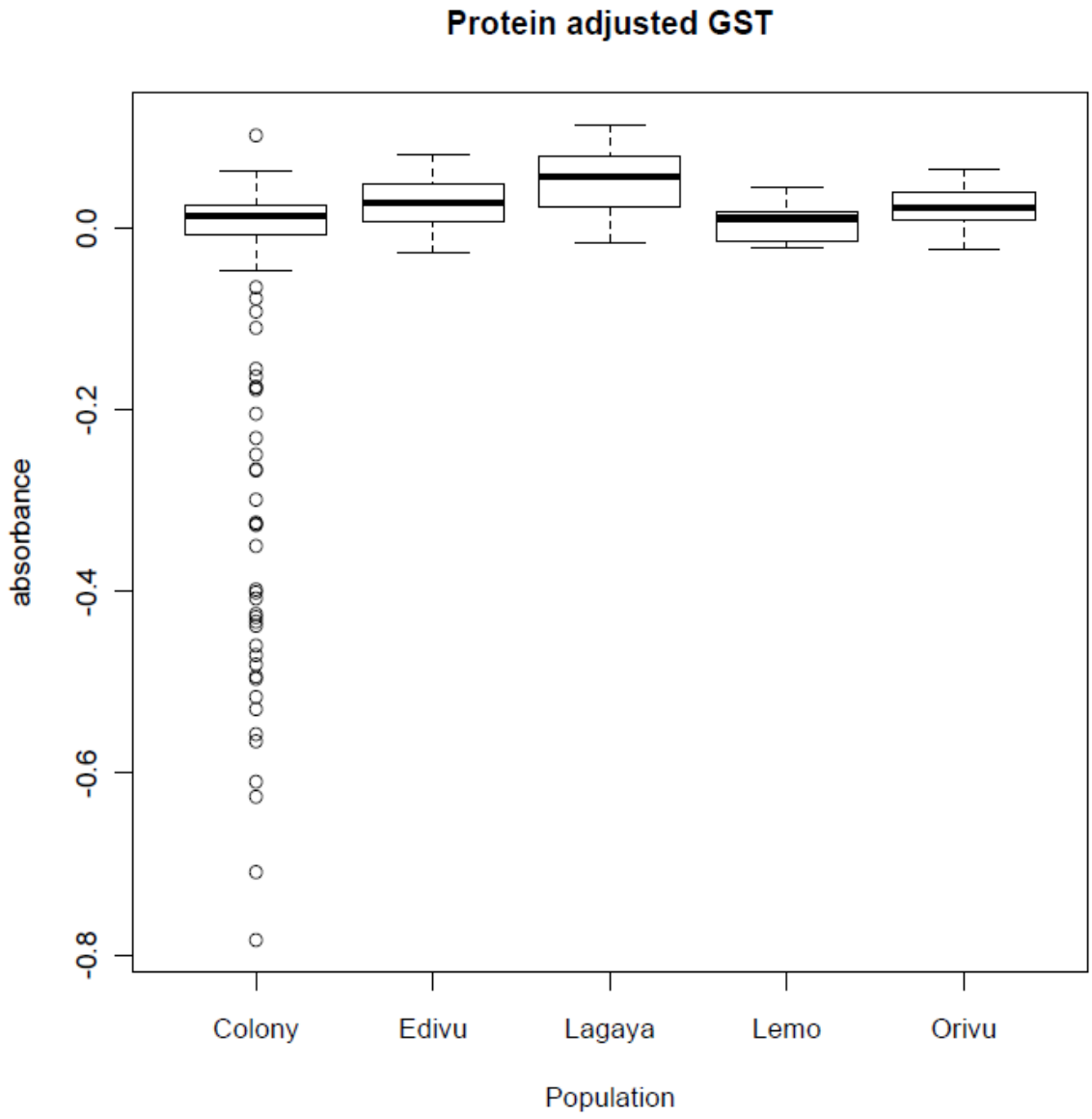

Figure 3.9. Box-and-whisker plot of protein adjusted glutathione S-transferase (GST) expression by five populations of *Xenopsylla cheopis.* Each single flea test is performed in triplicate, averaged, and divided by the protein value to adjust for body size variations. Open circles are values outside the 95% confidence interval (CI). The top and bottom bars are the maximum and minimum of the CI. Bar in the middle of the box is the 50th percentile (median) and the bottom and top of the box represent the 25th and 75th percentiles, respectively. The dashed vertical line is the whisker and is roughly 2 standard deviations above and below the mean.

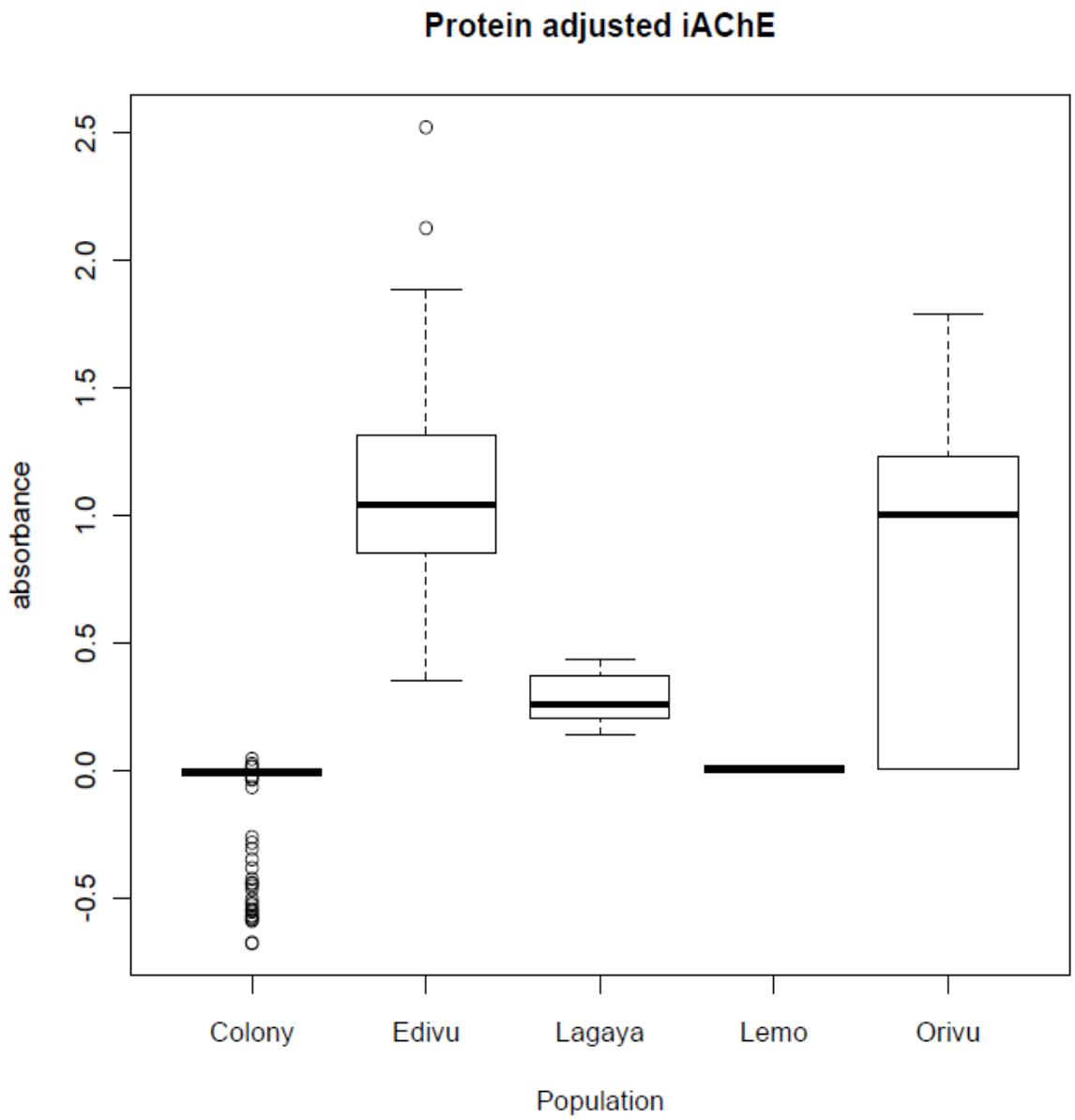

Figure 3.10. Box-and-whisker plot of protein adjusted insensitive acetylcholinesterase (iAChE) absorbance differences by five populations of *Xenopsylla cheopis.* Each single flea test is performed in triplicate, averaged, and divided by the protein value to adjust for body size variations. Open circles are values outside the 95% confidence interval (CI). The top and bottom bars are the maximum and minimum of the CI. Bar in the middle of the box is the 50th percentile (median) and the bottom and top of the box represent the 25th and 75th percentiles, respectively. The dashed vertical line is the whisker and is roughly 2 standard deviations above and below the mean.

Figures 3.6 through 3.10 are α-esterase, β-esterase, MFO, GST and iAChE box-and-whisker plot representations (respectively) of each of the enzymes analyzed with protein (equivalent to body mass) corrected for in each assay. Results indicate a significant increase in α and β-esterases in the Lagaya and Lemo populations, no differences in MFO or GST expression levels and increased presence of iAChE in Orivu and Edivu fleas.

Table 3.1 presents an analysis of variance (ANOVA) of the difference in means in each of the five enzyme analyses between the five different populations of *X. cheopis* tested. Results of the five population and enzyme comparisons show significant differences in α and β-esterase levels in the populations, no differences in the MFOs, field populations' expression of GST was different from the colony, and Edivu and Orivu were different from the colony, Lemo, Lagaya and each other in the iAChE assay of absorbance differences.

Figures 3.11a and b shows the group enzyme means for each level of each factor. For example, the vertical bar for the factor "enzyme" shows that β-esterase had the highest mean level of all the enzymes while the difference in glutathione-S-transferase had the smallest mean. Also, on average, field caught ("wild type") fleas have higher mean enzyme levels (approximately 0.34) than colony fleas (approximately 0.16). During exploratory data analysis we graphed group means for all data and group means for only the field caught fleas (Figure 3.11a-b, respectively). The long horizontal line is the grand mean. These figures suggest that the mean enzyme levels among the fleas potentially exposed to insecticide are higher than the mean enzyme levels among the unexposed fleas.

Table 3.1 Table of analysis of variance (ANOVA) of the difference in means in each of the five enzyme analyses performed comparing five populations of *Xenopsylla cheopis.* aEST is α-esterase, bEST is β-esterase, MFO is multi function oxidases, iAChE is insensitive acetylcholinesterase, and GST is glutathione S-transferase. Lemo, Lagaya, Orivu, and Edivu are villages in Uganda, Africa. Lemo and Lagaya have a history of indoor residual spraying (IRS) and Orivu and Edivu do not. Colony are laboratory fleas.

			Estimate	Std.Err	t	Pr(>|t|)		
aEST		(Intercept)	1.78676	0.02194	81.428	2.00E-16	***	yes
C1	Are the field populations different from the colony?	Pop1	1.08862	0.02563	42.47	2.00E-16	***	yes
C2	Are Edivu and Orivu different from Lagaya and Lemo?	Pop2	-0.89382	0.02709	-32.99	2.00E-16	***	yes
C3	Are Lagaya and Lemo different ?	Pop3	0.25443	0.0492	5.172	4.19E-07	***	yes
C4	Are Edivu and Orivu different?	Pop4	0.09844	0.02272	4.334	1.99E-05	***	yes
C5	Are the Edivu and Orivu different from the colony?	Pop5	0.3113	0.01896	16.421	2.00E-16	***	yes
bEST		(Intercept)	2.85223	0.04389	64.982	2.00E-16	***	yes
C1	Are the field populations different from the colony?	Pop1	1.69823	0.05127	33.121	2.00E-16	***	yes
C2	Are Edivu and Orivu different from Lagaya and Lemo?	Pop2	-1.51043	0.05419	-27.87	2.00E-16	***	yes
C3	Are Lagaya and Lemo different ?	Pop3	0.34509	0.09841	3.507	0.000521	***	yes
C4	Are Edivu and Orivu different?	Pop4	0.14936	0.04544	3.287	0.00113	**	yes
C5	Are the Edivu and Orivu different from the colony?	Pop5	0.40824	0.03792	10.766	2.00E-16	***	yes
MFO		(Intercept)	1.081392	0.091464	11.823	<2e-16	***	yes
C1	Are the field populations different from the colony?	Pop1	0.109554	0.106844	1.025	0.306		no
C2	Are Edivu and Orivu different from Lagaya and Lemo?	Pop2	0.030591	0.112932	0.271	0.787		no
C3	Are Lagaya and Lemo different ?	Pop3	-0.00133	0.205058	-0.006	0.995		no
C4	Are Edivu and Orivu different?	Pop4	0.026777	0.094686	0.283	0.778		no
C5	Are the Edivu and Orivu different from the colony?	Pop5	0.111688	0.079018	1.413	0.159		no
iAChE		(Intercept)	0.43066	0.033	13.049	2.00E-16	***	yes
C1	Are the field populations different from the colony?	Pop1	0.49799	0.03855	12.917	2.00E-16	***	yes
C2	Are Edivu and Orivu different from Lagaya and Lemo?	Pop2	0.41125	0.04075	10.092	2.00E-16	***	yes
C3	Are Lagaya and Lemo different ?	Pop3	0.13617	0.07399	1.84	0.0667	.	no
C4	Are Edivu and Orivu different?	Pop4	-0.14666	0.03416	-4.293	2.37E-05	***	yes
C5	Are the Edivu and Orivu different from the colony?	Pop5	0.68916	0.02851	24.171	2.00E-16	***	yes
GST		(Intercept)	0.009202	0.013233	0.695	0.487		no
C1	Are the field populations different from the colony?	Pop1	0.073199	0.015458	4.735	3.35E-06	***	yes
C2	Are Edivu and Orivu different from Lagaya and Lemo?	Pop2	-0.00179	0.016339	-0.11	0.913		no
C3	Are Lagaya and Lemo different ?	Pop3	0.02258	0.029668	0.761	0.447		no
C4	Are Edivu and Orivu different?	Pop4	-0.00231	0.013699	-0.169	0.866		no
C5	Are the Edivu and Orivu different from the colony?	Pop5	0.059806	0.011432	5.231	3.12E-07	***	yes

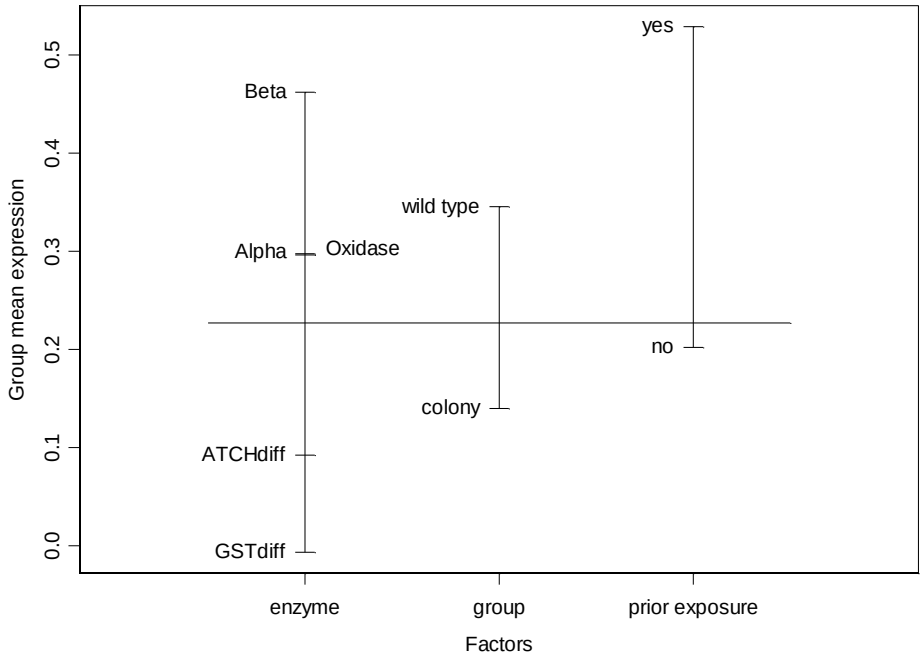

Fig. 3.11a. Group mean enzyme expression levels comparing colony and field caught *Xenopsylla cheopis* fleas and fleas potentially exposed to indoor residual spraying insecticides or not. "Wild type" means field caught. Long horizontal line is the grand mean.

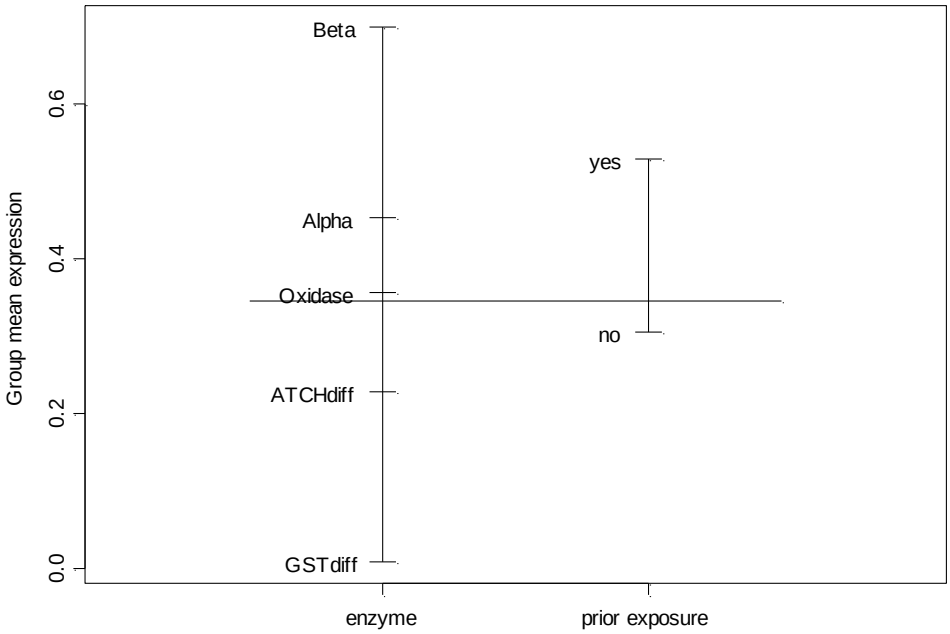

Fig. 3.11b. Group mean enzyme expression levels for only field caught *Xenopsylla cheopis* fleas, potentially exposed or unexposed to indoor residual spraying insecticides.

To test whether or not the differences in mean enzyme levels were statistically different in potentially exposed versus unexposed fleas, the data from colony fleas was excluded. This was done so that any effect of prior insecticide exposure would not be confounded with a colony/field caught flea effect. The data were assumed to follow a gamma distribution.

The results of the above analysis indicate that the interaction between enzyme and exposure is highly significant ($p<0.0001$). This can be observed in Figure 3.12 which shows that the relationship between potentially exposed fleas and unexposed fleas is not consistent across all enzymes but changes depending upon the enzyme. Multiple comparisons using Scheffe's method with an overall probability of a Type I error of 0.05 indicates that the mean α-esterase and β-esterase measurements were statistically higher for potentially exposed fleas than for unexposed fleas. While the mean difference the presence of AChE mutations was statistically lower for potentially exposed fleas than for unexposed fleas. Enzyme measurements for the potentially exposed fleas were adjusted by subtracting the terms associated with exposure from the linear component of the GLME and taking the reciprocal.

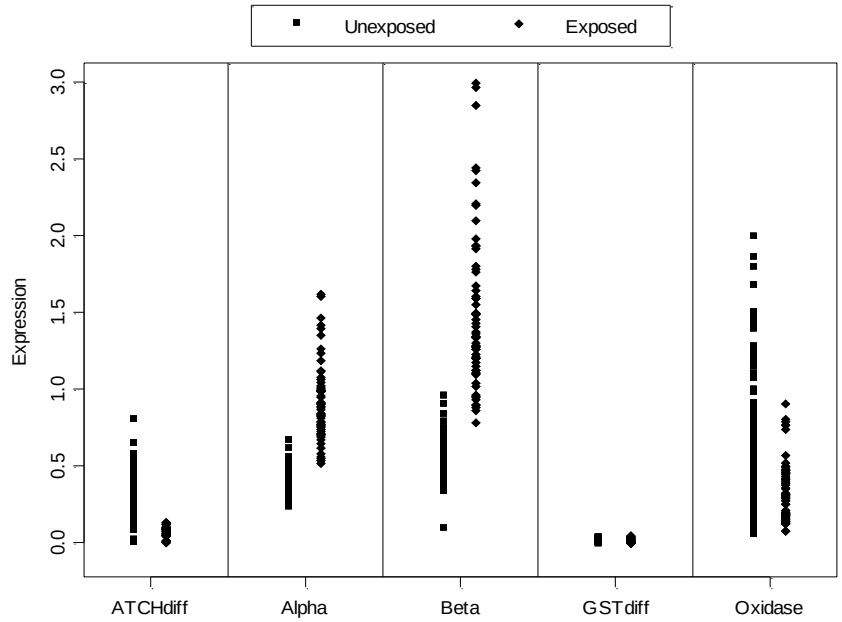

Figure 3.12. Comparison of *Xenopsylla cheopis* enzyme activity levels. Each point is an individual flea that was either potentially exposed to insecticides or not based on trapping location being in a dwelling with indoor residual spraying or not.

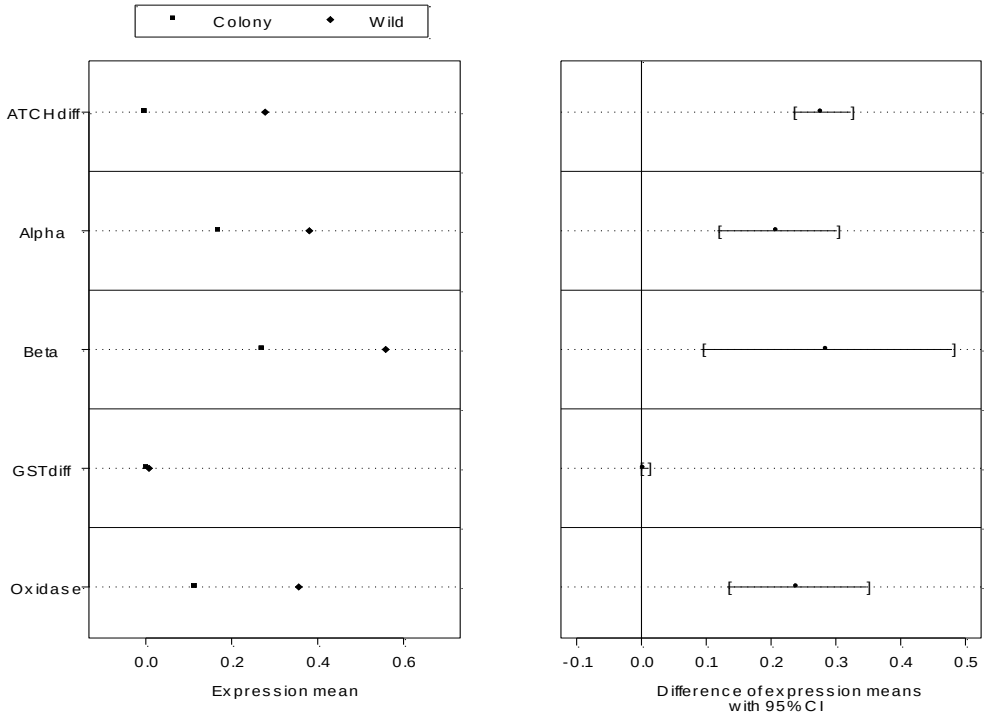

Figure 3.13. Comparison of mean levels of expression for each enzyme between colony and field caught *Xenopsylla cheopis* fleas (left side) and the amount of differences (wild minus colony) in those means (right side). The • on the right figure is the mean. The brackets and line on each side of the dot are the joint 95% confidence interval. "Wild" means field caught fleas.

The main results of the second analysis are shown in Figure 3.13. The left-hand side of Figure 3.13 shows the mean measurements for the field caught fleas and for the colony fleas for each of the enzymes. For all enzymes, the mean level is higher in the field caught fleas than in the colony fleas, although the GST amount is negligible. The right-hand side of Figure 3.13 shows the difference (field caught minus colony) in mean enzyme levels for each enzyme (indicated by the ●). The [--------] symbols represent the joint 95% confidence intervals for the differences using Scheffe's method. In joint 95% confidence intervals all assays together meet the 95% confidence interval. If a confidence interval crosses 0, then 0 is a plausible value for the difference in means for that enzyme. If a confidence interval does not cross 0, then there is little evidence that the mean levels for field caught fleas and colony fleas are equal. The right-hand side of Figure 3.13 shows that only the confidence interval for the difference in GST over time crosses 0; all of the other intervals are greater than 0. So, statistically the mean enzyme levels of field caught fleas are higher than the mean enzyme levels for colony fleas for the other 5 enzymes.

D. DISCUSSION

Monitoring of resistance to pesticides in vectors is an important component of vector control programs (WHOPES, 2006). Choice of insecticides should be based on susceptibility bioassay using a suitable array of methodologies including biochemical assays (Brooke, 2008). Biochemical assays measure the expression levels of enzymes possibly involved in the metabolism of insecticides by comparing field caught populations to known susceptible ones.(Brogdon, 1988; Brogdon & Barber, 1990). Thus,

enzyme-based resistance can be expressed as the proportion of adults sampled with higher enzyme activities than those of the susceptible population (Zayed et al, 2006).

When the average levels of expression of all enzymes are compared between the colony and field caught, the field caught Ugandan flea levels are over 2 times higher than the colony (0.34 vs 0.16) which meets the primary criterion for resistance. When analyzing Ugandan fleas alone, the mean enzyme levels were significantly higher in the fleas collected from dwellings with IRS history than those from dwellings with no spraying history (3.1 versus 4.9) which also strongly suggests a metabolic shift in response to potential exposure to the insecticides.

Enzymes with the highest mean expression in all populations were α-esterase and β-esterase and the lowest was GSTs. Fleas from areas with IRS use also had significantly higher mean expression levels of α-esterase and β-esterases when compared to the areas with no IRS, as did the field caught compared to the colony. Ugandan fleas were combed off rodents trapped in homes that had been sprayed with a pyrethroid. Although esterases are commonly associated with resistance to organophosphates and carbamates, and to a lesser extent, pyrethroids (Hemingway et al, 2004; Hemingway & Karunaratne, 1998; Yu, 2008), recent researchers working with *Anopheles* mosquitoes have found high levels of esterases associated with resistance to permethrin lambda-cyhalothrin and DDT. (Flores et al, 2005; Flores et al, 2006; Fonseca-Gonzalez et al, 2009a; Hamdan et al, 2005; Paeporn et al, 2004; Vulule et al, 1999). Zayed et al. (2006) observed an association between high esterases and pyrethroid resistance in populations of *Culex pipiens* (L) in Egypt. The increased esterase levels in the fleas field caught from IRS villages strongly suggest metabolic detoxification responses as a mechanism.

Not surprisingly, the Ugandan *X. cheopis* with no known history of exposure to DDT lacked elevated GST activity. Overexpression of GSTs is primarily associated with DDT resistance (Hemingway & Ranson, 2000; Yu, 2008). Zayed et al.(2006), though, noted a lack of GST activity in the face of high DDT resistance indicating knockdown resistance (Polson et al, 2010). DDT and pyrethroids act on the voltage-gated sodium channels in the nerve membrane and *kdr* is caused by a reduction in sensitivity at the target site to those insecticides (Davies et al, 2008). This may well be the case in many situations of insecticide resistance, and prior assessments that metabolic mechanisms are responsible for resistance may well be based on evidence obtained before sensitive molecular tools became routinely available to test for target site insensitivity or there could be multiple resistance. Further investigations using molecular techniques to determine the resistance mechanism should be performed.

The mean expression of AChE was higher for the field caught fleas than for the colony fleas, indicating an increased level of insensitive acetylcholinesterase in the field. Surprisingly, within the filed caught, the non-IRS villages' fleas had statistically higher AChE means. Organophosphates and carbamates target acetylcholinesterase (Hemingway & Ranson, 2000; Yu, 2008), and although the Ugandan fleas had no known exposure to these toxicants, they were not assessed for OP and carbamate resistance using our plate bioassay.

The MFO levels were moderate throughout all populations. Field caught fleas had higher levels than did the colony fleas, but the non-IRS village populations had a higher mean MFO levels than the exposed fleas. Accurate interpretation of this is difficult as the disparity could be a result of the feeding status of the fleas, rather than a response to

selective pressures. Colony fleas were collected 24 to 48 hours post bloodfeeding, but the field caught fleas were tested within 2 hours of collection off a rodent, so a bloodmeal was most likely still in the field caught insect's body as clearance takes days (Woods et al, 2009). Heme is a major component of red blood cells and is composed largely of cytochromes and peroxidases. The degradation of heme is, in large part, facilitated by heme oxygenase, an enzyme of the oxidoreductase class that catalyzes the cleavage of heme to form biliverdin (Dorland, 1957). This enzyme is also part of the family that includes the MFOs associated with resistance (Brogdon et al, 1997). The release of these components when rupturing the insect's body for the microplate assay could subsequently artificially elevate the amounts of MFOs detected by the assay.

Insecticide resistance surveillance is an integral aspect of vector management and enzyme-based assays should be included in the plan to characterize and understand the mechanisms insect vectors develop to circumvent toxic effects of chemicals. Our data provides *prima facie* evidence of metabolic resistance mechanisms present in populations of *X. cheopis* in northwestern Uganda near plague endemic areas. If the same chemicals used for malaria control are used for flea control during an epizootic, failure is a possible outcome of the efforts with, perhaps, disastrous consequences. Further work is needed to characterize the mechanisms of resistance on a molecular genetic basis.

IV. EVOLUTION OF *KDR* IN UGANDAN *XENOPSYLLA CHEOPIS* (ROTHSCHILD)

A. Introduction

Dichlorodiphenyltrichloroethane (DDT) and pyrethroids have a rich history in insect vector control. Ongoing indoor residual spraying (IRS) programs in Africa still utilize these chemicals and remain, with pyrethroid-treated bed nets, as the primary tools of Global Malaria Control strategy (Raghavendra et al, 2011). The target of both of these classes of insecticides is the insect's nervous system, where the insecticide binds to the voltage-gated sodium channel, leading to a delay in the closing of the channel, which causes excess neuroexcitation, tremors, rigid paralysis and death (Lund & Narahashi, 1983). Repeated primary and secondary (non-target species) exposure to pyrethroids and DDT have resulted in evidence of resistance in many species of insects (Curtis, 1985; Soderlund & Knipple, 2003; Tabashnik, 1989). Because insecticides with this mode of action are very effective, detection of resistance is imperative for developing effective vector control programs.

A well-known mechanism of resistance to DDT and pyrethroids is characterized by point mutation(s) in the insect *para*-type sodium channel gene which leads to nervous system receptor insecticide insensitivity. This mechanism was first explored with DDT in *Musca domestica* (L) (Busvine, 1951; Milani, 1954) and the term knockdown resistance (*kdr*) was coined for the trait. Further investigations have indicated that the most common mutation in the *para* segment of the sodium channel gene is a leucine to phenylalanine (L1014F, housefly numbering) substitution in domain II segment 6 of *para*-type sodium channel gene (Dong, 1997; Martinez-Torres et al, 1998; Martinez-Torres et al, 1999; Miyazaki et al, 1996; Williamson et al, 1996). Additionally, two other substitutions at this position have been found to confer resistance to pyrethroids and/or

DDT in several insect species (Soderlund & Knipple, 2003). A leucine to histidine mutation (L1014H) is associated with pyrethroid resistance in various species and a leucine to serine (L1014S) substitution confers resistance to DDT and low levels of permethrin (Martinez-Torres et al, 1999; Park & Taylor, 1997; Ranson et al, 2000a). An additional mutation, when found in conjunction with the L1014F substitution gives even greater resistance capabilities has been found in the domain II segment 5 of the sodium channel and has been designated as the super-*kdr* site (Soderlund & Knipple, 2003). Mutations vary in exact location in differing insect species, but are always 5´ to the L1014F site.

The rat flea, *Xenopsylla cheopis* (Rothschild), is a competent vector of *Yersinia pestis* (Yersin), the causative agent of plague. An analysis of a 44-year period of plague statistics by the World Health Organization indicated that the continent of Africa accounted for 27.8% of the reported cases (Tikhomirov, 1999). Resistance to DDT in *X. cheopis*, was first documented in the Poona District of India (Patel et al, 1960) as a result of intensive plague control measures targeting the rat flea. There is a risk for development of resistance in the rat flea in Africa as a result of secondary exposure to DDT and pyrethroid insecticides used in IRS programs. The status of resistance in *X. cheopis* is critical to the success of vector control programs to suppress plague outbreaks. To this end, the purpose of the present study was to characterize the *para* gene sequence in individual rat fleas, identify nucleotide substitutions in region II, segments 5 and 6 of *para*, and then test for associations between expression of resistance and single nucleotide polymorphisms (SNPs). We describe large amounts of linkage disequilibrium (LD) in populations tested indicating a classical selective sweep, high genetic diversity in

insecticide unexposed Ugandan *X. cheopis* but low genetic diversity in insecticide exposed Ugandan *X. cheopis.*

The hypothesis addressed in the last section of the study is that segregating sites in *para* are associated with natural variation in susceptibility to DDT and pyrethroids. Cosegregation of genotypes in a candidate gene with a phenotype provides direct genetic proof that a candidate gene constitutes a quantitative trait loci. Association mapping is a method that is being used to evaluate phenotypic associations with genotypes at candidate loci and has been proposed as a general method for detecting loci for susceptibility to complex human diseases (Risch, 2000). Evaluating association between markers at a candidate gene and a phenotype requires a sample of individuals from the field, each of whom has been evaluated for a phenotype (insecticide susceptibility) and in whom the genotypes at marker loci (SNPs in *para*) have been identified.

For discrete traits, such as susceptibility or resistance to an insecticide, the population sample is stratified according to susceptibility phenotype. An association between a SNP and the phenotypic trait is revealed as a significant difference in marker allele or genotype frequencies among fleas with alternative phenotypes. Taken to a finer level, if the genotypes at all SNPs in *para* are determined, one or a few of them should correspond to the site causing the phenotypic effect. These sites have come to be known as quantitative trait nucleotides (QTNs). Genetic epidemiologists have rapidly embraced QTN mapping as a powerful tool for identifying heritable genetic predisposition to human disease. Drosophila researchers have mapped QTNs in ectodermally expressed genes that control bristle number in Drosophila (Mackay, 2001). Domestic animal and plant breeders mapped QTNs associated with increased yield or other desirable characters

and then used these as selectable markers for more rapid crop and animal improvement (Winter et al, 2002). We use QTN mapping to test for an association between SNPs in *para* and susceptibility to DDT or pyrethroids.

B. Materials & Methods

1. Collection Methods and Sites

A laboratory colony of *X. cheopis* has been maintained at the Centers for Disease Control and Prevention (CDC) in Fort Collins, Colorado for nearly 20 years. The colony was originally established from a colony started in the mid-1980s at the University of Maryland taken from rats trapped in the Baltimore area. The colony was later shipped to Rocky Mountain Laboratories in Hamilton, MT. Some of that colony was shipped to CDC, Fort Collins in 1992. Fleas from northern Uganda were obtained by combing live-trapped rodents from locations in Fig. 4.1. Locations were chosen based on history of insecticide exposure during indoor residual spraying (IRS) programs for vector control and malaria prevention.

Trapping was performed in areas with either no history of insecticide exposure (Climate and Arua), or at sites with extensive history of IRS. In Lemo East, lambda-cyhalothrin in the Icon® 10WP (Syngenta East Africa Limited, Nairobi Kenya) formulation was sprayed from August-October 2008 and alpha-cypermethrin in the Fendona® 5WP (BASF, Waedenswil Switzerland) formulation (5.93% w/v (60 g/liter))

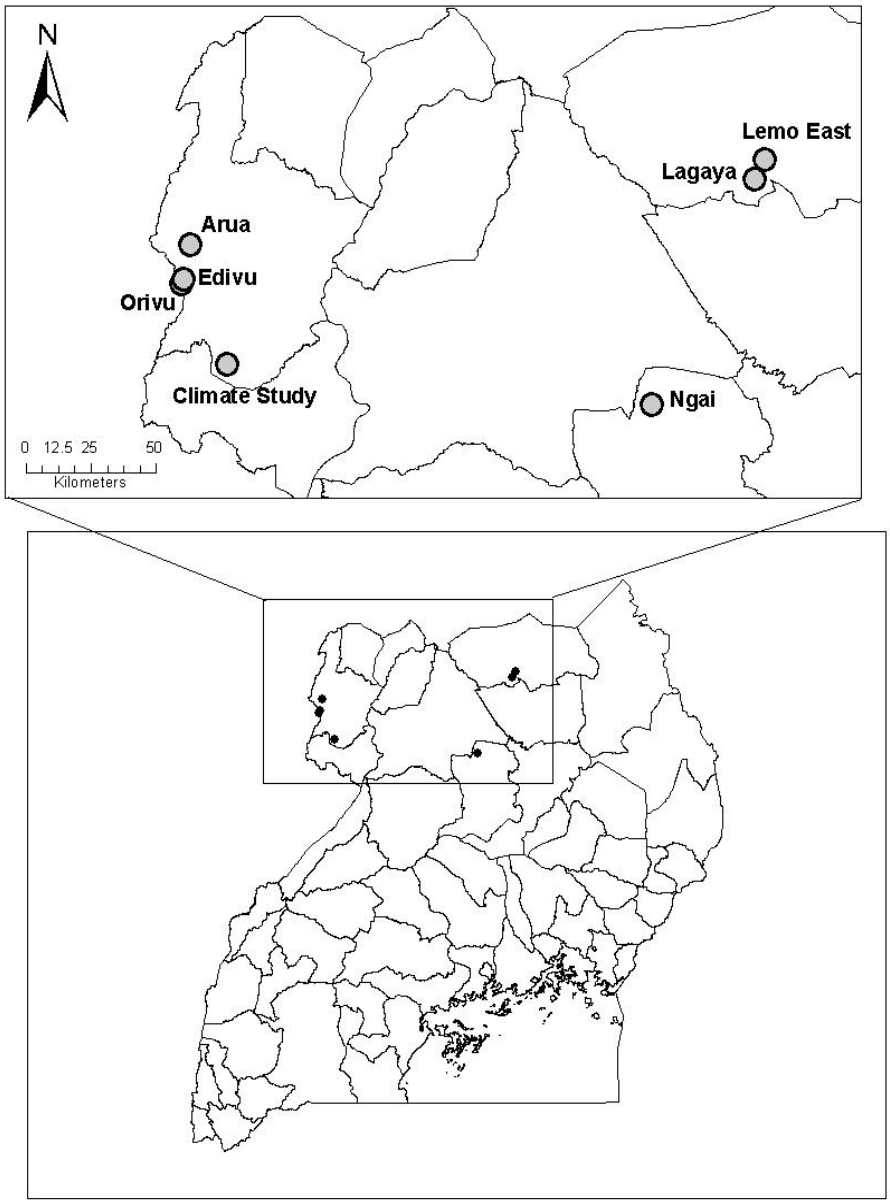

Figure 4.1. Map of Uganda, Africa with *Xenopsylla cheopis* collection sites identified. Populations from Edivu and Orivu were not used in this study.

was sprayed from August-October 2009. In Lagaya, Fendona® was used in October and November 2009. In the Ngai Trading Center Village both DDT and Icon® had been sprayed from March to May in 2008. Indoor residual spraying with Fendona® was taking place in the village at the time of trapping. The Climate Study fleas were supplied

by CDC Fort Collins personnel conducting separate studies in Uganda. This area has no history of prior insecticide use.

A total of 262 Tomahawk® live traps were set along the interior walls of cooking and sleeping huts following previously approved CDC IACUC protocols. Traps were collected early in the morning. Rodents were anesthetized with halothane gas by inserting a 4X4 gauze pad into a 50ml conical centrifuge tube, pouring a small amount of liquid halothane into the tube, placing the tube in a 1 liter zip top plastic bag, and inverting the cage with the rodent in it over the bag. After the rodent fell into the bag, the bag was sealed and the rodent was monitored for cessation of movement and response to stimuli. By holding the rat by its tail (still inside the bag) and vigorously shaking the plastic bag and the rat, fleas would drop off the rat and into the bottom of the bag; the rodent was subsequently removed, placed into a 2.5 inch deep plastic tray, and combed for fleas using a plastic commercial pet flea comb. After all trapping sessions, traps were vigorously agitated in soapy water and air dried.

2. Bioassay.

The plate bioassay previously described was used. Fleas collected at the Ugandan Lemo East and Lagaya sites were processed at the Ugandan Virology Research Institute lab in Arua. The fleas that did not survive the long, hot journey back to the lab were immersed in 75% EtOH for later DNA analyses. Three replicate Petri dishes of the discriminating dose of each insecticide were used. DDT (65ul of 1 mg/mL=65 ug) or lambda-cyhalothrin (20 ul of 100ug/mL=2ug) were added to 1 ml of acetone and the mixture was air dried for 30 minutes. Three prepared Petri dishes treated with DDT or lambda-cyhalothrin were transported to Ngai to conduct bioassays in the field. Fleas

were separately stored in EtOH according to collection site and survival status. Vials of fleas were later transported back to Colorado for further analyses.

3. DNA Isolation.

Single fleas were homogenized in 180µl of 1X PBS (Phosphate Buffered Saline: pH 7.4, 11.9mM phosphates, 137mM sodium chloride, 2.7mM potassium chloride) using 3 glass beads in a 2ml conical tip tube. Tubes were mounted in a Retsch® MM300 shaker and agitated for 2 minutes at a frequency of 20 vibrations per second. Further extraction was done using Qiagen DNeasy® Blood & Tissue Kit (Qiagen Sciences, MD) supplementary protocol. The DNA was eluted in final volume of 100µl AE (pH 9.0, 10mM Tris-HCl, 0.5mM ethylenediaminetetraacetic acid (EDTA)) buffer. Nanodrop quantification was performed on a Thermo Scientific (Fisher Scientific, PA) NanoDrop 1000 using software version 3.7.0 to verify the quality and quantity of nucleic acids. All samples were stored at -20°C. Template (10uL = 12-15 ng DNA) was used per PCR. Initial PCR conditions were established with pools of 10 *Oropsylla montana* (Baker), 10 *Xenopsylla cheopis*, or a pool of those two mixed with DNA from *Pulex irritans simulans* (L), and *Hoplopsyllus anomalus* (Baker).

4. PCR

A 50µl PCR contained 25 µl Promega Go Taq® Green Master Mix, 2 µl each primer at [10µM], 2µl template and 19ul water. Thermal cycling was run on a BioRad My Cycler™ thermal cycler (Bio-Rad Labs, CA) and program conditions were 1 cycle of 96°C for 5 minutes (initial denaturation) , 35 cycles of 95°C 30 sec (melting), 45°C for 1 min (annealing), 72°C 1 min (extension). This was followed by 72°C for 5 min (final extension) and held at 4°C. The annealing temperature was optimized with a thermal

gradient running at 45.0°C, 46.1°C, 48.0°C, 50.7°C, 54.5°C, 57.2°C, 59.0°C, and 60.0°C. Products were visualized on a 0.8% agarose gel (0.4g agarose, 50ml 1X TAE (Tris-Acetate-EDTA, pH 8.3, 40mM Tris-acetate, 1mM EDTA), 1ul Syber® Safe DNA gel stain (Invitrogen).

Development of PCR primers involved a great deal of trial and error. Primers Para1F, Para1R, Para4F and Para4R (Table 3.1) were designed using Primer Premier (Premier Biosoft International, Palo Alto, CA) and para gene sequences from six insect species retrieved from the National Center for Bioinformatics (NCBI) database (*Blattella germanica* (L) BGU73584; *Nasonia vitripennis* (Walker) NM_001134918; *Helicoverpa zea* (Boddie)GU574730; *Tribolium castaneum* (Herbst) NM_001165908; *Aedes aegypti* (L) AY663385; *Musca domestica* (L) AY834743). These primers all failed to produce any products with individual or pooled DNA samples.

Table 4.1.
Sequential list of primers used, listed in 5´ to 3´ order. Degenerate bases are in standard IUB code.

Primer name	Sequence
Para1F	GCAGYTBTTYGGCAARAACTA
Para1R	AGRTTDCCYATVACGACGGTVGC
Para4F	GGWGCBYTVGGKAAYYTGAC
Para4R	CSACVHRCATRCARTCCCAC
CF1-F	TGGCCAACGCTGAATTGC
CF2-R	TGTTTCATTATCCGCTGTTGG
SarF	CAACGCTGAATTTGCTTATATCC
SarR	CTCGAAGAACCGAAGTTCG
D1	AARYTNGCNAARTCNTGGCC
D5	GCNAARTCNTGGCCNAC
Dg2	GCDATYTTRTTNGTNTCRTTRTC
XC3A	CAGTGCCTGGGTAATCTAA
XC2	ACGTTTGTGTTGTGTATTATCAT
XC6	TTCGCCGTAATGGGTAT
XC5	TTGTGTTGTGTATTATCATCTTCAT

XC8R	AAAGCAACAGGCGACAA
XC9R	AAAAACCAAAGCAACAGG
AA1F	TTCGCCGTAATGGGTATGC
AA1R	KTCGTTGTCCGCTGTTGG
AA2F	ATGAGTGCCTTGGGTAATCTAA
AAC2F	GATGGAGTGCCTTGGGTAATCTAA
AAC2Fshort	GATGGAGTGCCTTGGGTAA
A1F	CATTATGGGTCGGACGAT
A1R	CGCTGTTGGAGCTGATAG
A2R	TCCGCTGTTGGAGCTGAT
SKDRF	ATGGGTCGGACGATGGAG
SKDRR	CGGCGAATATGAAGATGA

Next, the *para* gene primers designed for *Ctenocephalides felis* (Bouché) by Bass (2004b) (CF1-F and CF2-R, Table 4.1) were tried but failed with DNA from individual fleas. Primers SarF and SarR (Table 4.1) that were designed from the *C. felis para* gene sequence (EMBL/Genbank Accession No. AJ717585) also failed in pools and individual preparations.

Degenerate *para* primers D1, D5, and Dg2 (Table 4.1) (Bass et al, 2004b) were also tried. The PCR mixture was the same as above except that 10µl of pooled template was used. The first PCR contained primers D1 and Dg2. Program conditions were as above but the annealing temperature was 45°C for 1 min. A second PCR with 10µl template from the first PCR used the nested D5 and Dg2 primers (Table 4.1).

After 6 *para* gene sequence was determined for *X. cheopis*, species specific primers could be designed. These were XC3A, XC2, XC6, XC5, XC8R, and XC9R (Table 4.1). Program conditions were as above but the annealing temperature was raised to 55°C for 1 min. However, this series of primers failed with DNA preparations from individual fleas. The AAC2F, AAC2Fshort, A1F, A1R, A2R, SKDRF, and SKDRR (Table 3.1) primers were then redesigned and A1F, A1R yielded product of the 558 bp

predicted size with individual fleas. PCR product which was purified using the Qiagen QIAquick® PCR Purification Kit (Qiagen Sciences, Valencia, CA).

5. DNA sequencing.

For a 20µl reaction, 8µl of Applied Biosystems (Life Technologies Corp., Carlsbad, CA) Big Dye™, 2 µl of the forward or reverse primer for each reaction [3.3µM], 1ul of up to 10ng/ul of purified template, and 9ul of DNase free water was used. The thermal cycler program was one cycle of 96°C for 1 min, 25 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 1 min 15 sec. The program was run on an Applied Biosystems 9800 thermal cycler. Sequencing was performed on an Applied Biosystems 3130xl Genetic Analyzer.

When subjected to a BLAST (Basic Local Alignment Search Tool) search of the NCBI database, products were 93% identical to *C. felis* partial *para* gene (AJ717585). The ABI sequences were initially aligned using DNASTAR Lasergene 8 **(**DNASTAR, Inc., Madison, WI). Sequence ends were trimmed and aligned by hand using the *C. felis* codon annotation to identify exon boundaries. All nucleotide sites with double peaks in the trace file were scored as heterozygous. Contigs were converted into a text file with the Megalign program. ClustalW (Thompson et al, 1994) was used to align all sequences and to create an alignment file.

6. Population and phylogenetic analyses.

The alignment file contained diploid genotypes at polymorphic sites (IUPAC codes: adenine/guanine = R, adenine/guanine = M, adenine/thymine = W, cytosine/ guanine = S, cytosine/ thymine = Y, guanine/ thymine = K). Diploid genotype sequences cannot be analyzed using many of the statistical procedures for molecular evolutionary

analyses. Instead, the PHASE program in DnaSP5.1 (Rozas et al, 2003) was used to estimate the phase of nucleotides at each polymorphic site and created two haplotype sequences for each diploid genotype sequence. DnaSP5.1 was then used to identify numbers of segregating sites, haplotypes, nucleotide diversity (π) (Saitou & Nei, 1987,equations 10.5 or 10.6), theta per site (Saitou & Nei, 1987, equation 10.3) and the average number of nucleotide differences, k (Tajima, 1983, equation A3). DNAsp5.1 was also used to calculate the degree of linkage disequilibrium (LD), or nonrandom association between nucleotide variants at different polymorphic sites. The LD between all polymorphic sites were also tested for significance. Bioedit was used to identify identical sequences and thereby count the frequencies of individual haplotypes. All duplicated sequences were then removed to construct a dataset of unique haplotypes for phylogenetic analysis.

A Maximum Parsimony phylogeny was derived using PAUP* 4.0 (beta 10) (Swofford, 2003). Gaps were treated as a fifth character in this analysis. Bootstrapping was performed with 1000 pseudoreplicates to determine the consistency of individual branches. Association mapping between polymorphic sites in the para gene and insecticide resistance was performed using PGTheta (Gorrochotegui-Escalante et al, 2005).

C. RESULTS

1. Collection Methods and Sites

At the Arua market site the only rodents collected were two Gambian rats (*Cricetomys gambianus* Waterhouse) trapped in a storehouse room. The low trapping success rate was possibly due to the availability of foodstuffs throughout the marketplace

and therefore, rodents had little incentive to enter the traps. Visual tracking of rodents moving about the market made while placing the traps, confirmed that the population of rodents was robust. While the rodents were alive, large, brown bullet-shaped insects were observed moving unmolested about the rodents' fur. These relatively large dermapterans, identified as *Hemimerus* (Walker)spp, were the only insects found on each of the Gambian rats. *Cricetomys* is the principal host of *Hemimerus* and eight of the nine described species live on *Cricetomys* (Askew, 1971). All are confined to tropical Africa. The insects are wingless, eyeless and are generally distributed over the host's pelage.

Trapping results (Table 4.2) yielded a variety of rodents - Nile rats (*Arvicanthus niloticus* Desmarest), Gambian rats, and/or roof rats (*Rattus rattus* (L)). Roof rats are the target species as they most commonly host the Oriental rat flea *X. cheopis*. Flea infestations also fluctuated and the specific flea index was determined.

Table 4.2. Rodent trapping and *Xenopsylla cheopis* collection data in Uganda, Africa.

Trapping site	# Nile rats	# Gambian rats	# Roof rats	% success	# fleas	Flea index
Arua town	10	2	2	38.7	15	1.07
Arua airfield	43	0	0	50.0	31	0.72
Arua village	0	0	20	37.0	1	0.01
Lemo	0	0	37	7.0	85	2.29
Lagaya	0	0	52	9.9	271	5.21
Ngai	1	2	94	18.5	466	4.80
Orivu	0	0	52	27.9	12	0.62
Edivu	0	0	58	31.2	41	0.70

2. Bioassays

The laboratory derived insecticide discriminating doses on each Petri dish for *X. cheopis* used were: malathion 80ul @ [5.0ul/ml]; permethrin 2.5ug; lambda-cyhalothrin 2ug; DDT 65ug; deltamethrin 1ug.

Of the field caught populations tested by the plate assay with lambda-cyhalothrin, Lemo East fleas showed 57.7% survivorship at 60 minutes, Lagaya had 60.5% survivorship and Ngai averaged 58.0% survivorship. Ngai fleas tested with DDT had 81.8% survivorship.

3. PCR

Agarose gel electrophoresis of PCR products from the *para* primers in Table 4.1 on pooled samples identified faint products in the 500kb size. Products were more faint for pooled *X. cheopis* DNA and a double band was observed with *O. montana* DNA using the para4 primers. No product was seen with the para1 primers. PCR performed on single *X. cheopis* fleas yielded no products.

Single flea samples using CF1-F and CF1-R (Table 4.1) showed extremely faint bands in the 500kb range for the colony and one Lagaya sample at the 48°C, 46.1°C, and

45.0°C annealing temperatures. Nanodrop analysis of the products indicated a DNA concentration in a range of 4.6 - 22.5ng/ul. Sequenced products returned no readable data.

Nanodrop analysis on pooled colony *X. cheopis* extracted DNA indicated a range of 55.2 - 134.6ng/ul in the samples. The size, quality, and quantity of products were not correlated with the amount of starting template DNA. Agarose gel electrophoresis frequently revealed multiple bands, faint or no results and abundant primer-dimer pairs. Product was only present at the three lowest annealing temperatures.

Using the SarF and SarR primers (Table 4.1), only the positive control and *X. cheopis* at a concentration of 90.6ng/ul yielded products. *Ctenocephalides felis* DNA produced a band at every temperature and *X. cheopis* DNA yielded faint bands at 45°C and 46.1°C.

Degenerate primers designed from *C. felis para* gene sodium channel primers D1, D5, and Dg2 (Table 4.1) produced the first sequences from *X. cheopis*' with pooled colony DNA, but never successfully amplified from single flea DNA.

Primers XC3A, XC2, XC6, XC5, XC8R, and XC9R (Table 4.1) produced products in a single flea but sequencing product indicated no similarity to the known *para* gene sequence. Primer pair AA1F and AA1R (Table 4.1) yielded product with pooled DNA, and with DNA from a single flea DNA but purified product was too short. For primers AAC2F, AAC2Fshort, A1F, A1R, A2R, SKDRF, and SKDRR (Table 4.1), all primer combinations used yielded PCR product except SKDRF and SKDRR which targeted the putative super-*kdr* region. Comparing and contrasting sequenced product results from the primer combinations indicated the best pair was A1F and A1R.

4. Sequencing of *para* in individual fleas

Sequencing of the 558 bp *para* gene fragment in individual fleas proved problematic, even after the *para* sequence for *X. cheopis* had been determined with DNA from pooled fleas. Designing primers compatible with the 5′ end of the sequence that would result in consistent, readable nucleotides at the putative super-*kdr* site was difficult. PCR ingredients were altered, as were thermal cycling conditions. These modifications also failed to yield products from individual flea DNA. Primers that were selected directly from the sequence failed, as well as most of those designed by software. Using a mix-and-match strategy I finally obtained usable sequences using A1F and A1R (Table 4.1).

Figure 4.2 shows the 558bp sequence for the *para* gene domain II segments 5 and 6 of the voltage-gated sodium channel of *X. cheopis.* The fragment contained three exons and two introns. Five single nucleotide polymorphic (SNP) sites were found in exon 1; four of these produced amino acid replacement substitutions. Intron 1 contained 5 SNPs. Exon 2 contains six SNPs, three of which produced amino acid replacement substitutions. Two these are boxed in Figure 4.2. The second boxed SNP corresponds to the well established leucine to phenylalanine (CTT to TTT) L1014F mutation site associated with resistance to DDT and pyrethroids in other insects. The first boxed nucleotide was associated with DDT and lambda-cyhalothrin resistance but encodes a synonymous substitution. Nineteen SNPs were identified on intron 2. Exon 3 had 5 SNPs two of which produced amino acid replacement substitutions.

5. Sequence alignment

PCR products from 647 individual *X. cheopis* were sequenced, 33 of which were too short for further analyses. There were 467 characters in the sequence. Characters 1-91 were in exon1, characters 92-154 were in intron 1, exon 2 contained characters 155-343, intron 2 contained characters 344-414 and exon 3 contained characters 415-466. Each diploid genotype sequence was assigned in DNAsp5.1 as being in "A" or "B" haplotype phase for further analysis.

Figure 4.2. Nucleotide sequence of the domain II S5-S6 region of the *Xenopsylla cheopis para*-type voltage-gated sodium channel gene amplified from individual fleas. Primers are in bold. Segregating sites (SNPs) are underlined. Amino acids are above the second nucleotide of each codon. Introns are in lower case. Single polymorphic nucleotide sites corresponding to site 178 and 338 in the text are boxed. Site 356 in figure corresponds to L1014P *kdr* site in other insect species.

6. General properties of the gene

A total of 41 SNPs were found in the 467 sites analyzed in the Ugandan fleas These are catalogued in Table 4.3. Twenty five SNPs were located in introns and 16 SNPs were in exons. Exon segregating sites 178 and 338 were associated with resistance (see below) and also had the highest π (0.171 and 0.188 respectively). Ten of the 16 segregating sites in exon regions code for amino acid replacement, but occurred at much lower frequencies. Sequence variability analyses revealed 66 haplotypes (Table 4.4). One haplotype was predominant with a frequency of 0.813 and contained the *kdr* mutation as compared to the next most common with a frequency of only 0.061, which also contained the *kdr* mutation.

Table 4.5 provides detailed population genetic statistics. Predictably, the colony fleas, reared in the laboratory for nearly 25 years had the lowest nucleotide diversity, the lowest average number of differences and the lowest number of haplotypes. Conversely, the Climate Study Site fleas, presumably unexposed to insecticides had the highest nucleotide diversity, highest average differences and the most segregating sites. Among the exposed fleas, the Arua population (with the least pyrethroid exposure) had the highest nucleotide diversity. The Ngai fleas (extensively exposed to DDT and a pyrethroid) had the lowest nucleotide diversity among the field caught fleas. Figure 4.3 graphically displays nucleotide pairwise differences (π) within the populations. Most SNPs occurred in the introns, and were most abundant in the Climate Study area fleas. Fleas exposed to insecticides through IRS programs had the least amount of diversity.

Table 4.3. Polymorphic site mutations found in Uganda, Africa populations of *Xenopsylla cheopis.* π = nucleotide diversity. Bolded sites are QTNs.

Site#	Normal a.a.	Codon placement	Nucleic acid code	Replaced/ Synonymous	Replacement amino acid	π
7	Ala	3	M	S		0.0102
17	Leu	1	M	R	Ile	0.0017
39	Ile	2	W	R	Asn	0.0068
50	Phe	1	W	R	Ile	0.0034
86	Tyr	1	K	R	Asp	0.0017
99	Intron		K			0.1984
108	Intron		R			0.0017
122	Intron		R			0.0068
125	Intron		W			0.0068
135	Intron		Y			0.0068
144	Intron		R			0.0136
178	**Asp**	**3**	**Y**	**S**		**0.1714**
190	Pro	3	R	S		0.0051
216	His	2	R	R	Arg	0.0017
302	Ile	1	R	R	Val	0.0017
336	Asn	2	M	R	Thr	0.0017
338	**Leu**	**1**	**K**	**R**	**Phe**	**0.1878**
349	Intron		Y			0.1402
357	Intron		R			0.1782
358	Intron		Y			0.0203
359	Intron		K			0.1686
363	Intron		Y			0.0017
365	Intron		W			0.0186
370	Intron		R			0.1945
372	Intron		B			0.0068
378	Intron		R			0.0219
381	Intron		M			0.0236
382	Intron		W			0.0034
386	Intron		Y			0.0017
393	Intron		M			0.0017
396	Intron		M			0.0017
405	Intron		M			0.0051
408	Intron		M			0.0017
409	Intron		Y			0.0017
410	Intron		Y			0.0017
411	Intron		M			0.0051
421	Asn	3	W	R	Ile	0.0017
432	Ala	2	S	R	Gly	0.0017
433	Ala	3	S	S		0.0034
444	Ser	1	W	R	Thr	0.0017
446	Ser	3	R	S		0.0034

Table 4.4. Haplotype frequency data for *Xenopsylla cheopis*

Representative sequence	Overall frequency	Representative sequence	Overall Frequency
As364Lg	0.813	Bht125Le	0.001
Aht268Lg	0.061	Bs18Le	0.001
As334NgSI	0.024	Bs134Le	0.001
Bht283Lg	0.017	Bs137Le	0.001
Bht175Le	0.007	Bs130Le	0.001
Aht624NgRD	0.007	As127Le	0.001
Aht283Lg	0.003	Bs127Le	0.001
Bht233Lg	0.003	Bs144Le	0.001
As589NgSD	0.003	As16Le	0.001
Aht220Lg	0.002	Bs16Le	0.001
Aht494NgRD	0.002	As128Le	0.001
Aht66NgSD	0.002	Bs128Le	0.001
Aht624NgRD	0.002	Aht319NgRI	0.001
As35NgDIA	0.002	Bht319NgRI	0.001
Bs36NgDIA	0.002	Aht323NgRI	0.001
As455NgRI	0.002	Aht650NgSD	0.001
Bs455NgRI	0.002	Bht650NgSD	0.001
As49NgSI	0.002	Bht65NgSD	0.001
As420NgSI	0.002	As369NgSI	0.001
Bs420NgSI	0.002	As378NgSI	0.001
As67NgRD	0.002	Bs378NgSI	0.001
Bs665NgSD	0.002	As39NgDIA	0.001
Aht111Arua	0.002	Bs39NgDIA	0.001
Aht113Arua	0.002	As33NgDIA	0.001
Aht231Lg	0.001	Bs33NgDIA	0.001
Bht231Lg	0.001	As36NgDIA	0.001
Aht233Lg	0.001	As422NgSI	0.001
Aht234Lg	0.001	As42NgSI	0.001
As226Lg	0.001	Bs42NgSI	0.001
As241Lg	0.001	Bs410NgSI	0.001
Aht194Le	0.001	As59NgSD	0.001
Aht126Le	0.001	As536NgSI	0.001
Bht126Le	0.001	As1Arua13	0.001

Sequence code: A/B= representative of each diploid sequence, ht= heterozygote, Lg= Lagaya, Le= Lemo East, Ng= Ngai, Arua= Arua, DIA= died before plate assay, SI= susceptible to lambda-cyhalothrin, RI= resistant to lambda-cyhalothrin, SD= susceptible to DDT, RD= resistant to DDT

Table 4.5. Molecular genetic variability statistics in *Xenopsylla cheopis* from Uganda, Africa.

	Lg	Le	Ng	NgD	NgSD	NgRD	NgSI	NgRI	Arua	Colony	Climate	All
Number of haplotypes analysed	166	156	738	58	172	166	268	74	36	60	54	1228
Number of segregating sites	13	24	58	11	14	10	16	7	15	1	46	69
Number of unique Haplotypes	12	20	48	6	13	8	15	6	8	2	34	87
Nucleotide diversity(π)	0.0026	0.0031	0.0011	0.0025	0.0019	0.0006	0.0005	0.0016	0.0078	0.0004	0.0125	0.0042
Theta per site	0.0049	0.0090	0.0072	0.0052	0.0053	0.0038	0.0056	0.0031	0.0078	0.00046	0.0255	0.0195
Average # differences (k)	1.187	1.407	0.511	1.151	0.922	0.263	0.215	0.723	3.598	0.183	5.788	1.926

Lg- Lagaya, Le-Lemo East, Ng-Ngai Trading Village (all samples), NgD-Ngai died while handling, NgSD-Ngai suseceptible to DDT, NgRD-Ngai resistant to DDT, NgSI-Ngai susceptible to lambda-cyhalothrin, NgRI-Ngai resistant to lambda-cyhalothrin.

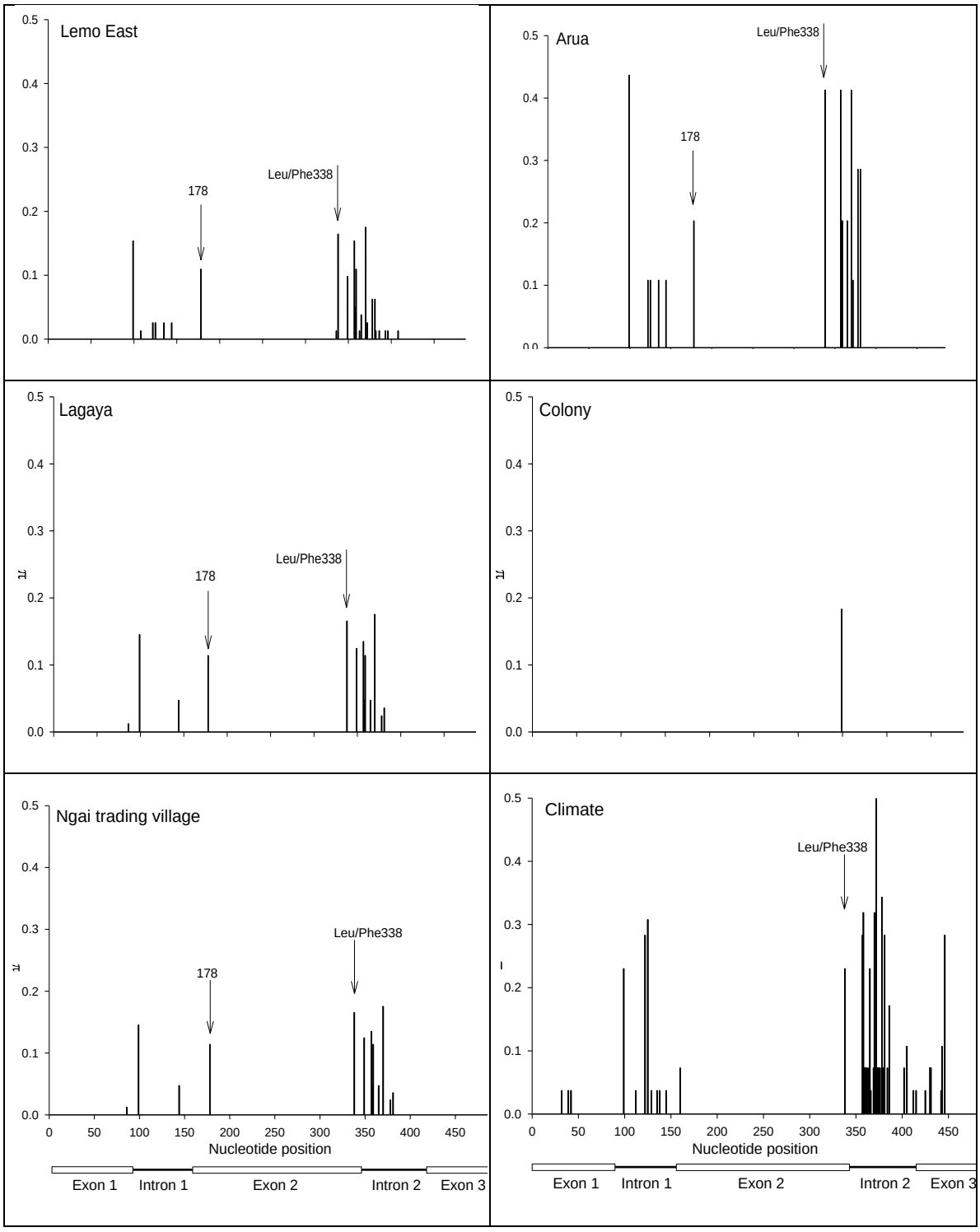

Figure 4.3. Measure of nucleotide pairwise differences (π) within each population of *Xenopsylla cheopis* tested.

7. Phylogeny

Figure 4.4 is the maximum parsimony phylogeny of the *X. cheopis* haplotypes. *Xenopsylla nubica* was used as the outgroup because it is closely related. Haplotype labels ending with the character "C" are genotype CTT at the *kdr* site (susceptible allele) coding for a leucine. Labels ending with a "T" are genotype TTT (resistant allele) coding for a phenylalanine. The most frequent sequence is located at the top of the tree in bold. The base of the tree is populated entirely by the wild type, leucine haplotypes (C). The upper branch consists mostly of the resistant haplotypes (T). The base of the upper branch between the two distinct groups contains a majority of phenylalanine genotypes, and the wild type (leucine) haplotypes are indicated with an asterisk.

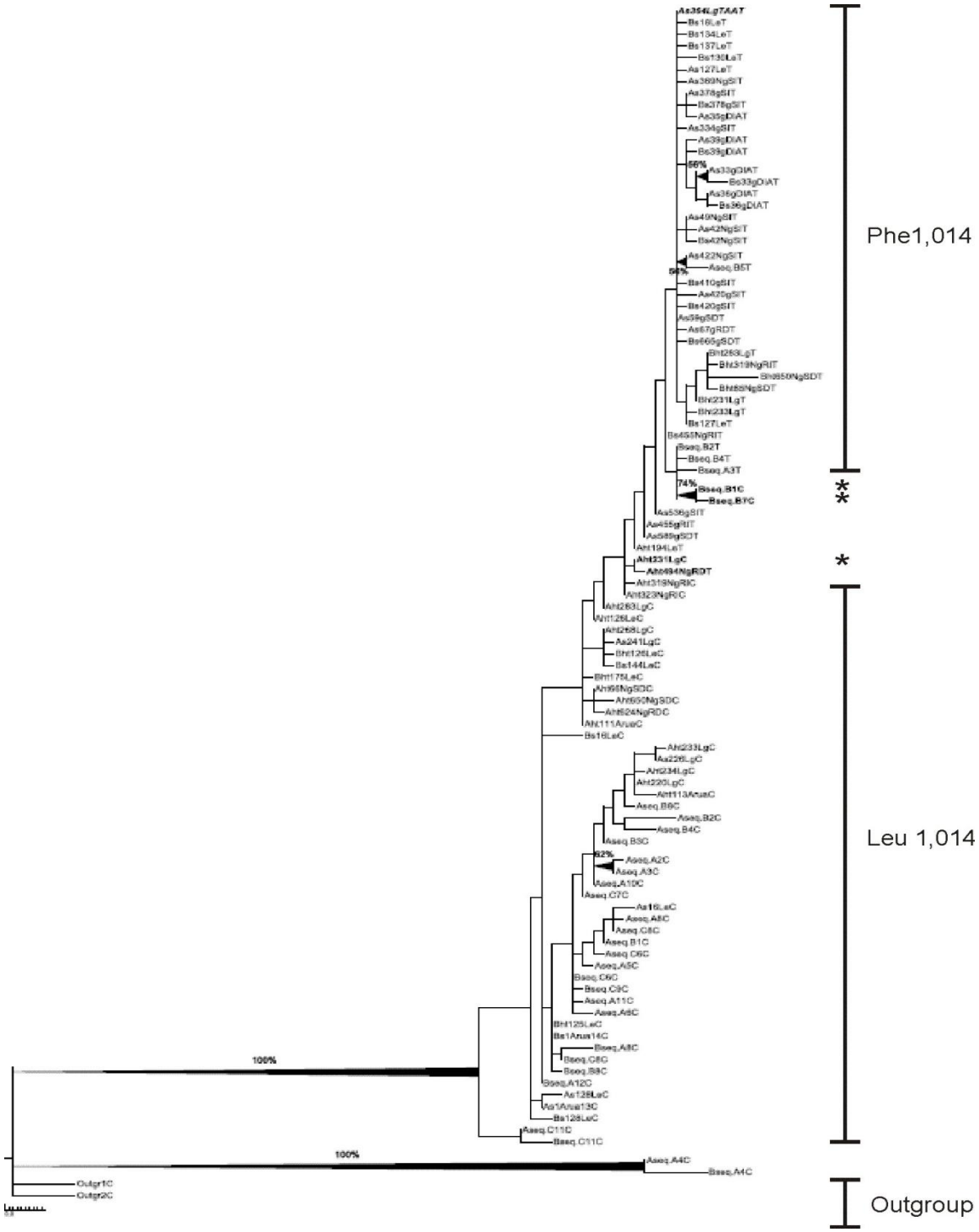

Figure 4.4. Maximum parsimony phylogeny of *para* sequences in *Xenopsylla cheopis* haplotypes. *Xenopsylla nubica* is the outgroup. Haplotypes with an unmutated *kdr* site end with a C and are contained with in the Leu 1,014 bracket. Haplotypes with a mutated *kdr* site end with a T and are contained within the Phe 1,014 bracket. Asterisks in between the brackets indicate a haplotype ending with a C.

8. Analysis of linkage disequilibrium

Linkage disequilibrium analysis tests whether alleles at different loci randomly and independently assort (Black et al, 2008). If so, they are considered to be in linkage equilibrium. If not, allele are said to be in linkage disequilibrium. Disequilibrium can be positive when alleles occur together more often than expected based upon their independent frequencies or negative when they occur together less often than expected. Linkage disequilibrium analysis was performed in DNAsp5.1 wherein a Fisher's Exact test was used to test for significance.

Figures (4.5a-f) are graphical representations of linkage disequilibrium analyses among all pairs of SNPs in field collected fleas. Introns are outlined with boxes. The solid black boxes on the principal diagonal represent each SNP site compared with itself. Disequilibrium results between loci appear above the principal diagonal. SNPs in disequilibrium appear as a solid black box while boxes containing SNPs in equilibrium are empty.

Linkage disequilibrium among all Ugandan collections appears in Fig. 4.5a. There were 780 pairwise disequilibrium tests performed and 119 (15.3%) were significant. The majority of significant disequilibrium was detected between intron SNPs (77/119 = 64.7%) while 13 (10.9%) of these were between exons and 29 (24.3%) were between exon and intron sites. However, linkage disequilibrium can result from either lack of recombination or mixing of genotypes from structured populations (Wahlund's effect). This can be avoided by analyzing individual populations alone (Black & Tabashnik, 2005).

This analysis was repeated on the Climate fleas alone (Fig. 4.5b). There are 668 pairwise disequilibrium tests performed and 82 (12.3%) were significant. The majority of significant disequilibrium was again detected between intron SNPs (69/82 = 84.1%), while 7 (8.5%) of these were between exon sites and 6 (7.3%) were between exon and intron sites. Thus the disequilibrium noted in Fig. 4.5a is not entirely due to mixing of genotypes. Instead it is likely that mutations in the second intron accumulated faster on a chromosome (cis) than chromosomes recombined (trans).

Linkage disequilibrium is seen in most of the populations, but is especially evident in the DDT (Fig. 4.5c) and lambda-cyhalothrin (Fig. 4.5e) resistant fleas. In the DDT resistant population (Fig.4.5c) there were 28 pairwise disequilibrium sites and 21 (42%) were significant. Of those significant sites, 1 (8.3%) was between exons, 6 (50%) were between introns and exons, and 5 (41.7%) were between introns. Disequilibrium in the lambda-cyhalothrin resistant (Fig 4.5e) fleas indicated 21 pairwise disequilibrium sites and 21 (100%) were significant. This tight cluster indicates selection for resistance is occurring faster than recombination or mutation. Exon disequilibrium accounted for 1 (4.8%) site, exon to intron disequilibrium involved 10 (47.6%) sites and introns had 10 sites (47.6%) in disequilibrium.

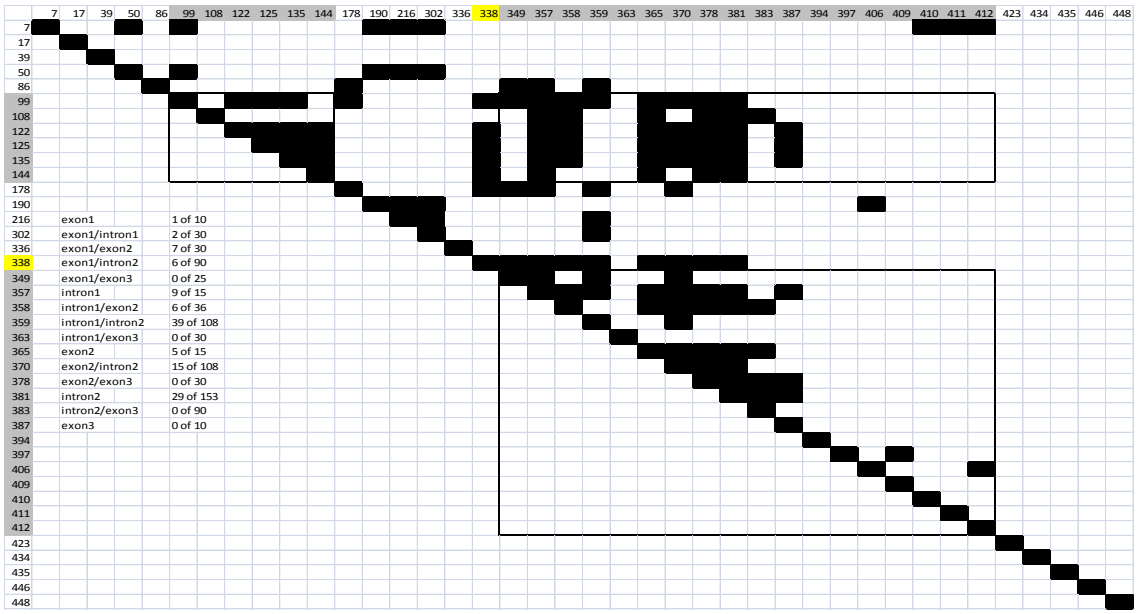

Figure 4.5a. Linkage disequilibrium among all *Xenopsylla cheopis* fleas collected in Uganda, Africa. Pairwise numbers are segregating sites on the *para* gene. Solid black boxes are sites that are in linkage disequilibrium. Intron areas are boxed and segregating sites in introns are grey. Highlighted exon is the *kdr* equivalent site.

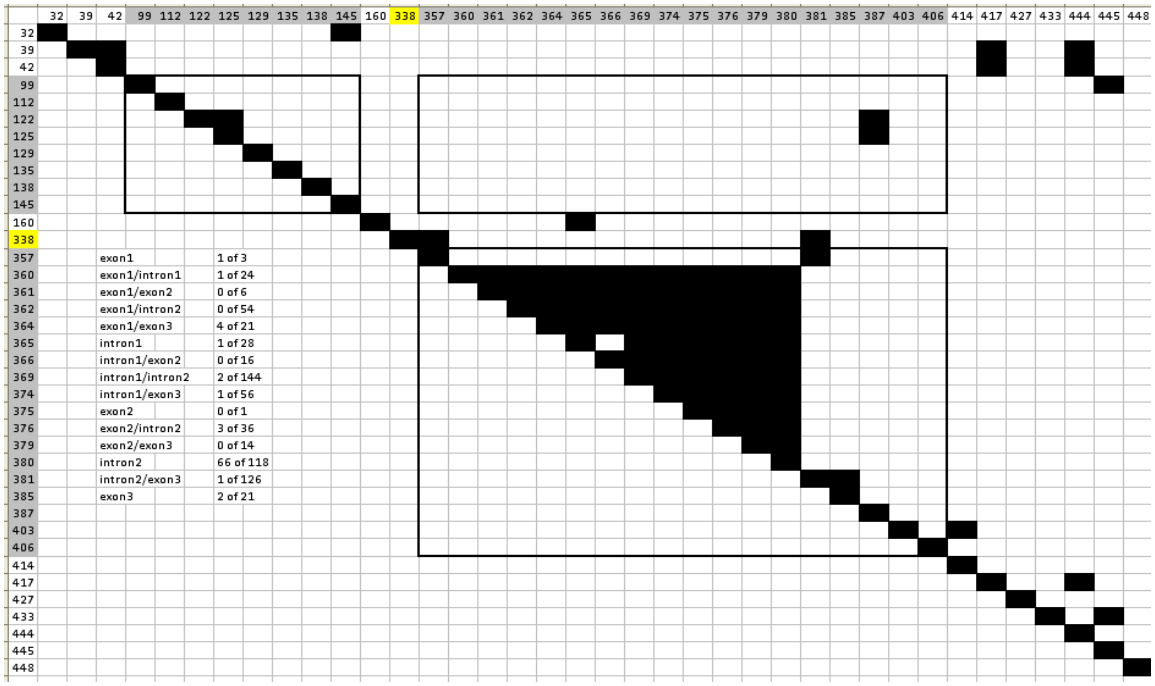

Figure 4.5b. Linkage disequilibrium among Climate Study population *Xenopsylla cheopis* fleas collected in Uganda, Africa. Pairwise numbers are segregating sites on the *para* gene. Solid black boxes are sites that are in linkage disequilibrium. Intron areas are boxed and segregating sites in introns are grey. Highlighted exon is the *kdr* equivalent site.

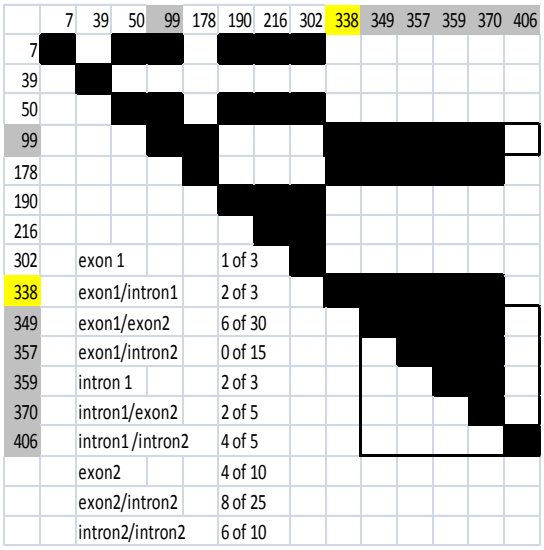

Figure 4.5c. Linkage disequilibrium among DDT resistant *Xenopsylla cheopis* collected in Uganda, Africa. Pairwise numbers are segregating sites on the *para* gene. Solid black boxes are sites that are in linkage disequilibrium. Intron areas are boxed and segregating sites in introns are grey. Highlighted exon is the *kdr* equivalent site.

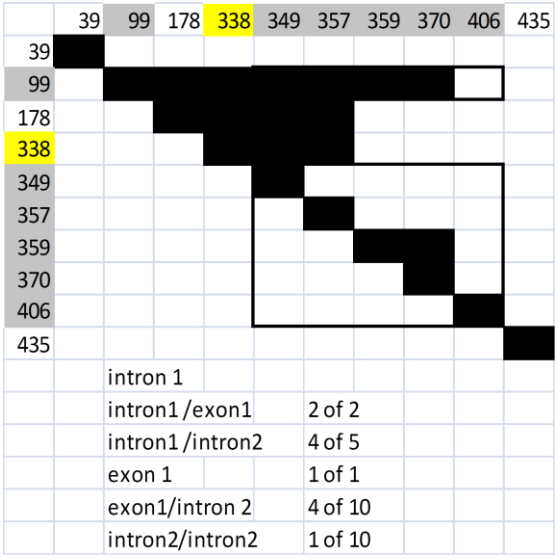

Figure 4.5d. Linkage disequilibrium among DDT susceptible *Xenopsylla cheopis* collected in Uganda, Africa. Pairwise numbers are segregating sites on the *para* gene. Solid black boxes are sites that are in linkage disequilibrium. Intron areas are boxed and segregating sites in introns are grey. Highlighted exon is the *kdr* equivalent site.

.

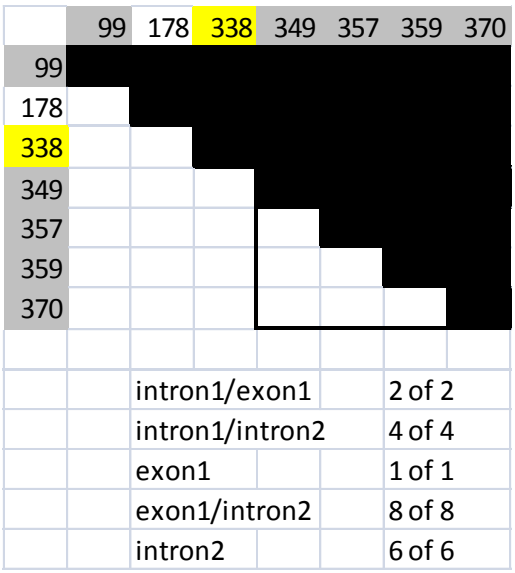

Figure 4.5e. Linkage disequilibrium among lambda-cyhalothrin resistant *Xenopsylla cheopis* collected in Uganda, Africa. Pairwise numbers are segregating sites on the *para* gene. Solid black boxes are sites that are in linkage disequilibrium. Intron areas are boxed and segregating sites in introns are grey. Highlighted exon is the *kdr* equivalent site.

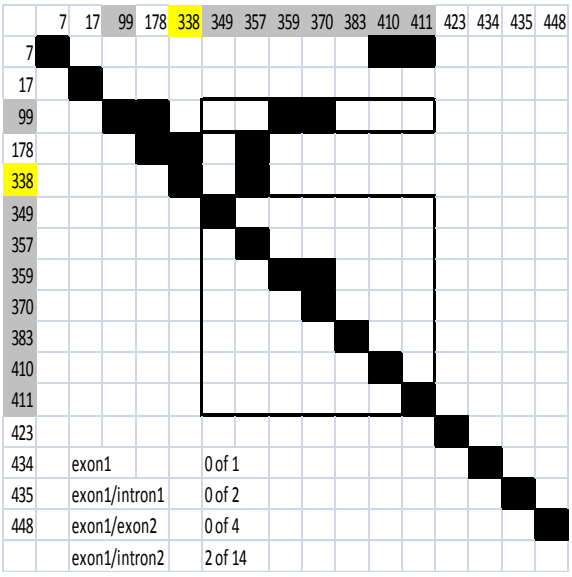

Figure 4.5f. Linkage disequilibrium among lambda-cyhalothrin susceptible *Xenopsylla cheopis* collected in Uganda, Africa. Pairwise numbers are segregating sites on the *para* gene. Solid black boxes are sites that are in linkage disequilibrium. Intron areas are boxed and segregating sites in introns are grey. Highlighted exon is the *kdr* equivalent site.

The DDT susceptible phenotype (Fig. 4.5d) shows a high degree of linkage disequilibrium in the exon regions, but less clustering of the sites. Pairwise testing 35/109 (32%) of sites were in significant disequilibrium. Of these, 11 (31.4%) were between exons, 12 (34.2%) were between introns and exons and 12 (34.2%) were between introns. The lambda-cyhalothrin susceptible (Fig.4.5f) population indicates less evidence of linkage disequilibrium. Of the 126 pairwise test sites found in disequilibrium, 9 (7.14%) were significant. Exon disequilibrium accounted for 1 (11%) site, there were 5 (56%) sites in disequilibrium between introns and exons, and 3 (33%) between introns.

9. QTN mapping of SNPs with DDT and pyrethroid resistance

PGTheta performed a series of statistical tests of association between specific nucleotides and resistant phenotypes (Gorrochotegui-Escalante et al, 2005). For all analyses, the para sequence file was partitioned according to whether the flea from which the sequence was obtained was susceptible (dead) or resistant (alive) in bioassay. The frequency of a nucleotide i at a segregating site is p_i. For Quantitiative Trait Nucleotide (QTN) mapping of resistance, p_i in susceptible fleas was compared to p_i in resistant fleas. For each alternative nucleotide at a segregating site, θ was estimated and the consistency of θ was evaluated with a permutation test (Doerge & Churchill, 1996; Weir & Cockerham, 1984). Next each sequence is randomly assigned to a phenotype and θ_i is calculated for the randomized dataset. This randomization is repeated 10,000 times. All values of θ_i are then sorted and the 9,500th largest value at each SNP site defines the 95% threshold. Figure 4.6 shows result of QTN mapping along the *para* gene with the permutation test for DDT resistance in Ngai flea populations. Figure 4.7 illustrates QTN mapping of lambda-cyhalothrin resistance in Ngai fleas. Filled circles are the θ in the

original dataset. Open circles are the 95% threshold. Any SNP in which the filled circle is greater than the open circle is considered a QTN.

For DDT the SNPs, or segregating sites, at nucleotide 178 and 338 (*kdr*) are putative QTNs. For lambda-cyhalothrin, SNPs 178, 338, 349, 359, and 369 are QTNs, even though SNPs 349, 359 and 369 are located in the second intron.

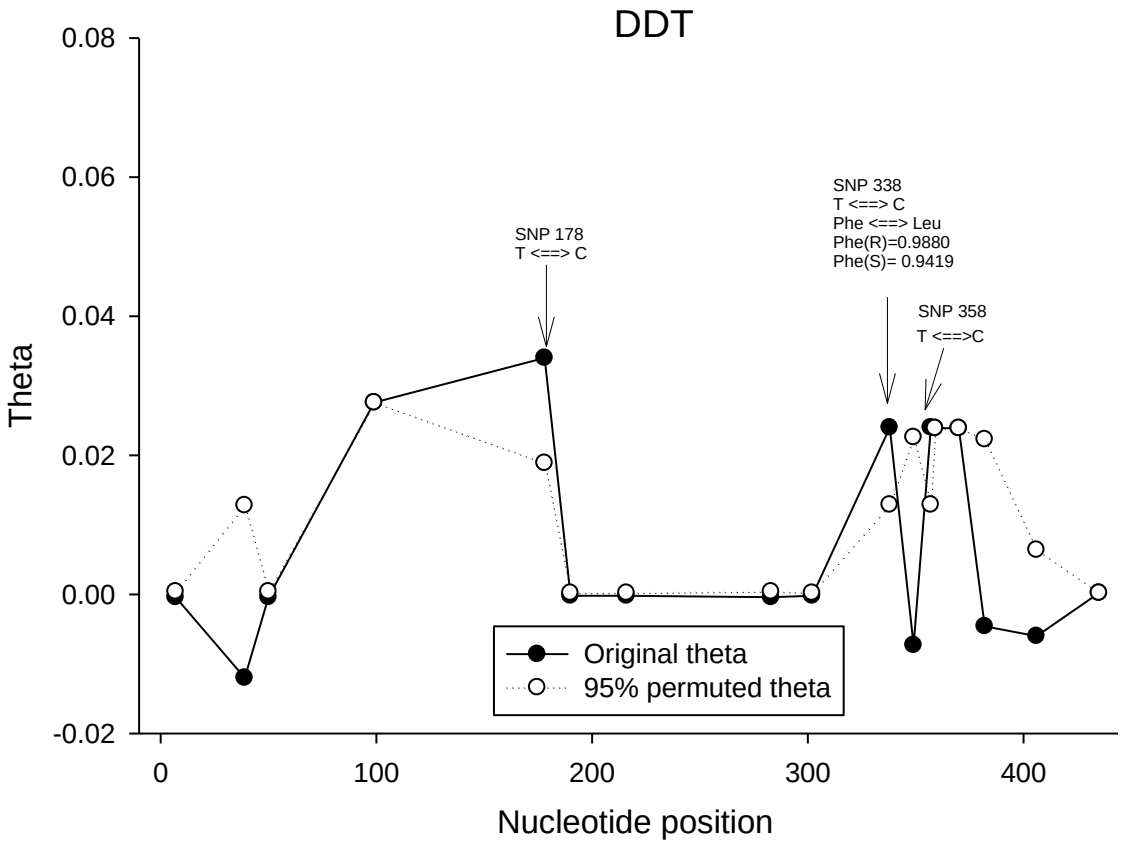

Figure 4.6. Theta permutations of Ngai populations of *Xenopsylla cheopis* tested with DDT. Single nucleotide polymorphisms (SNPs) are identified and the nucleotide shifts are indicated with double arrows. SNP 338 is the *kdr* site with phenylalanine frequencies expressed by resistant (R) and susceptible (S) populations indicated.

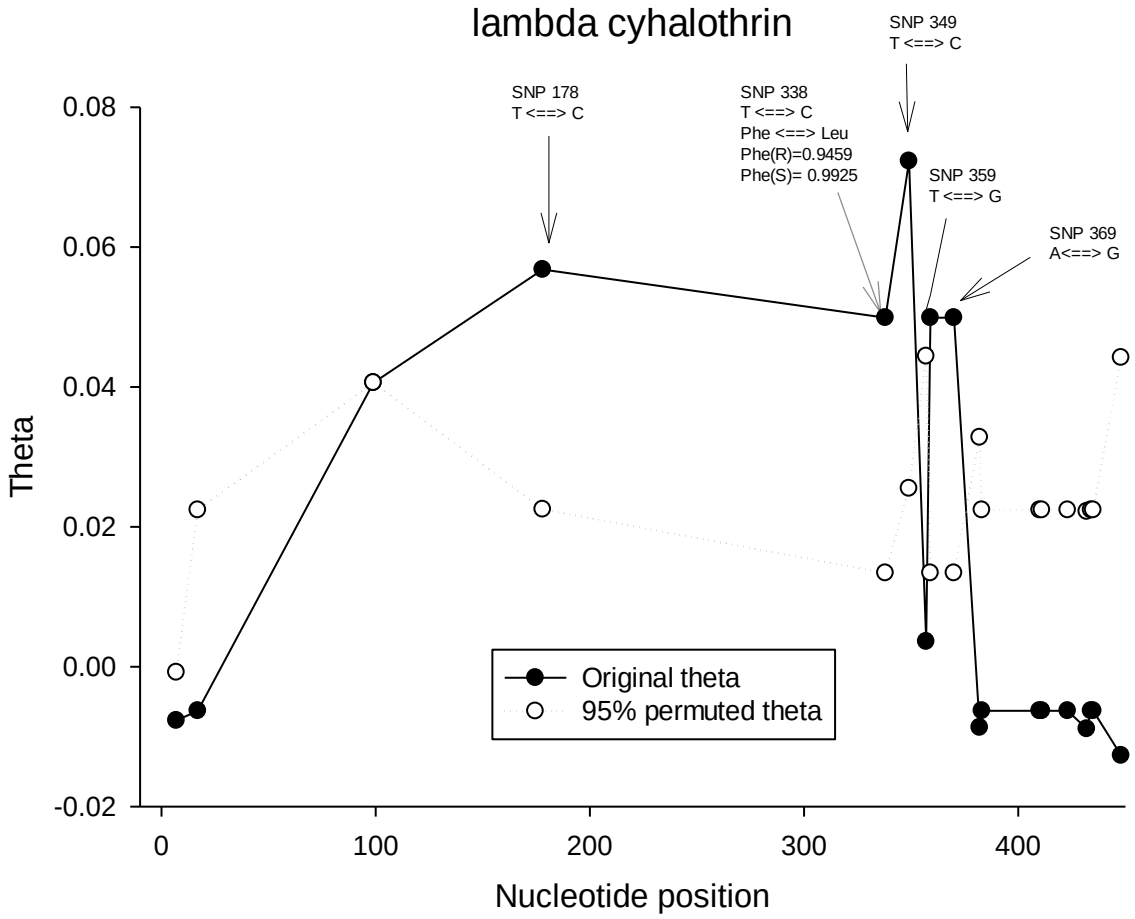

Figure 4.7. Theta permutations of Ngai population of *Xenopsylla cheopis* tested with lambda-cyhalothrin. Single nucleotide polymorphisms (SNPs) are identified and the nucleotide shifts are indicated with double arrows. SNP 338 is the *kdr* site with phenylalanine frequencies expressed by resistant (R) and susceptible (S) populations indicated.

10. Analysis of QTN 178 and 338 with respect to mortality rate

Table 4.6 shows mortality relative to the genotypes at loci 338 and 178 respectively. Despite the fact that only one Leu1014 homozygote was identified, the trend is that Leu1014 homozygotes had greater mortality than Leu1014/Phe1014 heterozygotes which had greater mortality than Phe1014 homozygotes. This trend suggests that

Phe1014 has an additive effect in conferring DDT resistance, indicated by the survivorship gradually increasing with the addition of just one Phe allele. Interestingly, mortality associated with the alternative alleles at SNP 178 from lambda-cyhalothrin exposure was counter to expectations with the lowest mortality in C homozygotes, intermediate mortality in C/T heterozygotes and the greater mortality in T homozygotes. However this observation is weak because of the very few C homozygotes and heterozygotes available.

Table 4.6. Mortality statistics for polymorphic nucleotide sites 338 and 178 associated with six different genotypes of *Xenopsylla cheopis* potentially exposed to DDT and/or lambda-cyhalothrin. Leu=leucine, Phe=phenylalanine, C=un mutated nucleotide, T= mutated nucleotide. Numbers in parentheses are died/tested

		DDT	Lambda-cyhalothrin
Site 338 Genotype	Leu/Leu	100% (1/1)	0% (0/1)
	Leu/Phe	80% (8/10)	50% (2/4)
	Phe/Phe	49% (77/158)	80% (132/166)
Site 178 Genotype	C/C	100% (2/2)	0% (0/1)
	C/T	80% (8/10)	50% (3/6)
	T/T	48% (76/157)	80% (131/164)

.

Table 4.7 shows the frequency of the distribution of the Phe 1014 allele in all the populations. These were tested for their fit to Hardy-Weinberg expectations and F_{IS} was calculated as 1-(heterozygotes(observed) / heterozygotes (expected)). F_{IS} is positive when there is an excess of homozygotes and negative when there is an excess of heterozygotes. Colony fleas had the lowest Phe1014 frequency with zero while the Ngai fleas had the

highest frequency with 0.96. The Ngai population has an insecticide exposure history to both DDT and lambda-cyhalothrin. The Arua population, allele frequency 0.187, which had no IRS exposure but had the potential of secondary exposure via daily pyrethroid treatments of visitor lodging quarters proximal to the rodent hosts. The Lemo and Lagaya populations with IRS histories of pyrethroid exposure had an average frequency of 0.95.

F_{IS} was positive and significant in Arua, Lemo and Ngai. In each case this was due to an excess of Leu1014 homozygotes suggesting the possibility of reverse selection acting on Phe1014 heterozygotes and homozygotes.

Table 4.7. Frequency of *kdr* allelic combinations at the 1014-equivalent site in the tested *Xenopsylla cheopis* populations. Leu=leucine allele. Phe= phenalalnine allele. F_{IS} is the correlation among uniting gametes from individuals (I) in each subpopulation (S).

Population	Leu/Leu	Leu/Phe	Phe/Phe	Total	Phe Allele frequency	F_{IS}	Prob
Colony	30	0	0	30	0.000	0.000	1.000
Climate	23	6	1	30	0.133	0.135	0.461
Arua	12	2	2	16	0.187	0.590	0.018
Lemo	3	4	81	88	0.943	0.576	0.000
Lagaya	1	7	82	90	0.950	0.181	0.085
Ngai	5	21	366	392	0.960	0.295	0.000

D. DISCUSSION

When strongly beneficial SNPs arise in a population, for instance those conferring insecticide resistance in the presence of exposure to insecticides, the frequency of the

beneficial allele will increase. When selection for the beneficial allele is strong, neutral loci linked to the beneficial allele will also increase in frequency because selection will increase the frequency of the beneficial SNP and all linked SNPs on the same chromosome faster than recombination can place them on opposite chromosomes (Pavlidis et al, 2008). This "genetic hitch-hiking" results in decreased allelic variation near the site of selection since only the chromosome bearing the novel beneficial mutation will survive (Ennis, 1984). As the beneficial allele goes to fixation, this phenomenon is best known as a selective sweep, which will also increase linkage disequilibrium (Black et al, 2008). Most genes subject to selective sweeps are associated with strong ecological adaptation.(Pavlidis et al, 2008). Studies with *Anopheles gambiae* Giles linking insecticide resistance to reductions in genetic diversity due to selective sweep pressures typify current findings (Lynd et al, 2010; Ranson et al, 2000b; Ranson et al, 2004) .

Four trends in our data suggest that the Phe1014 allele has lead to a selective sweep of the *X. cheopis* genome in regions of Uganda where insecticide exposure has occurred. First, the Phe1014 SNP was most frequent in regions with the greatest insecticide exposure (e.g. Ngai) and lowest in populations with the least insecticide exposure (e.g. Climate). Second, haplotype and genetic diversity indices were greatest in regions with the least insecticide exposure (e.g. Climate) and the least diversity was observed in regions with the greatest exposure (e.g. Ngai). Third, linkage disequilibrium was greatest in regions with the greatest insecticide exposure (e.g. Ngai) and lowest in populations with the least insecticide exposure (e.g. Climate). Fourth, phylogenetic analysis also exhibited a pattern highly characteristic of selective sweep pressures. The

susceptible haplotypes were clustered at the basal location on the tree and the resistant haplotypes were all located at the distant branches of the tree. There were very few sequences where the two haplotypes overlapped on the tree.

Phe1014 appeared to have an additive effect in that the Leu/Phe heterozygotes demonstrated a stronger knockdown recovery to DDT, used 2008 in Ngai IRS, than the Leu/Leu homozygotes, but not as strong as the Phe/Phe homozygotes. Curiously, the opposite effect occurred for the mutation(s) in lambda-cyhalothrin exposed fleas where a decreased survival was associated with the Phe mutation in both heterozygotes and homozygotes. Possibly this is a result of the more persistent insecticide, DDT, selecting for mutational resistance development more quickly and selection of other mechanisms in pyrethroid resistance leading to selection in lambda-cyhalothrin. Maybe there is an initial fitness cost associated with the mutation. The number of fleas collected with the susceptible genotype was very low, which could have artificially skewed the data. Further work needs to be done to better understand the results.

The specific flea index, or number of fleas per rodent is an indicator of the risk of plague. An index over 1 is regarded as indicative of high potential for plague transmission (Bahmanyar & Cavanaugh, 1976). Flea indices in collection sites with a high potential for exposure from IRS programs ranged from 2.29 - 5.21 while sites with little or no exposure had a values from 0.01 - 1.07. Clearly, while IRS possibly mitigates the risk of malaria, it has the reverse effect on the potential risk of plague.

Flea control is likely to be the method of choice for controlling plague outbreaks in Uganda and other plague-endemic regions. An alarming aspect of my results suggests that resistance can develop quickly in Ugandan populations of *X. cheopis.* Further

investigations need to be done to elucidate the temporal aspect of development of resistance, the mechanisms and degree to which the fleas are actually exposed to insecticides and to sequence other possible target site mutations.

SUMMARY

Insects have been developing resistance to insecticides as long as humans have been trying to poison them. Scientific developments have enable us to better monitor and understand the mechanisms insects use to circumvent us. Integrated management systems must be implemented to prevent or delay resistance to the toxins. In the case of insect vectors of disease, proper management could literally be a life-or-death situation.

Plague a potentially fatal disease vectored by fleas infected with *Yersinia pestis*, overlaps with malaria in some parts of the world. If the fleas develop resistance secondarily as a result of exposure to insecticides used in indoor residual spraying (IRS) programs, controlling an outbreak could be difficult or even impossible.

To this end, this study undertook the development and field testing of a new resistance bioassay for fleas, tested for detection of metabolic resistance mechanisms in *Xenopsylla cheopis*, a very competent flea vector of plague, and then characterized and analyzed the genetic ramifications of knockdown (*kdr)* resistance in the sodium channel *para* gene of *X. cheopis* in northern Uganda. Some of the fleas had a known potential for exposure to insecticides from IRS in dwellings.

The bioassay used glass insecticide treated Petri dishes as the medium for evaluating phenotypic expression of resistance. The Ugandan fleas were tested against DDT and a pyrethroid based on recent local IRS program initiatives. This test, after refinement, proved to be rapid, inexpensive, field-expedient, easy to perform, and provided fairly accurate information regarding resistance in the fleas. This type of assay is well suited for developing countries where maintaining a cold-chain is difficult, and electricity and funding are in low supply. Discriminating doses of insecticides tailored to reflect local use history should be assessed on a local naïve population of fleas to give a

better true baseline value. Assay could also be run longer than the 60 minutes used in this study to better evaluate severity of possible knockdown resistance recovery events.

Measurement of the detoxifying enzyme profiles expressed by the tested flea populations showed that field caught fleas had increased levels of all enzymes as compared to colony fleas. Within field caught fleas the population potentially exposed to insecticides had an increased mean level of expression of enzymes over the unexposed, especially α and β-esterases. The one exception was the serendipitous discovery that the (putatively) unexposed fleas had increased levels of insensitive acetylcholinesterase (iAChE). The possibility of exposure and resistance to organophosphates and/or carbamates in this population needs further investigation. With the exception of iAChE, there is evidence of the Ugandan fleas exposed to DDT and pyrethroids having increased levels of detoxification enzymes.

Genetic analysis of the *para* voltage gated sodium channel gene in *X. cheopis* focused on knockdown resistance (*kdr)* and associated segregating sites. The classic *kdr* site mutation was found to dominate the haplotypes of the exposed flea populations. There was ample evidence of reduced nucleotide diversity, numerous segregating sites, clustering of linkage disequilibrium sites, - all evidence of classic selective sweep mechanisms at work. Genotypic analyses compared with phenotypic data collected with the Petri dish assay clearly linked the resistance associations. Curiously, *kdr* mutation did not confer increased survivorship to lambda-cyhalothrin as it did for DDT. DDT also demonstrated an additive effect for survival. Perhaps this is a function of higher selective pressure from the more persistent insecticide (DDT), a longer history of potential exposure, or alternate mechanisms working in concert The number of susceptible

homozygotic fleas tested was very low, so that could have a confounding effect. This phenomenon needs further investigation.

Although the biochemical assay gives a gross analysis of enzyme activity in the fleas, it should be used to buttress other findings of resistance as ways to definitively ascertain their role in resistance are more difficult to process.

The plate assay if a good "quick and dirty" field assessment tool, especially for developing countries with limited resources, enabling real-time monitoring of resistance. For irrefutable evidence of *kdr* and the extent to which it has permeated the population's genetics, PCR based assays are superior. Unfortunately, this technology might be out of the reach of the countries that need it the most.

Clearly, resistance is developing rapidly in the non-target Ugandan flea populations. If it is here, where else is it and how are we going to monitor and manage it? Ongoing malaria control programs continue to have the potential to put selective pressures on fleas to develop resistance. If funding were no option, a multiple faceted approach could be taken starting with education and enabling of local populations of people at risk. Mitigation of rodent/human interface attractants would reduce the potential for exposure to insecticides by the fleas that live in rodent nests in roof thatch. Dish assays could be performed on a regular basis by trained trapping crews. Local vector control officers could send fleas to a central well-stocked lab for PCR analysis. Decisions on what insecticide to use in IRS programs by public health officials could be adjusted accordingly to the results of these tests.

In the event of a plague outbreak, rapid reduction of the flea vector is the first concern. Even if there had been no local ongoing monitoring of fleas for resistance, a

rapid reaction team (e.g. the U.S. Army) could use the dish assay with different insecticides to quickly give a rough evaluation of susceptibility/resistance in the fleas. This information could guide the choice of insecticide(s) chosen to suppress the outbreak rapidly enough to protect the humans at risk.

The triumvirate of tests described in this study provides evidence for the importance of detecting and monitoring for development of insecticide resistance in fleas and public health interests should provide the impetus. The risks associated with the lack of ability to control an outbreak of plague could be addressed in a proactive manner.

APPENDIX 1

INDEPENDENT STUDY

TABLE OF CONTENTS

September 11, 2009
With Jeff Borchert and Scott Bernhart

Rodent Trapping and Release
Flea Collection

Traps used: Tomahawk live trap.

Bait: Creamy peanut butter mixed with raw oats. About 2 tablespoons was loosely wrapped in wax paper. Packet was placed in rear of traps.

Area: Local (Masonville) active rock quarry.

Weather: Fair, mild temperatures (~ 73°F) partly cloudy.

Rodent species of interest: *Spermophilis variegatus* (Rock squirrel). Preferred habitat is open rocky areas; oak-juniper growth in canyons.

Traps were placed on and around rock piles with either used-looking burrow runs (presence of feces and disturbed soil) or in the area of an actual rock squirrel sighting. Traps were checked approximately every 30 minutes. The area was plentiful with suitable habitat and a mild, wet summer resulted in significant food availability. Sunflower seed hulls were observed on rocks in areas with known activity.

Ultimately, three squirrels were trapped in eight hours. They were put into a tool box modified to act as an anesthetic chamber, complete with a plexiglass observation window. Isoflurane vapors were pumped into the box after a rodent, still in the cage, was placed into it. An adequate level of sedation was assessed at one breath per second. The squirrel was then removed from the chamber and then from the cage. Personnel wearing Personal Protective Equipment (PPE) then combed the fleas off the squirrel by suspending it by its tail over a plastic wash basin, combing vigorously with a commercial pet flea comb and allowing the fleas to fall into the basin. The squirrel was released at the trap site when it started to recover from the anesthesia. Fleas were collected with forceps from the anesthetic chamber and the wash basin, and then put into tubes of 70% alcohol for future identification and processing.

Observations: Colorado is the northern edge of the rock squirrel's range and Masonville seems to be on the eastern fringe of its distribution. Even though numerous rock squirrels were seen in the various habitat zones in the quarry, very few were actually caught in the traps. The squirrels did not seem overly cautious about human encroachment though. I think perhaps pre-baiting to accustom the rodents to the novel food source might increase chances of success. Some experienced rodent trappers swear by the addition of bacon grease to the bait.

Some traps were set on top of a rock with no surrounding cover by one experienced person. None of these traps caught a squirrel, but squirrels that were observed eating seeds were out in plain sight. Squirrels that were caught were of good body condition, and their pelage was thick. Combing met with mixed results. The lead researcher was hoping for an average of 10 fleas per rodent, but the numbers ended up with more like 4 or 5 on the average.

Figure 1. Tomahawk® wire traps used in the study are loaded with wax paper packets of bait. Plastic flagging tape on the traps increases visibility for recovery and does not seem to negatively affect rodent attraction to the traps.

Figure 2. Masonville, Colorado rock quarry. Typical habitat of *Spermophilis variegatus* (rock squirrel) in its northeastern fringe of its distribution in Colorado. The open rocky areas of the quarry were ideal for this rodent's environmental preferences. Cages were placed on unprotected, open areas of these rocks after observing rock squirrels feeding on top of them.

Figure 3. Trapped *Spermophilis variegatus* (rock squirrel). This shows the typical size and habitat of the rock squirrel in northern Colorado. Behavior in the cage was erratic but not aggressive.

Figure 4. Homemade anesthetic box. A plastic toolbox was modified by drilling holes, inserting plastic tubing attached to a hollow cylinder for cotton balls infused with inhalant anesthetic, which was circulated by a small air pump attached to the alternate side of the cylinder. Power was provided through the cigarette lighter attachment of the vehicle. The box could accommodate the entire wire trap so rodent handling prior to anesthetization was minimized.

Figure 5. Combing fleas off an anesthetized rodent. Rodents are carefully removed from the anesthetic box and the trap, quickly inspected for general health, held by the rear quarters, and combed for fleas. Small plastic human comb was used in a downward fashion into a deep, light colored basin. Personal protective equipment includes, at a minimum, thick gloves.

September 14, 2009
With Leah Colton

Filling Insecticide Treated Bait Stations
(part of a Bartonella control study)

Stations: Protecta brand bait stations in preset areas

Bait: Imidacloprid-laced blocks with whole oats

Area: Foothills behind CDC campus

Weather: Fair, quite warm (~82°F)

Rodent species of interest: *Neotoma mexicana*, Mexican woodrat. There is a thin strip of habitat in central Colorado along the foothills of the Rockies. Preferred habitat type is rocky situations in mountains; ponderosa pine forests and pinon-juniper areas.

Traps were located on the slopes by using marker tape and flags. The amount of bait consumed was annotated, as well as the presence or absence of nesting material in the station. Bait was then replaced in the stations with an unsecured piece added in to encourage the rodents to transport the bait back to the nest.

The researcher's hypothesis is that eliminating or controlling the flea population on Mexican woodrats will reduce the prevalence of Bartonella spp. in the rodent population. A prior study performed last year indicated a 100% infection rate in the woodrats. (unidentified species of Bartonella)

Researcher's protocol is based on prior trapping of the woodrats, assessing levels of flea infestations and the extent of Bartonella infections. Bartonella is detected by retro-orbital bleeding of the rodents and then the blood is later tested in the lab. The trapped woodrats are released. The imidacloprid (an insecticide) laced (at 250 ppm) bait is then put out for constant consumption. Rodents are captured monthly during the study. Levels of flea infestation and Bartonella infection are again assessed.

Week of 2-5 Nov

Actual Rodent Trapping and Flea and Blood collection

Traps: Tomahawk filled with a generous amount of polyfill for nesting (cold protection)

Bait: Fresh carrots, shell corn

Area: Same area as had bait stations behind CDC

Weather: Cool evenings (never lower than 28°F), sunny days

Rodents trapped : *N. mexicana*

Traps were filled with polyfill and bait and set open overnight. Traps were then checked for presence/absence of wood rats at daybreak. Three areas were trapped. Two were areas that had been populated with imidicloprid-laced bait stations all summer and one (the control) had no bait fed. Tomahawk traps had been in place all summer for rodents to acclimate to their presence.

Trapped rats were then transported (in the cage) to the processing area nearby. The rats were first anesthetized with isoflurane gas in a box and then maintained with a flow-by mask mixture of oxygen and isoflurane. Rats were combed for fleas and fleas were collected. Rats were then sexed (all were males), weighed, and scanned for the presence of a microchip. All trapped rats have an Avid microchip inserted subcutaneously, dorsad, behind the head before being released. Presence of a microchip indicated rat had previously been captured (either during the current week or early in the summer when another trapping program was run).

Rats were then bled peri-ocularly with a micro hematocrit tube. Blood was to be analyzed later for presence/absence of *Bartonella* spp.. Rats were recovered in their cage at the processing site and then returned to the same area in which they were trapped.

Traps were set out three sequential nights. Only woodrats in the unbaited control area were caught for the first two nights. Some were re-captures. On the last night, one woodrat from baited area ‘C’ was captured. Several theories abounded for the dearth of captured ‘treated’ rats. One was that there was a plague die-off. There had been 3

incidental woodrat deaths in the trapping project, so, as I had formal necropsy training, I performed necropsies (report included) on each rodent and then harvested the liver and spleen from each of the deceased rats. They tested negative for *Yersinia* by IFA. They were all from the untreated area that was resulting in live captures, so the results could realistically not reflect possible problems in the baited areas. The excellent body condition of the remainder of the organs, except the heart and lungs, of the rats indicated that cause of death was probably cardio-respiratory arrest.

My personal theory for the trapping failure is twofold. One, they had no incentive to enter the traps. Loose bait had been left in the Protecta traps quite frequently and I think they had stockpiled large amounts of it in their nests and the carrots were not all that enticing. (woodrats are a pack rat) Second, the Tomahawk traps had been left out all summer and the foot pans were not as responsive to pressure as they should have been. Some of the bait and stuffing had actually disappeared out of the traps in the baited areas without the trap being sprung. Maybe mice had taken the contents or maybe rats did, but the footpan was not activated.

The changes I would have made to increase the chances of success are: 1) Service the Tomahawk traps to ensure proper function, 2) Not to have left loose bait, 3) Pull up the Protecta bait stations at least 1.5-2 weeks prior to trapping, and, 4) Use more attractive bait in a less portable package (e.g. peanut butter and raw oats twisted up in a wax paper packet like was used for the rock squirrels).

4 Nov 2009

Dr. Abbe D. Ames, DVM

Necropsy Report for Three Wild-Caught Woodrats (*Neotoma mexicana*)

All three woodrats were caught in humane type Tomahawk traps in the Foothills area immediately adjacent, and to the west, of the CDC Campus, Fort Collins, Colorado and only one was alive when brought to the processing area. Woodrat #1 was caught on the night of 2-3 Nov and had expired in the trap. The remaining two were caught on the night of 3-4 Nov and expired that date. Woodrat #2 had expired in the trap and woodrat #3 expired after processing. Ambient temperatures were in the mid-to-low 50*'s. Cloud cover was thin. The third rodent expired after having been subjected to box and face mask delivered isoflurane gas anesthesia. It was not conscious at the time of death. All were necropsied on 4 November, 2009 by a veterinarian present (the author).

Woodrat #1: Rodent was a male, approximately 6.5 inches long. Pelage was thick and even. Teeth were yellow but unbroken and of normal length. Body condition score was normal. No lymph node enlargement. No obvious external signs of trauma evident. As rodent had been expired approximately 24 hours and left in chilly ambient conditions, body was slightly into rigor, but was still malleable and no obvious signs of necrosis were evident. Only a ventral longitudinal incision was made from the pharyngeal area to the anus.

Thoracic cavity: Right atrium of heart was occluded with a large hemorrhage, otherwise normal. Lungs had disseminated foci of deeply reddened patches, were diffusely red and heavy. Surface glossy. No foam or fluid oozed on cut surfaces. Discolorations penetrated the parenchyma. Large area of hemorrhagic tissue likely dependant postmortem change. No other abnormalities noted.

Abdominal cavity: No gross abnormalities noted. Stomach was moderately full and contents were partially digested food material. Liver, spleen, kidneys, remainder of intestinal tract within normal limits (WNL). Fecal pellets noted in colon. Small amount

of retroperitoneal fat present lateral to spinal column. Liver and spleen were sampled for testing.

Woodrat #2: Rodent was a male, approximately 6 inches long. Pelage was thick and even. Teeth were yellow but unbroken and of slightly shorter length. Body condition score was normal. No lymph node enlargement. No obvious external signs of trauma evident. Ectoparasites had been removed. One incision on the ventrum from the pharyngeal area to the anus was made.

Thoracic cavity: Heart was WNL. Lungs were glossy, heavy, and diffusely light red. Patches of locally extensive hemorrhage penetrating the parenchyma were present bilaterally. No liquid or foam oozed on the cut surface.

Abdominal cavity: No gross abnormalities noted. Stomach was very full and contents were partially digested plant material and carrot. Liver, spleen, kidneys, remainder of intestinal tract (WNL), all were transected. Fecal pellets noted in colon. Small amount of retroperitoneal fat present lateral to spinal column. Liver and spleen were sampled for testing.

Woodrat #3: Rodent was a male, approximately 6 inches long. Pelage was thick and even. Teeth were yellow but unbroken and fairly long. Body condition score was normal. No lymph node enlargement. No obvious external signs of trauma evident. Ectoparasites had been removed. One incision on the ventrum from the pharyngeal area to the anus was made.

Thoracic cavity: Heart was WNL. Lungs were glossy, heavy, and diffusely light red. Frequent patches of locally extensive hemorrhage were present bilaterally. No liquid or foam oozed on the cut surface.

Abdominal cavity: No gross abnormalities noted. Stomach was moderately full and contents were partially digested plant material. Liver, spleen, kidneys, remainder of intestinal tract WNL. Fecal pellets noted in colon. Small amount of retroperitoneal fat present lateral to spinal column. Liver and spleen were sampled for testing.

Figure 6. Rodent processing station in the field.

Figure 7. Woodrat (*Neotoma mexicana)* in Tomahawk trap.

Figure 8. Rodent in Tomahawk trap with cotton batting for thermal protection, and carrot pieces for sustenance.

Figure 9. Performing peri-ocular blood collection on a rodent with a microhematocrit glass tube into a 1.5 ml plastic screw-top tube.

Figure 10. One researcher combing rodent while another holds small funnel mask for inhalation of anesthetic gas.

14 Sept 2009

With Marv Godsey

Mosquito Trapping in Loveland

Traps: CDC miniature light trap altered to a Model 1512 John Huck rotator bottle trap.
Bait: CO2 and light. CO2 rate of flow is 0.5 L/min from a 20lb. tank.
Area: SE Loveland, low drainage area with willow thickets and scrub trees.
Weather: Fair, temperature ~ 75°F, calm.
Species of interest: *Culex tarsalis, Aedes vexans, Ae. dorsalis,* and *Ae. melanimon*.

Traps were set in late afternoon/early evening. There were 2 light traps and one weather station per site. There were 2 sites. Traps were a CDC miniature light trap modified to accommodate rotating plastic bottles under the collection fan apparatus. The jars are set to rotate every 28 minutes. This time is variable with the time available between sunset and civil twilight. A small patch impregnated with insecticide is in the bottom of each bottle. The weather station records temperature, humidity and wind speed on a constant basis. This juxtaposition of data gathered in a predictable temporal sequence allows the researcher to correlate time, weather conditions and relative mosquito abundance in each collection site. Traps are left in place 48 hours with bottles changed out in 24 hours. Identification and processing of mosquitos will take place at a later date. Researcher is assessing the levels of West Nile virus in the local mosquito populations.

Figure 11. Rotator bottle trap assembled. Compressed air bottle contains carbon dioxide.

Figure 12. Weather station attached to traps.

Figure 13. Close-up of bottle rotator with light attached to the top.

16 Sept 2009

With Marv Godsey

Mosquito Trapping Fort Collins

Traps: CDC miniature light trap altered to a Model 1512 John Huck rotator bottle trap.

Bait: CO2 and light. CO2 rate of flow is 0.5 L/min from a 20lb. tank.

Areas: Central Fort Collins by Mulberry and Riverside; NW Fort Collins, residential area at the edge of Redwing Marsh.

Weather: Warm, temperature ~ 86°F, calm.

....Species of interest: *Culex tarsalis, Aedes vexans, Ae. dorsalis,* and *Ae. melanimon*

Traps were set in late afternoon/early evening. There were 2 light traps and one weather station per site. There was 1 site. Traps were a CDC miniature light trap modified to accommodate rotating plastic bottles under the collection fan apparatus. The jars are set to rotate every 28 minutes. This time is variable with the time available between sunset and civil twilight. A small patch impregnated with insecticide is in the bottom of each bottle. The weather station records temperature, humidity and wind speed on a constant basis. This juxtaposition of data gathered in a predictable temporal sequence allows the researcher to correlate time, weather conditions and relative mosquito abundance in each collection site. Traps are left in place 48 hours with bottles changed out in 24 hours. Identification of mosquitos and processing will take place at a later date. Researcher is assessing the levels of West Nile virus in the local mosquito populations.

Figure 14. Researcher assembling modified rotator traps at wooded/cleared zone.

Figure 15. Power line biome where traps were set in northeast Fort Collins, Colorado.

Figure 16. Homeowner observing mosquito collection traps being assembled in their backyard.

Figure 17. Rotator bottle traps set at the yard/marsh interface zone.

16 Nov 2009

With Marvin Godsey

Mosquito Identification

Prior to meeting with Marv, I did both the larval and adult mosquito identification DVD from the AFPMB. The larval ID was pretty straightforward and understandable. The adult DVD was packed with a lot of morphological/anatomical terms and information and I spent a considerable amount of time on learning that before I continued onto the practice IDs. I found the instant feedback particularly helpful and gratifying!

Marv started out by instructing me on proper handling and preservation of the mosquitoes in the lab. He uses a dissection microscope on a cold table (protected with a white towel). The insects are put in a petri dish with a moistened filter paper on the bottom. This protects from premature dessication and reduces the possibility of them blowing off with a stray breeze. After they are identified, they are sorted into smaller petri dishes with similarly moistened filter paper. (I labeled my petri dishes so I could keep track of the different species).

Since Marv has locally collected mosquitoes, we used the Darcie and Ward key as the reference. Marv started the session with a home-made tutorial that he drew out for me (included). He starts keying out the mosquitoes by identifying the abdomen as pointed or blunt. He had examples of each to illustrate the characteristics. Common *Aedes* spp. and *Psorophora* spp. have the pointed abdomens and *Culex* spp., *Culiseta* spp., and *Coquillettidia* spp. have the blunt abdomen. We reviewed several examples under the microscope prior to proceeding more in-depth. We then went over the anatomy of what he considers the most important identifying characteristics for mosquitoes here (e.g. pre- and post-spiracular setae, setae on the scutellum, basal vs apical banding on the legs and abdomen, nuances of scale patterns, etc.). Then Marv brought out several frozen mosquitoes collected in Larimer County. We initially used the Darcie and Ward key to become more familiar with it and the couplets leading us to the identifications. Then we used his homemade 'key' to identify the same mosquitoes. His quick-and-dirty key worked very well! Then he turned me loose with a vial of mosquitoes and let me ID

them by myself. He was happy to help me with any questions I had. After I got them done he checked my results. The one I had a problem with was *Culex pipiens* vs *Cx. restuans*. The abdominal banding patterns and scutellar spots interpretation was a bit subjective, I thought. The predominant mosquitoes he has collected here are *Aedes vexans*, *Culex tarsalis*, and *Cx. pipiens*. I continued to identify mosquitoes for about another hour and a half. I returned on the 18th and ID'd for three hours. I got very comfortable with identifying the local mosquitoes. I liked Marv's quick ID key and then it was all about familiarization by repetition after that! Processing a lot of mosquitoes also improved my handling skills so I could manipulate them without damaging them.

18 November

With Marvin Godsey

Mosquito Processing

After we identified the mosquitoes, Marv assigned them to ‘lots’ according to their ID and time and location collected. The lot sizes were up to 50 mosquitoes. Each batch was put in to a 2.0 ml tube with one copper BB (the kind that is ammunition for air guns) and 1.75 ml of BA1 media is added. In a biosafety hood, the tubes were loaded into a rack that was clamped into a Qiagen Mixer Mill (now called a Qiagen Tissuelyser) and agitated for 4 minutes at 21cycles/second. Samples are removed and then cold centrifuged. Protocol is included in workbook. Aliquots of 200 microliters were drawn off each sample for virus testing later and the remainder was frozen at -70*C.

CDC Protocol for Mosquito Grinding
Using the Qiagen Mixer Mill
Last updated 3-15-2007

Supplies Needed

1. 2.0 ml AXYGEN microtubes, clear, homoplymer, boil-proof. VWR International, 1310 Goshen Parkway, West Chester, PA 19380. 1-800-932-5000. 5,000 tubes per box at $225.73, catalog #10011-742.

2. BB'sCopperhead (2500 premium grade BBs) Airgun Shot, Steel BB Cal. (4.5 mm) East Bloomfield, NewYork 14443 (Can be bought at sporting goods stores, WalMart, Kmart ect.).

3. Qiagen Mixer Mill 300 (now sold as Qiagen Tissuelyser) with 2 x 24 adapter plates for 2 ml microcentrifuge tubes (will not hold Fisher brand 2.0 ml microtubes).

PROTOCOL (must be done in a biosafety cabinet):

1. Put one BB and up to 50 mosquitoes in a labeled 2.0 ml tube with 1.75 ml of cold diluent (we use BA-1 media to allow isolation of live virus). With large-bodied mosquitoes you may have to load half of the individuals, half of the media, then the remainder of the mosquitoes and media.
2. Load the sealed tubes into the appropriate Qiagen plastic tube racks (one container holds 24 samples) and install on mixer mill unit according to the equipment instructions. Be sure to put the mixer mill in a hood for grinding mosquitoes and unloading containers. Make sure to cap the tubes tightly-we have had leaks in the past. Plastic tube racks can be refrigerated before use to help maintain the cold chain, do not refrigerate lids and this will ease later removal.
3. Run mixer mill for 4 min at 25.0 cycles/second.
4. Centrifuge 3 minutes at 8,000 rpm in a refrigerated microcentrifuge (We have tried higher RPMs and longer times and have found the BB may protrude from the bottom of the tube and cause it to leak). Centrifuge using an aerosol-block rotor or in a biosafety cabinet. EEA (Ecology & Entomology Activity) also has the use of a Qiagen Sigma Centrifuge 4K15. Protocol has been modified for this centrifuge as follows: 5700 RPM, 4 minutes at 4°C.

5. Original samples are stored at -80°C. We do not remove the BB from the tube for subsequent processing. We have found that it does not interfere with live-virus isolation, RT-PCR, or long-term storage at -70ºC or -80°C.

Protocols for mosquito trituration

Copper Clad (BB) Grinding (must be done in a biosafety cabinet)

1. Place up to 50 mosquitoes in plastic, capped culture tubes. We use 6 ml, 12x75 mm polypropylene (not polystyrene, they will break) round bottom tubes (Falcon #2063 from Becton Dickinson).

2. Place 4 copper-clad steel beads (BBs) in tube. BBs are round copper clad air gun pellets available at most discount, hardware, or sporting goods stores. BBs may be autoclaved and dried thoroughly prior to use, though we have had no problems using BBs that were not autoclaved.

3. Add 2.0 ml of BA-1 or other grinding diluent into tube with mosquitoes. Close cap tightly. Vortex 45 sec to 1 min until slurry is formed. Hold tube at a 45 degree angle.

4. BBs may be removed from the homogenate with a magnet or homogenate may be decanted into a centrifuge tube by holding a magnet to the tube while pouring off. However, BB removal from the ground pool material is optional. Preliminary testing indicates that long-term frozen storage of the homogenate with the BBs does not affect virus titer or detection by EIA, RT-PCR, or virus isolation in cell culture.

5. Centrifuge tubes at 6,000 rpm for 15 minutes to separate solid material from supernatant. Aliquot clarified suspension as required for virus testing.

6. At this point, mosquito homogenate may be frozen at -70 C until tested. Preferably, testing should be done prior to freezing to avoid titer reduction from freeze/thaw.

Mortar and Pestle Grinding (must be done in a biosafety cabinet)

1. Place 1-50 mosquitoes in a sterile, cold mortar. Add a small amount of alumdum, an inert abrasive, to aid in grinding.

2. Have ready a rack with aliquots of 2.0 ml of cold diluent.

3. Add ~ 0.5 ml of cold dilute to mortar and grind mosquitoes for approximately 30 seconds. Add remaining diluent and continue grinding until mosquitoes are completely macerated.

4. Pour material into 1.7 ml microcentrifuge tubes.

5. Spin tube at 6,000 rpm for 20 min in refrigerated centrifuge.

6. Transfer aliquots of clarified supernatant as desired for virus testing. Store aliquots and pellets with remaining supernatant at -70ºC.

Plaque Reduction Neutralization Test (PRNT) 1:10 Antibody screen

Principle: If neutralizing antibody is present in sera and mixed with a known concentration of virus, the virus cannot attach to cells; infectivity is blocked

Methods:

1. Calculate appropriate virus dilution (working dilution)
    a. Want challenge dose to be 100-200 pfu/0.1ml

2. Mix working dilution of virus with sera creating a final 1:10 dilution of seurm in a 96-well plate
    Example: 60 ul of whole serum
    15 ul of BA-1
    75 ul of working virus dilution

3. Set up controls
    a. **Positive antibody control:** use 60 ul of a known anti-"virus" antibody (use either 1:10 or 1:100 anti-"virus") and mix with 15ul of BA-1 and 75ul of working virus dilution
    b. **Negative antibody control:** mix 75ul of working virus dilution with 75ul of BA-1
    c. **Back titration:** dilute virus to yield 100pfu/.1ml, 10pfu/.1ml, and 1pfu/.1 ml. (This will be plated in duplicate….so need at least 200ul of each dilution)

4. Incubate 96-well plate
    a. 37 degrees for 1 hour **OR**
    b. 4 degrees (fridge) overnight

5. Add 100ul of each mixed sample to Vero cells in 6-well plate. (Add back titration in duplicate)

6. Incubate Vero plates at 37 degrees Celsius for 1 hour

7. Make 1st overlay (M199 or Barry's). Directions are in the tissue culture room.
    TIP: If the total volume is 1000mls, then make 500mls of 1% agarose and 500mls of media including sodium bicarb. Microwave agarose until it dissolves into solution. Place the media and agarose solutions in 44 degree water bath. DO NOT MIX MEDIA AND AGAROSE UNTIL AGAROSE IS COOLED DOWN…..AT LEAST 15 MIN. Otherwise the boiling agarose will denature all of the proteins in the media.

Plaque Reduction Neutralization Test (PRNT) continued…

8. Add 3 mls of overlay to each Vero well. Be careful not to disrupt the cell monolayer.

9. Incubate the plates at 37 degrees.

10. Add 3 mls of 2nd overlay (same recipe as 1st overlay with the addition of neutral red) on appropriate day:
    a. West Nile: add 2nd overlay on Day 2 post-infection
    b. SLE: add 2nd overlay on Day 5 post-infection

11. For two days after the 2nd overlay was added, count plaques. Roughly, anything with > or = 90% neutralization could be considered "antibody positive"
    ie. if challenge dose of virus was 100pfu…anything with 10 or less plaques might be considered antibody positive)

Titrating Antibody Positive Samples

Principle: It is necessary to find the antibody titer of your samples. This will help to determine if your sample is definitely WN antibody positive vs. "flavivirus" positive, etc.

Methods:

1. Make 2-fold serial dilutions of your serum (use enough volume to plate in duplicate).

2. Start with 1:10 and dilute out to 1:320

3. Mix dilutions with virus following same protocol as the 1:10 antibody screen.

4. When interpreting the results, the reciprocal of the dilution of serum that neutralizes the challenge inoculum represents the titer of antibody.
    EXAMPLE: if 1:40 antibody dilution neutralizes virus but 1:80 does not, then your antibody titer would be 40

Mosquito Pool Plaque Assay

Principle: isolating "live" virus from mosquito pools

Methods:

1. Fill out cell culture sheets with information about your samples (See attached for an example).

2. Label each plate with a number matched with the number on the cell culture sheet and put the date of inoculation on one plate from each stack.

3. Prepare hood for inoculations. Pour off media from 6-well Vero plates into sink or discard pan in the hood.
   -leave a "crescent moon" of media on the plate so the cells don't dry out (approximately 300ul)

4. Inoculate 100ul of sample per well (single or duplicate wells)

5. Incubate plates at 37 degrees Celsius for 1 hour in CO2 incubation.
   -this is when the virus attaches to the cells

6. Add 3mls of 1st overlay (M199 or Ye-Lah) per well
   -recipe in tissue culture room and attached

7. Incubate plates in the CO2 incubator at 37 degrees Celsius for:
   -120 hours (SLE)
   -48 hours (West Nile)
   -24 hours (other fast growing viruses)
   (This is when the virus is actually infecting the cells)

8. Add 3mls of 2nd overlay per well
   - this overlay contains Neutral Red
   - recipe in tissue culture room and attached

9. Put plates back in 37 degrees Celsius incubator. Invert the plates when the agarose has hardened.

10. For the next 10 days check the wells and count plaques when they appear

    - Plaques can occur anywhere a virus infects and kills the cells.
    - West Nile plaques are usually somewhat large and appear between days two and four.

- SLE plaques are usually quite small and will appear at about day six.
- Alphaviruses can appear on days two and three.

11. Record the plaque assay results on the cell culture sheets

    a. TCD = Total Cell Death (entire monolayer is dead or lysed)

    b. TNTC = Too Numerous To Count

    c. Or number of plaques counted in each well

    d. If a well is contaminated with mold or bacteria, note the date on the cell culture paperwork at the time of first observation.

12. If necessary, harvest the plaque(s). Cryovials should be labeled according to what you are harvesting. For example, LA03 ####; V1 (refers to first passage on Vero cells); then the harvest date.

    a. If you have only one or two plaques:
        i. Take pipettor with pipet tip and "pick" plaques by scraping around in the well where the plaques are
        ii. Place in 0.5 – 1.0mls BA-1with 20% FBS (AKA Harvest Media)

    b. If you have a lot of plaques
        I. Remove the agarose overlay from the wells with a cell scraper, taking care not to dislodge the cell layer.
        II. Add 1.0ml of BA-1with 20% FBS to each well
        III. Use the scraper to scrape off the cell monolayer in the wells
        IV. Combine the media and scraped cells from both wells into one of the wells (if you are only harvesting one well this is not necessary)
        V. Pipet up the cells and media and divide it (1.0ml each) between two cryovials

13. Fill out a viral harvest card

14. The identity of the virus will be confirmed by RT-PCR.

    - Take the original pool material + the two cryovials of harvested virus and place them in the *isolated viruses* box in the -86C ULT Freezer in 213C

10 December 2009

With Marvin Godsey

West Nile RNA Purification and Real Time PCR

Marv and I sat down to the BioRobot 9604 machine to process samples previously extracted from local, rotator trapped mosquitoes. The machine is capable of processing a 96 well plate in about 2.5 hours. This procedure is used to purify viral RNA from samples of ground up mosquitoes. Marv will then process the samples with real time PCR looking for West Nile virus in the mosquitoes. (Each sample represents a 'batch' of up to 50 mosquitoes)

We used a Qiagen kit. We mixed a lyophilized protease with a commercial buffer. We then mixed a lysis buffer and carrier RNA (aids precipitation). The samples had 200ul of supernatant in them. The machine will automatically use wash buffers 1 and 2 and ethanol during the process. No phenol or chloroform extraction is used.

We placed the 4 tubes of protease buffer in the appropriate slots and the 4 tubes of lysis buffer in their slots. The samples (#88) and controls (#4) (positive and negative) are opened and placed in a metal block. Ethanol and the wash buffer bottles were topped off. Special tips are stocked in their spot. Three special tube trays are placed in their blocks. These will eventually hold the purified samples. Two are simple tube trays and the other is a vacuum membrane tube tray. The machine is capable of several different programs and Marv ensured that the right one was chosen.

The machine then cycles through several rinse cycles with de-ionized water to purge the lines. It withdraws 50ul of sample from each tube, puts them into another well and then subjects them to a cycle of lysing, binding, washing twice, and eluting. Literature claims >90% nucleic acid recovery at any virus titer.

Purified samples were then used for real-time PCR. We mixed the master mix (kit), primers, probe (both manufactured by the CDC), and sample according to the recipe. The mix was then loaded (50ul) into another tray and placed on the thermal cycler with the proper program. For a sample to be deemed positive for West Nile virus, its

curve must be above the established level at 34.8 cycles. Our particular batch had no positive samples, the controls worked well, but there was a very unusual erratic curve from one sample, which will be rerun later. Marv said he is not surprised there are no positive samples, as the virus seems to be present at lower levels this year.

04 Nov 2009

With John Montenieri

"Flagging" for Fleas

Materials: 12 foot plumber's snake with a 4"x4" piece of white diaper flannel attached (clamped) to an end

Area: Field on the east side of Jax Sporting Goods store on N. College Ave.

Weather: Temperature 56*F, sunny, slight breeze

Target species: Fleas: *Oropsylla hirsuta* and *O. tuburculata cynomuris* collected from black-tailed prairie dog ((*Cynomys ludovicianus*) burrows.

The field by Jax had a robust population of black-tailed prairie dogs. We either looked for holes that had just had a prairie dog in it or located holes that showed fresh signs of usage (scat, dark colored loose earth around the hole, etc.). I inserted the flannel end of the plumber's 'snake' in the prairie dog hole and pushed it into the hole while simultaneously waving the snake side-to-side. This side-to-side motion serves two purposes: 1) wiggle the 'snake' as far into the hole as possible and, 2) create vibrations that attract fleas by mimicking host movement.

I waited 30 seconds and then withdrew the 'snake', making sure to keep the flannel patch on the ground. I positioned myself upwind of the flannel (so as to not accidentally have any fleas blow onto me) and gently unfolded the flannel, looking for fleas. Then I turned the flannel over and checked the other side.

Collection of the fleas at this point can consist of dropping the entire flannel piece into a ziplock bag. Bags can then be put on ice to maintain 'live' fleas. Care should be taken to keep the fleas from overheating. Collection can also be performed by picking fleas off the flannel with forceps and dropping then into tubes of 2% saline (or a preservative relevant to your experimental procedure).

Although we surveyed several holes, we did not collect any fleas. The instructor speculated that the colony was not prone to a high flea population in the first place, as all

inhabitants appeared to be very healthy and of good body condition. He also intimated the random nature of successful flea collection was inherent to large prairie dog colonies, as was this one. Flagging works well for collecting nidiculous fleas of the prairie dog, but is not practicable for target rodents whose nest is not easily located or penetrated by a plumber's 'snake'. Trapping live rodents and combing them for fleas seems to be the procedure of choice in that case.

Figure 18. Researcher inserting plumbing ‘snake’ into rodent burrow.

Figure 19. Flagging material attached to plumbing ‘snake’ used to insert into burrows to collect fleas from rodent burrows.

21 Oct 2009

With John Montinieri

Flea Identification

John and I sat down with a copy of the chapter 'Order Siphonaptera' from the Furham and Katts key in the insect identification room at the CDC. There is a huge picture of an enlargement of a generalized flea on the wall. He spent time discussing and pointing out anatomical features he considered important and/or difficult to visualize on a real flea. We then keyed out a flea to the genus *Oropsylla* together. I then did a *Ctenocephalides felis* by myself. The visual signature of the antenna surprised me so we reviewed that. I then worked on a few more fleas by myself (*Pulex irritans*, *Diamanus montanus* and *Monopsyllus wagneri*). The limitation I found with the key I was supplied was that it only went down to genus for a majority of fleas. For my personal needs related to my project, though, it keys *Xenopsylla* to either *cheopis* or *braziliensis*. I will need proficiency with that as I am only going to focus on *X. cheopis* in Uganda.

20 November 2009

With Scott Bernhardt

Flea Processing

Before we went to the lab, Scott discussed with me the basic premise of his project and the sequencing challenges he has encountered. He is working with *Oropsylla montana* wild type fleas combed from rock squirrels trapped in Colorado, Nevada, Utah, Arizona, and New Mexico. He has spent a great deal of time designing primers to target the ND4 mitochondrial gene and a nuclear gene for sequencing. The primers are 20-25 base pairs long.

He is currently performing DNA extractions on the fleas and is amassing the samples for PCR later. We went into his lab and he walked me through the extraction as I performed it. He macerates pools of 10-15 fleas with the same Qiagen Tissuelyser that Marv uses for mosquitos, with the exception of using 3 smaller glass beads instead of copper BBs. He runs the cycle for 8 minutes at 20 cycles/second. The maceration step seemed to disintegrate the fleas nicely. The Quigen protocol Scott uses is a Qiagen Supplementary Protocol: Purification of total DNA from insects using the DNeasy® Blood & Tissue Kit. It is a standard digestion/purification/extraction protocol. Scott uses centrifugal separation instead of the manifold Andrias uses for ticks.

We spoke at length about my project and extrapolated that these techniques will probably be used in my project too. I will be assisting with the PCR (and sequencing) part of his research when he gets enough samples extracted to do them.

9 December 2009

With Scott Bernhart

Flea DNA Purification and PCR

Using DNA samples previously extracted, Scott and I then purified the DNA prior to performing PCR in order to reduce the impurities and increase the efficiency of the PCR reaction.

I used a Qiagen purification kit that used a series of buffers in spin columns to bind the DNA to the membrane and wash out the impurities. Then an elution solution was spun through that released the DNA into the tube.

Samples were than mixed with a Master Mix consisting of Taq polymerase, forward and reverse primers, and purified water to make a total sample volume of 50 microliters (ul). Samples were than loaded onto a thermal cycler and PCR was performed. When the cycle had finished, 5 ul of each sample was loaded onto an agarose gel that had a non-carcinogenic UV reactive marker added. Ladder flanked the sample on both sides. Results were visualized in a UV hood. Sample amplification was weak, so 4 of the poor performers were chosen for a gradient PCR to ascertain the optimal annealing temperature, as that is why Scott thought the PCR products were of low volume. Temperature gradients programmed into the thermal cycler went from 48°C to 60°C.

PCR product was also quantified on a micro spectrophotometer. This nanodrop machine gives the results as an amount of DNA in ng/ul. Those results concurred with the visual results from the gel.

12 Nov 2009

With Marc Dolan

Tick Collection

I met with Marc Dolan. Since the majority of his tick collection (and research) is based on the tick population at Earle Naval Weapons Station in Coltsneck, New Jersey and the collection 'season' is over, he showed me powerpoint slides of the area they collect from and the field techniques used. He also showed me the materials they use, as they are stored at the CDC. He uses a 1 meter squared cloth drag. This material is white Levi's Cotton Docker material purchased at a sewing store. A wooden dowel is inserted through the top with a cord handle for the operator. The bottom of the drag is weighted to assure even coverage by the cloth. Lead washers or heavy cording can be used to this end. The area of interest is dragged by measured transect or by time. The disease of interest to him is *Borrelia burgdorferi* and ticks are processed for presence of disease after they are transported to the CDC in Fort Collins. Marc uses permanone® on his clothing to protect him when he drags for ticks. He is also working on natural tick repellants/insecticides. One is nootkatone, a derivative of grapefruit essence. Statistics have shown that high pressure spraying of tick environments is more effective in controlling tick populations (better penetration of the leaf litter) than backpack type sprayers. Marc would like to test more of his nootkatone formulations, but has been experiencing a low number of homeowners that will allow spraying (of anything) on their property and the weapons station area is very large with minimal migration control.

The nymphs they collect in the Spring have virtually 0% disease as *B. burgdorferi* is not transovarily transmitted and they typically have not taken a bloodmeal yet, but (desperate) adults have nearly a 30% infection rate. Adults are usually collected in the Fall though. Mice are the primary disease transmitting hosts (nymph and larval stage tick food source) and deer (adult tick bloodmeal source) are amplifying hosts. Marc said the temperature cutoff for successful collection is around 50°F at the ground leaf litter. He also stated that he thinks about 3-5% of the population is questing at one time. He

expects to collect mostly *Dermacentor* spp. ticks in marshy areas and *Ixodes scapularis* and *Amblyomma* in deciduous forest leaf litter.

Marc has developed bait stations with a Topspot® wick inside for antiectoparasite treatment of the rodents. But that did not eliminate potentially infected rodents from the population at large. So he took the concept one step further and put doxycycline-laced bait in the stations too to eliminate the spirochete from the rodent host. The station has had to evolve to a metal type due to squirrels gnawing on the plastic stations, but the concept seems rather effective. The lab Marc is in is currently in the process of assessing this year's level of tick infection.

After the ticks are collected they are put into small glass vial that have the bottom coated with a layer of plaster of Paris mixed with activated charcoal. The tops of the vials are covered with a fine mesh organza material. Ticks returned to the CDC are kept in a 21*C chamber with a 16hr light/8hr dark cycle. They are fed at 24*C so they moult faster. Adults are fed on rabbits and larvae and nymphs are fed on mice. One can also use a dessicator bell jar with distilled water in it. The addition of potassium chloride keeps the humidity at 80% in the jar.

3 Nov 2009

With Gabrielle Dietrich

Tick Identification

Prior to meeting with Gabrielle, I did the tick identification tutorial from the AFPMB CD. I found it quite helpful and easy to navigate.

Gabrielle and I used the insect identification room at the CDC. We identified ticks that were recently collected and preserved in alcohol, and a couple from the slide-mounted CDC collection. The keys we used were: Ixodes keys: Keirans and Clifford; Kierans and Litwack (Ixodes east of the Mississippi River); Cooley and Kohls. Dermacentor keys: Armed Forces Pest Management Board; Brinton, Beck and Allred; Yunker, Keirans, Clifford, and Easton; Cooley.

The ticks I identified were: *Ixodes scapularis*, deer tick

Amblyomma maculatum, Gulf Coast tick

Amblyomma americanum, lone star tick

Riphicephalus sanguineus, brown dog tick

Dermacentor variabilis, American dog tick

Gabrielle did a good job of helping me see the differences between the genera, gave me manipulation tips to better visualize difficult to see characteristics and really took her time to point out the relativity of some of the key descriptors. Compare and contrast, i.e. She also emphasized being familiar with all the different keys because they do not agree on all the characteristics associated with a certain tick!

6 Nov 2009

With Andrias Hojgaard

Tick Processing

Tick samples are *Ixodes scapularis* collected from Earle Naval Weapons Station in New Jersey. The first step is to extract the tick DNA from the whole tick. A Qiagen kit is used and the published protocol is enclosed. I caught up with Andrias after he had macerated the whole ticks with a pestle in a 1.5 microcentrifuge tube and had mixed them with buffer and proteinase K and allowed them to incubate overnight.

Samples were collected and vortexed. A commercial buffer was added and mixed . Samples were incubated 10 minutes. Then ethanol was added to each sample to precipitate the DNA and then mixed. Samples were loaded into spin column collection tubes and then onto a vacuum manifold where 250 microliters of a different buffer was pulled through twice. This same procedure was repeated twice more with a different buffer. Spin column samples were removed from the manifold and put into a clean 1.5 ml tube which had another different buffer added to it. Samples were incubated 1 minute. This step is repeated, samples are centrifuged and the eluted fluids contain the tick DNA. These extracted samples are first run on a real-time PCR to ensure that tick DNA is actually present in the sample before running it for the presence of Borrelia spp.

For the real-time PCR, Andrias used a Roche product, 'FastStart Universal Probe Master (ROX). He used their master mix and added his actin probe (supplied by the CDC) and oligonucleotides to it. This amount per sample was 23 microliters. Two microliters of sample was added for a total of 25 microliters per sample. Positive controls were commercial samples of *I. scapularis* DNA and negative control was water. His probe is a cyber green fluorescent tag that is held between the double-stranded DNA. The bonds melt at 83*C so when the DNA is unwound the signal decreases (and vice-versa). He uses the signal curve signature on the readout to confirm that he has the desired product present in the amplified mix. He also has a cutoff of >12 copies to accept the PCR product amplification as acceptable (i.e positive). Andrias uses the same basic procedure on the tick DNA product to then detect the presence of *Borrellia* spp..

REFERENCES

AFPMB. (2002) Guide to pest surveillance during contingency operations. In Defense Do (ed.), *Armed Forces Pest Management Board*, Vol. Technical Guide No. 43, p. 166.

AFPMB. (2011) Living Hazards Database. Armed Forces Pest Management Board, Vol. www.afpmb.org.

Ahmad M, Denholm I, Bromilow RH (2006) Delayed cuticular penetration and enhanced metabolism of deltamethrin in pyrethroid-resistant strains of *Helicoverpa armigera* from China and Pakistan. *Pest Manag Sci* 62: 805-810

Aiello S (ed) (2010) *The Merck veterinary manual*. Whitehouse Station, N.J.: Merck

Askew RR (1971) *Parasitic Insects*, London, United Kingdom: Heinemann Educational.

AVMA. (2001) Plague Fact Sheet. American Veterinary Medical Association.

Bacot AW, Martin CJ (1914) Observations on the mechanism of the transmission of plague by fleas. *Journal of Hygiene* 13 (Plague Suppl. III): 423-439

Bahmanyar M, Cavanaugh DC (1976) In *Plague Manual*, pp 45-54. Geneva: World Health Organization

Bass C, Lansdell SJ, Millar NS, Schroeder I, Turberg A, Field LM, Williamson MS (2006) Molecular characterisation of nicotinic acetylcholine receptor subunits from the cat flea, *Ctenocephalides felis* (Siphonaptera: Pulicidae). *Insect Biochem Mol Biol* 36: 86-96

Bass C, Schroeder I, Turberg A, Field LM, Williamson MS (2004a) Identification of the Rdl mutation in laboratory and field strains of the cat flea, *Ctenocephalides felis* (Siphonaptera: Pulicidae). *Pest Manag Sci* 60: 1157-1162

Bass C, Schroeder I, Turberg A, L MF, Williamson MS (2004b) Identification of mutations associated with pyrethroid resistance in the para-type sodium channel of the cat flea, *Ctenocephalides felis*. *Insect Biochem Mol Biol* 34: 1305-1313

Binder S, Levitt AM, Sacks JJ, Hughes JM (1999) Emerging infectious diseases: Public health issues for the 21st century. *Science* 284: 1311-1313

Black WC, Goffochetegai-Escalante N, Randle NP, Donnelly MJ (2008) The yin and yang of linkage disequilibrium: Mapping of genes and nucleotides conferring insecticide resistance in insect disease vectors. *Adv Exp Med Biol* 627: 71-83

Black WCI, Tabashnik BE (2005) Population Genetics of Disease Vectors. In *Biology of Disease Vectors*, Marquardt WC (ed), pp 465-450. Oxford, UK: Elsevier

Bossard R. (1997) Evaluation and use of bioassays for surveying insecticide susceptibility for cat fleas, *Ctenocephalides felis felis* (Bouche), in relation to resistance. Kansas State University.

Bossard RL, Hinkle NC, Rust MK (1998) Review of insecticide resistance in cat fleas (Siphonaptera: Pulicidae). *J Med Entomol* 35: 415-422

Brogdon WG (1984) Mosquito protein microassay. I. Protein determinations from small portions of single-mosquito homogenates. *Comp Biochem Physiol B* 79: 457-459

Brogdon WG (1988) Microassay of acetylcholinesterase activity in small portions of single mosquito homogenates. *Comp Biochem Physiol C* 90: 145-150

Brogdon WG (1989) Biochemical resistance detection: an alternative to bioassay. *Parasitol Today* 5: 56-60

Brogdon WG, Barber AM (1990) Microplate assay of glutathione s-transferase activity for resistance detection in single-mosquito triturates. *Comp Biochem Physiol B* 96: 339-342

Brogdon WG, Beach RF, Barber AM, Cordon-Rosales C (1992) A generalized approach to detection of organophosphate resistance in mosquitoes. *Med Vet Entomol* 6: 110-114

Brogdon WG, Dickinson CM (1983) A microassay system for measuring esterase activity and protein concentration in small samples and in high-pressure liquid chromatography eluate fractions. *Anal Biochem* 131: 499-503

Brogdon WG, McAllister JC (1998a) Insecticide resistance and vector control. *Emerg Infect Dis* 4: 605-613

Brogdon WG, McAllister JC (1998b) Simplification of adult mosquito bioassays through use of time-mortality determinations in glass bottles. *J Am Mosq Control Assoc* 14: 159-164

Brogdon WG, McAllister JC, Vulule J (1997) Heme peroxidase activity measured in single mosquitoes identifies individuals expressing an elevated oxidase for insecticide resistance. *J Am Mosq Control Assoc* 13: 233-237

Brooke BD (2008) kdr: can a single mutation produce an entire insecticide resistance phenotype? *T Roy Soc Trop Med H* 102: 524-525

Brown TM, Brogdon WG (1987) Improved detection of insecticide resistance through conventional and molecular techniques. *Annu Rev Entomol* 32: 145-162

Busvine JR (1951) Mechanism of resistance to insecticide in houseflies. *Nature* 168: 193-195

Cantor NF (2001) *In the wake of the plague the Black Death and the world it made,* New York: Free Press.

Casida JE (2009) Pest Toxicology: The Primary Mechanisms of Pesticide Action. *Chemical Research in Toxicology* 22: 609-619

Chanteau S, Ratsifasoamanana L, Rasoamanana B, Rahalison L, Randriambelosoa J, Roux J, Rabeson D. (1998) Plague, a Reemerging Disease in Madagascar. *Emerging Infectious Diseases*. Centers for Disease Control and Prevention, Atlanta , GA, Vol. 4, pp. 101-103.

Chow CY (1965) Plague in Viet Nam and DDT resistance in the flea vector (*Xenopsylla cheopsis*). . *WHO Inf Circ Insecticide Resistance* 54: 6

Coleman M, Hemingway J (2007) Insecticide resistance monitoring and evaluation in disease transmitting mosquitoes. *J Pestic Sci* 32: 69-76

Curtis CF (1985) Theoretical-models of the use of insecticide mixtures for the management of resistance. *B Entomol Res* 75: 259-265

Dary O, Georghiou GP, Parsons E, Pasteur N (1991) Dot-blot test for identification of insecticide-resistant acetylcholinesterase in single insects. *J Econ Entomol* 84: 28-33

Dash AP, Raghavendra K, Pillai MKK (2007) Resurrection of DDT: a critical appraisal. *Indian J Med Res* 126: 1-3

David J-P, Strode C, Vontas J, Nikou D, Vaughan A, Pignatelli PM, Louis C, Hemingway J, Ranson H (2005) The Anopheles gambiae detoxification chip: A highly specific microarray to study metabolic-based insecticide resistance in malaria vectors. *P Natl Acad Sci USA* 102: 4080-4084

Davies TGE, Field LM, Usherwood PNR, Williamson MS (2007) DDT, pyrethrins, pyrethroids and insect sodium channels. *International Union of Biochemistry and Molecular Biology Life* 59: 151-162

Davies TGE, O'Reilly AO, Field LM, Wallace BA, Williamson MS (2008) Knockdown resistance to DDT and pyrethroids: from target-site mutations to molecular modelling. *Pest Manag Sci* 64: 1126-1130

Doerge RW, Churchill GA (1996) Permutation tests for multiple loci affecting a quantitative character. *Genetics* 142: 285-294

Dong K (1997) A single amino acid change in the para sodium channel protein is associated with knockdown-resistance (kdr) to pyrethroid insecticides in German cockroach. *Insect Biochem Molec* 27: 93-100

Dorland WAN (1957) Dorland's illustrated medical dictionary. *Dorland's illustrated medical dictionary*

Duplaix N. (1988) Fleas. The lethal leapers. *National Geographic*, Vol. 173, pp. 672-694.

Dutt AK, Akhtar R, McVeigh M (2006) Surat plague of 1994 re-examined. *Southeast Asian J Trop Med Public Health* 37: 755-760

Eidson M, Thilsted JP, Rollag OJ (1991) Clinical, clinicopathological, and pathological features of plague in cats - 119 cases (1977-1988). *Journal of the American Veterinary Medical Association* 199: 1191-1197

Eisen RJ, Bearden SW, Wilder AP, Montenieri JA, Antolin MF, Gage KL (2006) Early-phase transmission of *Yersinia pestis* by unblocked fleas as a mechanism explaining rapidly spreading plague epizootics. *Proc Natl Acad Sci U S A* 103: 15380-15385

Eisen RJ, Borchert JN, Holmes JL, Amatre G, Van Wyk K, Enscore RE, Babi N, Atiku LA, Wilder AP, Vetter SM, Bearden SW, Montenieri JA, Gage KL (2008) Early-phase transmission of *Yersinia pestis* by cat fleas (*Ctenocephalides felis*) and their potential role as vectors in a plague-endemic region of Uganda. *Am J Trop Med Hyg* 78: 949-956

Eisen RJ, Eisen L, Gage KL (2009) Studies of Vector Competency and Efficiency of North American Fleas for *Yersinia pestis*: State of the Field and Future Research Needs. *J Med Entomol* 46: 737-744

Eisen RJ, Lowell JL, Montenieri JA, Bearden SW, Gage KL (2007a) Temporal dynamics of early-phase transmission of *Yersinia pestis* by unblocked fleas: secondary infectious feeds prolong efficient transmission by *Oropsylla montana* (Siphonaptera: Ceratophyllidae). *J Med Entomol* 44: 672-677

Eisen RJ, Wilder AP, Bearden SW, Montenieri JA, Gage KL (2007b) Early-phase transmission of *Yersinia pestis* by unblocked *Xenopsylla cheopis* (Siphonaptera: Pulicidae) is as efficient as transmission by blocked fleas. *J Med Entomol* 44: 678-682

Ennis S (1984) Linkage Disequilibrium as a Tool for Detecting Signatures of Natural Selection. In *Methods in molecular biology*, Collins AR (ed), pp 59-70. Clifton, New Jersey: Humana Press

Fitt GP, Mares CL, Llewellyn DJ (1994) Field-evaluation and potential ecological impact of transgenic cottons (*Gossypium-hirsutum*) in Australia. *Biocontrol Science and Technology* 4: 535-548

Flores AE, Albeldano-Vazquez W, Salas IF, Badii MH, Becerra HL, Garcia GP, Fuentes SL, Brogdon WG, Black WC, Beaty B (2005) Elevated alpha-esterase levels associated

with permethrin tolerance in *Aedes aegypti* (L.) from Baja California, Mexico. *Pesticide Biochemistry and Physiology* 82: 66-78

Flores AE, Grajales JS, Salas IF, Garcia GP, Becerra MH, Lozano S, Brogdon WG, Black WCt, Beaty B (2006) Mechanisms of insecticide resistance in field populations of *Aedes aegypti* (L.) from Quintana Roo, Southern Mexico. *J Am Mosq Control Assoc* 22: 672-677

Fonseca-Gonzalez I, Cardenas R, Quinones ML, McAllister J, Brogdon WG (2009a) Pyrethroid and organophosphates resistance in *Anopheles (N.) nuneztovar*i Gabaldon populations from malaria endemic areas in Colombia. *Parasitol Res* 105: 1399-1409

Fonseca-Gonzalez I, Quinones ML, McAllister J, Brogdon WG (2009b) Mixed-function oxidases and esterases associated with cross-resistance between DDT and lambda-cyhalothrin in *Anopheles darlingi* Root 1926 populations from Colombia. *Mem Inst Oswaldo Cruz* 104: 18-26

Gage K (2005) Fleas, the Siphonaptera. In *Biology of Disease Vectors*, Marquardt WC (ed), 7, pp 77-92. Elsevier

Gage K, Kosoy M (2006) Recent Trends in Plague Ecology. In *Recovery of the black-footed ferret: progress and continuing changes*, Roelle J, Miller BE, Godbey J, Biggins DE (eds), pp 213-231. Fort Collins, CO: US Geological Survey

Gage KL, Dennis DT, Orloski KA, Ettestad P, Brown TL, Reynolds PJ, Pape WJ, Fritz CL, Carter LG, Stein JD (2000) Cases of cat-associated human plague in the Western US, 1977-1998. *Clin Infect Dis* 30: 893-900

Gage KL, Kosoy MY (2005) Natural history of plague: perspectives from more than a century of research. *Annu Rev Entomol* 50: 505-528

Gage KL, Ostfeld RS, Olson JG (1995) Nonviral vector-borne zoonoses associated with mammals in the United States. *Journal of Mammalogy* 76: 695-715

Gorrochotegui-Escalante N, Lozano-Fuentes S, Bennett KE, Molina-Cruz A, Beaty BJ, Blackiv WCt (2005) Association mapping of segregating sites in the early trypsin gene and susceptibility to dengue-2 virus in the mosquito *Aedes aegypti*. *Insect Biochem Molec* 35: 771-788

Gould EA, Higgs S (2009) Impact of climate change and other factors on emerging arbovirus diseases. *T Roy Soc Trop Med H* 103: 109-121

Gratz N (1977). Problems and developments in the control of flea vectors of disease. *Fleas: Proceedings of the International Conference on Fleas*; Peterborough, United Kingdom. A.A. Balkema, Rotterdam.

Gratz N (1999) Rodent reservoirs and flea vectors of natural foci of plague. In *Plague manual: Epidemiology, distribution, surveillance and control*, Organization WH (ed), pp 63-96. Geneva: World Health Organization

Hamdan H, Sofian-Azirun M, Nazni W, Lee HL (2005) Insecticide resistance development in *Culex quinquefasciatus* (Say), *Aedes aegypti* (L.) and *Aedes albopictus* (Skuse) larvae against malathion, permethrin and temephos. *Trop Biomed* 22: 45-52

Hemingway J, Hawkes NJ, McCarroll L, Ranson H (2004) The molecular basis of insecticide resistance in mosquitoes. *Insect Biochem Molec* 34: 653-665

Hemingway J, Karunaratne S (1998) Mosquito carboxylesterases: a review of the molecular biology and biochemistry of a major insecticide resistance mechanism. *Med Vet Entomol* 12: 1-12

Hemingway J, Ranson H (2000) Insecticide resistance in insect vectors of human disease. *Annu Rev Entomol* 45: 371-391

Hemingway J, Ranson H (2005) Chemical Control of Vectors and Mechanisms of Resistance. In *Biology of Disease Vectors*, Marquardt WC (ed), 41, pp 627-638. Elsevier

Hinkle NC, Wadleigh RW, Koehler PG, Patterson RS (1995) Mechanisms of Resistance in a Strain of Cat Fleas (Siphonaptera:Pulicidae). *Journal of Entomological Science* 30: 43-48

Husseneder C, Garner SP, Foil LD, Macaluso KR (2010) Development of Microsatellites for Genetic Analyses and Population Assignment of the Cat Flea (Siphonaptera: Pulicidae). *J Med Entomol* 47: 1028-1033

Ilg T, Schmalz S, Werr M, Cramer J (2010) Acetylcholinesterases of the cat flea *Ctenocephalides felis*: Identification of two distinct genes and biochemical characterization of recombinant and in vivo enzyme activities. *Insect Biochem Molec* 40: 153-164

IRAC. (2011) Prevention and Management of Insecticide Resistance in Vectors of Public Health Importance. Insecticide Resistance Action Committee.

Kambhampati S, Bossard R, Dryden MW (1997) Rapid assay for the detection of esterases in the cat flea, *Ctenocephalides felis* (Siphonaptera : Pulicidae). *Journal of the Kansas Entomological Society* 70: 129-132

Keiding J (1975) Problems of housefly (*Musca-domestica*) control due to multiresistance to insecticides. *Journal of Hygiene Epidemiology Microbiology and Immunology* 19: 340-355

Kelly J (2005) *The great mortality an intimate history of the Black Death, the most devastating plague of all time*, New York: HarperCollins Publishers.

Kilpatrick JW, Fay RW (1952) DDT-resistance studies with the Oriental rat flea. *J Econ Ent* 42: 284-288

Krishnamurthy BS, Joshi GC (1962) Susceptibility status of rat fleas (*X. cheopis*) to DDT, BHC and dieldrin in Punjab, Bihar and Uttar Pradesh. *Indian Journal of Malariology* 16: 137-142

Li X, Schuler MA, Berenbaum MR (2007) Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. *Annu Rev Entomol* 52: 231-253

Lorange EA, Race BL, Sebbane F, Hinnebusch BJ (2005) Poor vector competence of fleas and the evolution of hypervirulence in *Yersinia pestis*. *Journal of Infectious Diseases* 191: 1907-1912

Lund AE, Narahashi T (1983) Kinetics of sodium-channel modification as the basis for the variation in the nerve membrane effects of pyrethroids and DDT analogs. *Pesticide Biochemistry and Physiology* 20: 203-216

Lynd A, Weetman D, Barbosa S, Egyir Yawson A, Mitchell S, Pinto J, Hastings I, Donnelly MJ (2010) Field, Genetic, and Modeling Approaches Show Strong Positive Selection Acting upon an Insecticide Resistance Mutation in *Anopheles gambiae* s.s. *Mol Biol Evol* 27: 1117-1125

Mackay TFC (2001) Quantitative trait loci in Drosophila. *Nature Reviews Genetics* 2: 11-20

Martinez-Torres D, Chandre F, Williamson MS, Darriet F, Bergé JB, Devonshire AL, Guillet P, Pasteur N, Pauron D (1998) Molecular characterization of pyrethroid knockdown resistance (kdr) in the major malaria vector *Anopheles gambiae* s.s. *Insect Mol Biol* 7: 179-184

Martinez-Torres D, Foster SP, Field LM, Devonshire AL, Williamson MS (1999) A sodium channel point mutation is associated with resistance to DDT and pyrethroid insecticides in the peach-potato aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae). *Insect Mol Biol* 8: 339-346

Mears S, Clark F, Greenwood M, Larsen KS (2002) Host location, survival and fecundity of the Oriental rat flea *Xenopsylla cheopis* (Siphonaptera:Pulicidae) in relation to black rat *Rattus rattus* (Rodentia:Muridae) host age and sex. *B Entomol Res* 92: 375-384

Mee CL, Jr. (1990) How a mysterious disease laid low Europe's masses. *Smithsonian*, Vol. 20, pp. 66-79.

Milani R (1954) Comportamento mendeliano della resistenza alla azione abbattante del DDT: correlazione tran abbattimento e mortalita in *Musca domestica* L. *Riv Parassitol* 15: 513-542

Miyazaki M, Ohyama K, Dunlap DY, Matsumura F (1996) Cloning and sequencing of the para-type sodium channel gene from susceptible and kdr-resistant German cockroaches (*Blattella germanica*) and house fly (*Musca domestica*). *Mol Gen Genet* 252: 61-68

Morens DM, Folkers GK, Fauci AS (2004) The challenge of emerging and re-emerging infectious diseases. *Nature* 430: 242-249

Mullen GR, Durden LA (2009) *Medical and veterinary entomology*, Burlington, MA: Academic Press/Elsevier.

Nauen R (2007) Insecticide resistance in disease vectors of public health importance. *Pest Manag Sci* 63: 628-633

Paeporn P, Supaphathom K, Srisawat R, Komalamisra N, Deesin V, Ya-umphan P, Leeming S (2004) Biochemical detection of pyrethroid resistance mechanism in Aedes aegypti in Ratchaburi province, Thailand. *Trop Biomed* 21: 145-151

Park Y, Taylor MFJ (1997) A novel mutation L1029H in sodium channel gene hscp associated with pyrethroid resistance for *Heliothis virescens* (Lepidoptera: Noctuidae). *Insect Biochem Molec* 27: 9-13

Patel TB, Bhatia SC, Deobhanker RB (1960) A confirmed case of DDT-resistance in *Xenopsylla cheopis* in India. *B World Health Organ* 23: 301-312

Pathology AFIo. (2008) Outbreak of Bubonic Plague in Uganda. In Defense Do (ed.), *DoD Directory of Public Health Laboratory Services Newsletter*.

Patil VL, Guthrie FE (1979) Cuticular lipids of 2 resistant and a susceptible strain of houseflies. *Pesticide Science* 10: 399-406

Pavlidis P, Hutter S, Stephan W (2008) A population genomic approach to map recent positive selection in model species. *Mol Ecol* 17: 3585-3598

Pham H, Dang D, Minh N, Nguyen N, Nguyen T (2009) Correlates of environmental factors and human plague: an ecological study in Vietnam. *International Journal of Epidemiology* 38: 1634-1641

Piesman J, Gage K (2000) Bacterial and Rickettsial Diseases. In *Medical Entomology A Textbook on Public Health and Veterinary Problems Caused by Arthropods*, Eldridge BF, Edman JD (eds), 10, pp 390-395. Netherlands: Kluwer

Plimmer JRE (2003) *Encyclopedia of agrochemicals*, Hoboken, New Jersey: Wiley.

Poland JD, Dennis DT (1999) Diagnosis and Clinical Manifestations. In *Plague Manual Epidemiology, Distribution, Surveillance and Control*, Gage K, Dennis DT (eds), Vol. WHO/CDS/CSR/EDC/99.2, 2, pp 43-53. Geneva: WHO

Pollitzer R (1954) *Plague. World Health Organization Monograph Series No. 22*, Geneva, Switzerland: World Health Organization.

Pollitzer R, Meyer KF (1961) The Ecology of Plague. In *Studies in Disease Ecology*, May JF (ed), pp 433-590. New York: Hafner

Polson KA, Brogdon WG, Rawlins SC, Chadee DD (2010) Characterization of insecticide resistance in Trinidadian strains of *Aedes aegypti* mosquitoes. *Acta Trop*: 1-8

Prevention CfDCa (2009) Bubonic and pneumonic plague- Uganda, 2006. *MMWR Morbidity Mortality Weekly Report* 58: 778-781

Radcliffe EB, Hutchison WD, Cancelado REE (2009) *Integrated pest management concepts, tactics, strategies and case studies*, Cambridge, UK ;: Cambridge University Press.

Raghavendra K, Barik TK, Reddy BPN, Sharma P, Dash AP (2011) Malaria vector control: from past to future. *Parasitol Res* 108: 757-779

Ranson H, Jensen B, Vulule JM, Wang X, Hemingway J, Collins FH (2000a) Identification of a point mutation in the voltage-gated sodium channel gene of Kenyan *Anopheles gambiae* associated with resistance to DDT and pyrethroids. *Insect Mol Biol* 9: 491-497

Ranson H, Jensen B, Wang X, Prapanthadara L, Hemingway J, Collins FH (2000b) Genetic mapping of two loci affecting DDT resistance in the malaria vector *Anopheles gambiae*. *Insect Mol Biol* 9: 499-507

Ranson H, Paton MG, Jensen B, McCarroll L, Vaughan A, Hogan JR, Hemingway J, Collins FH (2004) Genetic mapping of genes conferring permethrin resistance in the malaria vector, Anopheles gambiae. *Insect Mol Biol* 13: 379-386

Renapurkar DM (1990) Distribution and insecticide resistance of the plague flea *Xenopsylla cheopis* in Maharashtra State, India. *Med Vet Entomol* 4: 89-96

Risch NJ (2000) Searching for genetic determinants in the new millennium. *Nature* 405: 847-856

Roberts DR, Andre RG (1994) Insecticide Resistance Issues in Vector-Borne Disease-Control. *Am J Trop Med Hyg* 50: 21-34

Rozas J, Sanchez-DelBarrio JC, Messeguer X, Rozas R (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* 19: 2496-2497

Saitou N, Nei M (1987) The neighbor-joining method - a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4: 406-425

Salyers AA (2002) *Bacterial pathogenesis a molecular approach*, Washington, D.C.: ASM Press.

Sawicki RM (1987) Definition, detection, and documentation of insecticide resistance In *Combating resistance to xenobiotics: biological and chemical approaches*, Ford MG (ed), pp 105-107. Chichester, UK: Ellis Horwood

Sawicki RM, Devonshire AL, Rice AD, Moores GD, Petzing SM, Cameron A (1978) Detection and distribution of organophosphorus and carbamate insecticide-resistant *Myzus persicae* (Sulz) in Britain in 1976. *Pesticide Science* 9: 189-201

Service MW (1991) Agricultural development and arthropod-borne diseases: a review. *Revista de SaÃºde PÃºblica* 25: 165-178

Shyamal B, Ravi Kumar R, Sohan L, Balakrishnan N, Veena M, Shiv L (2008) Present susceptibility status of rat flea Xenopsylla cheopis (Siphonaptera: Pulicidae), vector of plague against organochlorine, organophosphate and synthetic pyrethroids 1. The Nilgiris District, Tamil Nadu, India. *J Commun Dis* 40: 41-45

Singchai C, Srisawat R, Phanphuwong T, Vimutisunthorn E, Deesin V, Yamput S, Pongwatanaklusiri P, Puthavathana P (2003) Surveillance of Commensal Rat and Shrew Populations in the Bangkok Area with Referneces to Flea Index as the Risk Indicator of Plague. *J Med Assoc Thai* 86: 795-801

Smolinski MS, Hamburg MA, Lederberg J. (2003) Microbial Threats to Health: Emergence, Detection, and Response. Nature Publishing Group.

Soderlund DM, Knipple DC (1999) Knockdown resistance to DDT and Pyrethroids in the house fly (Diptera : muscidae): From genetic trait to molecular mechanism. *Ann Entomol Soc Am* 92: 909-915

Soderlund DM, Knipple DC (2003) The molecular biology of knockdown resistance to pyrethroid insecticides. *Insect Biochem Molec* 33: 563-577

Sustriayu N, Sudomo M, Kusharyono C, Liat LB (1980) Susceptibility to DDT, malathion, fenitrothion and dieldrin of three flea species in the boyolali plague endemic area, Central Java, Indonesia. *Southeast Asian J Trop Med Public Health* 11: 108-112

Swofford DL (2003) *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4.*: Sinauer Associates.

Tabashnik BE (1989) Managing Resistance with Multiple Pesticide Tactics - Theory, Evidence, and Recommendations. *J Econ Entomol* 82: 1263-1269

Tajima F (1983) Evolutionary relationship of DNA-sequences in finite populations. *Genetics* 105: 437-460

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL-W - improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22: 4673-4680

Tikhomirov E (1999) Epidemiology and Distribution of Plague. In *Plague Manual Epidemiology, Distribution, Surveillance and Control*, Gage K, Dennis DT (eds), Vol. WHO/CDS/CSR/EDC/99.2, pp 11-42. Geneva: WHO

USACHPPM. (1995) Prevention and Control of Plague. U.S. Army Center for Health Promotion and Preventative Medicine, Vol. Technical Guide 103.

Vinson SB, Law PK (1971) Cuticular composition and DDT resistance in tobacco budworm Lepidoptera-Noctuidae. *J Econ Entomol* 64: 1387-1393

Vulule JM, Beach RF, Atieli FK, McAllister JC, Brogdon WG, Roberts JM, Mwangi RW, Hawley WA (1999) Elevated oxidase and esterase levels associated with permethrin tolerance in *Anopheles gambiae* from Kenyan villages using permethrin-impregnated nets. *Med Vet Entomol* 13: 239-244

Webb CT, Brooks CP, Gage KL, Antolin MF (2006) Classic flea-borne transmission does not drive plague epizootics in prairie dogs. *P Natl Acad Sci USA* 103: 6236-6241

Weir BS, Cockerham CC (1984) Estimating F-stastics for the analysis of population-structure. *Evolution* 38: 1358-1370

WHO (1966) Summary of tests reported to ME/RTI from 1 September 1965 to 28 February 1966 showing abnormal physiological responses of anophelines to insecticides. *WHO Inf Circ Insecticide Resistance* 56

WHO (1966a) Summary of test reported to ME/RTI from 1 March 1966 to 31 August 1966, showing abnormal physiological responses of anophelines to insecticides. . *WHO Inf Circ Insecticide Resistance* 58&59: 9-11

WHO. (1970) Criteria and meaning of tests for determining susecptibility or resistance of insects to insecticide. World Health Organization, Vol. Tech. Rep. Ser. 443.

WHO (1976) Resistance of vectors and reservoirs of diesease to pesticides. 22nd Rep. WHO Expert Committee on Insecticides. . *Tech Rep Ser* No. 585: 88 pp

WHO (1980) Resistance of vectors of disease to pesticides. Fifth Rep. WHO Expert Committee on Vector Biology and Control. *Tech Rep Ser* No. 655: 82 pp

WHO. (1983) International Health Regulations (1969). World Health Organization, Geneva, Vol. Third annotated edition.

WHO (1986) Resistance of vectors and reservoirs of disease to pesticides. Tenth report of the WHO Expert Comminttee on Vector Biology and Control. . *Tech Rep Ser* No. 737: 87 pp

WHOPES. (2006) World Health Organization Pesticide Evaluation Scheme. *WHO/CDS/NTD/WHOPES/GCDPP/20061*. World Health Organization Pesticide Evaluation Scheme.

Wilder AP, Eisen RJ, Bearden SW, Montenieri JA, Tripp DW, Brinkerhoff RJ, Gage KL, Antolin MF (2008) Transmission efficiency of two flea species (*Oropsylla tuberculata cynomuris* and *Oropsylla hirsuta*) involved in plague epizootics among prairie dogs. *Ecohealth* 5: 205-212

Williamson M, Martinez-Torres D, Hick C, Devonshire A (1996) Identification of mutations in the housefly para-type sodium channel gene associated with knockdown resistance (kdr) to pyrethroid insecticides. *Molecular and General Genetics MGG* 252: 51-60

Wimsatt J, Biggins DE (2009) A review of plague persistence with special emphasis on fleas. *Journal of Vector Borne Diseases* 46: 85-99

Winter A, Kramer W, Werner FAO, Kollers S, Kata S, Durstewitz G, Buitkamp J, Womack JE, Thaller G, Fries R (2002) Association of a lysine-232/alanine polymorphism in a bovine gene encoding acyl-CoA : diacylglycerol acyltransferase (DGAT1) with variation at a quantitative trait locus for milk fat content. *P Natl Acad Sci USA* 99: 9300-9305

Woods ME, Montenieri JA, Eisen RJ, Zeidner NS, Borchert JN, Laudisoit A, Babi N, Atiku LA, Enscore RE, Gage KL (2009) Identification of Flea Blood Meals Using Multiplexed Real-Time Polymerase Chain Reaction Targeting Mitochondrial Gene Fragments. *The American Journal of Tropical Medicine and Hygiene* 80: 998-1003

Yu SJ (2008) *The toxicology and biochemistry of insecticides*, Boca Raton, Florida: CRC Press/Taylor & Francis.

Zaramba S. (2005) Uganda Malaria Control Strategic Plan 2005/06-2009/10. In Programme MC (ed.). Ministry of Health, Kampala, Uganda, pp. 14-15.

Zayed AB, Szumlas DE, Hanafi HA, Fryauff DJ, Mostafa AA, Allam KM, Brogdon WG (2006) Use of bioassay and microplate assay to detect and measure insecticide resistance in field populations of Culex pipiens from filariasis endemic areas of Egypt. *J Am Mosq Control Assoc* 22: 473-48**2**