2013

Environmental Specific Effects On Life History And Energy Metabolism Traits In A Permethrin Resistant Strain Of Anopheles Gambiae

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ENVIRONMENTAL SPECIFIC EFFECTS ON LIFE HISTORY AND ENERGY METABOLISM TRAITS IN A PERMETHRIN RESISTANT STRAIN OF *ANOPHELES GAMBIAE*

by

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A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2013

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ABSTRACT

Widespread use of pyrethroids for control of malaria vectors together with use in agriculture for pest control has led to an increase in emergence of populations of pyrethroid resistant phenotypes. Insecticide resistance can likely alter vector development time, reproduction, behavior, and longevity, all of which can affect the potential for disease transmission. In this study we assessed whether the genetic modifications (*kdr* mutation, cytochrome P450 and esterase detoxification) in a permethrin resistant strain of *An. gambiae* influence traits related to fitness. The performances of these traits were compared between two strains of *An. gambiae,* one permethrin susceptible (ASEMBO1) and the other permethrin resistant (RSP) when held under identical conditions. First we assessed several life history traits and energy metabolism traits in females. We found that the RSP larvae had a longer developmental time, but a reduced normal lifespan compared to the ASEMBO1 strain. We also measured metabolic rate and mitochondrial ROS production and found that RSP adult females had reduced metabolic rate and mitochondrial coupling efficiency but higher mitochondrial ROS production. Temperature is an important abiotic factor which influences mosquito life cycle. Next, we compared developmental time from L1 to adult emergence and adult body size between the two strains cultured at 25˚C or 30˚C. Both strains showed reduced survivorship at 30˚C. RSP had a

longer mean developmental time than ASEMBO1 strain. The mean dry weight was significantly lower in mosquitoes cultured at 30˚C than at 25˚C. Lastly, we assessed the effect of temperature on glycogen utilization to understand effects of rearing temperature on survival strategies between the two strains. Glycogen content was measured in teneral adults and in adults reared at 25˚C or 30˚C and exposed to two nutritional states (honey - fed or starved). Glycogen content in teneral adults fed and in starved adults was significantly higher in the RSP strain than in the ASEMBO1 strain. Under starvation conditions, the RSP strain lived significantly longer than the ASEMBO1 strains at both 25˚C and 30˚C. This dissertation provides the first insights into life history and energy metabolism traits in *An. gambiae* with co-occurrence of *kdr* mutation and metabolic detoxification mechanisms.

Key words: Insecticide resistance, *Anopheles gambiae*, energy metabolism, life history traits, temperature, glycogen utilization

ACKNOWLEDGEMENTS

I would like to thank my mentors: Dr. Robert Novak and Dr. Maria DeLuca for their guidance in completion of this dissertation. I am thankful to Dr. Robert Novak for introducing me to mosquito biology. I am greatly indebted to Dr. DeLuca for her hard work that helped bring this project to fruition. This included all the time she took learning about mosquitoes, away from Drosophila her main research organism. I cannot say “thank you”, enough. Accepting me into her lab helped enrich my dissertation greatly.

I do thank members of my dissertation committee: Dr. Asim Bej, Dr. Pauline Jolly, Dr. Douglas Watson and Dr. Stephen Watts for the great insight in their comments towards completion of this dissertation. I am especially thankful to Dr. Stephen Watts who, as a member of my thesis committee steered me towards further pursuit in education and he did this with patience, hard work and a lot of grace. He was determined to see this dissertation to completion.

My deep thanks goes to the former Chair of the Department of Biology Dr. Bud Fischer for the great support at a time when the journey had become very tough, and to, Dr. Edward W. Hook III and Mrs. Sally Fried of the Division of Infectious Diseases, William C. Gorgas Center for Geographic Medicine, for granting me permission to use the mosquito facility and their support of our research. I am thankful to the Department of Biology for the generous support they gave me during my study.

I am deeply grateful to Dr. William Grizzle for his steadfast faith in me. The foresight and encouragement he extended to me throughout this study was a great pillar that I could not have done without.

I acknowledge with gratitude the statistical help I received from Drs. Wen Wan and Jeff Leips from Virginia Commonwealth University and University of Maryland at Baltimore County, respectively.

I also acknowledge Dr. Douglas Moellering and Dr. Gin Chuang now at LSU for the much needed help they extended to me with mitochondrial respiration and mitochondrial ROS production experiments. I am thankful to the lab members of De Luca; Michelle Chambers Moses together with Dr. Su Bu for the metabolic measurements and the several other laboratory methods they trained me in. I am grateful to the former members of the Dr. Novak lab; Drs. Raymond Kim, Joel Morris and Eric Camano for the exposure they availed to me. The encouragement I received from Ms. Denise Oelschlager and Dr. Evrim Gurpinar is invaluable.

I would not have embarked on this journey if Makerere University had not granted me a study leave, and so I am thankful to that institution for the opportunity they availed to me to take time off work and come to this country and get immersed in this study.

Last but not least, I would like to thank my family, especially my wife, Deborah for her unwavering loyalty to this cause. She graciously held the family together and ably filled in for me the many times I was away from the family. I would also like to thank my children, for hanging in there, for braving the many weekends without their daddy home with them, the numerous evenings he was not there to help them with homework. After some time they adjusted and stopped asking why I could never take time off and be with them.

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INTRODUCTION

Females of the genus *Anopheles* are vectors of Plasmodium parasites that cause human malaria, a disease implicated in nearly 800,000 deaths a year worldwide of mostly children under 5 years of age (W.H.O., 2011). Malaria infection is caused by one or more of four species of micro-organisms that belong to the genus *Plasmodium*: *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae,* and *Plasmodium ovale.* (CDC, 2012). Among tools to curb transmission cycle of this disease is the use of insecticide treated bednets and indoor residual spraying with pyrethroids. While this intervention has reduced malaria transmission, the extensive use of pyrethroids for control has led to an increase in the incidence of insecticide resistance. This study was undertaken to understand the effects of insecticide resistant phenotype in the key life history traits of *An. gambiae*. Also evaluated were the effects of temperature on development time, body size, glycogen utilization and survival under starvation conditions.

ANOPHELES GAMBIAE

Anopheles gambiae refers to a complex of seven morphologically indistinguishable species of mosquitoes of the genus Anopheles endemic within the tropics. Species identification is by species specific nucleotide sequences of ribosomal DNA polymerase chain reaction (PCR) (Scott et al., 1993). The species complex consists of *An. arabiensis*, *An. bwambae, An. merus, An. melas, An. quadriannulatus* and *An. gambiae* sensu stricto (s.s.) (Davidson, 1964). Although these species can co-exist, individual species of *An. gambiae* complex exhibit different behavioral traits. For example, the *An. quadriannulatus* is generally considered to be zoophilic; whereas *An. gambiae* s.s. is mainly anthro-

pophilic and *An. arabiensis* is more adapted to arid environments (Lehmann and Diabate, 2008).

Anopheles gambiae life cycle

Anopheles gambiae mosquitoes are holometabous i.e., they undergo a complete metamorphosis from egg through four larval stages L1-L4, molting into pupae and finally adults. Adult females undergo a gonotrophic cycle in which they seek a blood meal from a vertebrate host and search for a suitable aquatic site to lay eggs (Ts and Gillies, 1964). Each gonotrophic cycle lasts 2 to 4 days depending on factors such as temperature (Afrane et al., 2005), availability of breeding sites (Gu et al., 2006) and number of previous gonotrophic cycles (Gillies and Wilkes, 1965). The larvae and pupae are aquatic while the adults are terrestrial.

Insecticides used in control of *An. gambiae*

The main classes of insecticides used in control of disease vectors include pyrethroids, organophosphates (OPs), carbamates, organochlorides, insect growth regulators (IGR) and bacterial toxins. Of these pyrethroids are preferred because of their low mammalian toxicity but high potency. Thus, these chemicals are a major constituent of insecticide-treated bednets used to prevent parasite transmission when an infected female mosquitoes seeks a blood meal.

Organophosphates are organic compounds that contain a phosphorus atom, with examples including Malathion and Dimethoate (Krieger, 2001a). These compounds are widely used as insecticides in the control of agricultural pests and vectors of medical importance.

Carbamates are esters of carbamic acids NHR.COOR'. In the majority of insecticidal carbamates, the constituent R' is a methyl group. Examples of carbamates are Bendiocarb and Aldicarb (Krieger, 2001b). They are less toxic than organophosphates and they are not broad spectrum pesticides. However, like organophosphates, carbamates are inhibitors of cholinesterase and relatively non persistent in the environment (Krieger, 2001b).

Mode of action of pyrethroids

As this work focuses on pyrethroid resistant phenotype, only the mode of action of pyethroids will be discussed. Pyrethroids are a class of synthetic axionic poison which target insect nerve tissues particularly the voltage-gated sodium channel. Examples of pyrethroids include permethrin, allethrin and bifenthrin (Krieger, 2001c). In an inactive state, when the membrane is at the resting potential, the sodium channel is closed. However, upon channel activation, the membrane becomes depolarized, and opens allowing nerve impulses to flow along the axon. This process occurs rapidly (one hundredth of a sec). In the susceptible mosquitoes, exposure to pyrethroids leaves the sodium ion channel open in a continuous state of depolarization leading to paralysis and death usually within a short time.

History of Development of Resistance to Insecticides

Effective indoor residual spraying against malaria vectors depends on whether mosquitoes rest indoors (i.e., endophilic behavior) and whether they are susceptible to the compounds in use. The mosquito needs to rest on the insecticide-treated walls for a suffi-

cient time to pick up a lethal dose. Naturally endophilic species include *An. gambiae* s.s. and *An. funestus* in Africa, *An. culicifacies* in India, and *An. minimus* in East and South-east Asia (Pates and Curtis, 2005). Exophilic behavior has evolved in certain populations exposed to prolonged spraying programs. Spraying of the walls and ceilings of houses with residual insecticides such as DDT sufficiently reduces the survival prospects of indoor resting *Anopheles* mosquitoes to greatly decrease malaria transmission (MacDonald, 1957).

Insecticide resistance

Insecticide resistance is an inherited attribute that comes to characterize an insect population following prolonged application of insecticides. Resistance is used to define a population where insecticide dosages formerly effective now meet with control failure. This is a major obstacle in control of diseases of medical, veterinary and agricultural importance. Insecticide resistance is not only a problem in medical entomology but in agricultural entomology as well (Brown, 1958).

Resistance is due to selection of hereditary factors such that the selected insecticide traits alone or in combination alter the physiology or biochemistry of an insect in such a way that higher amounts are required for killing them, even up to the point where the insect tolerates any given dose. These changes can also lead to resistance of other compounds other than the one used for the selective insect – this is referred to as cross resistance (Oppenoorth, 1976).

Understanding the nature of resistance can be helpful in attempts to prevent or overcome resistance as well as give indications of properties that are desirable in new insecticides to reduce the danger of resistance development.

Resistance occurs as a result of a genetic response to population selection pressure and is due to a selection of hereditary factors. Factors influencing development of resistance to insecticides can be classified into the following categories: a) genetic, b) reproductive, c) behavioral/ecological, and d) operational (Corbel and N'Guessan, 2013).

Mechanisms of insecticide resistance

Target site modification

Voltage gated sodium channel mutations (kdr)

Reduced nervous sensitivity to DDT and pyrethroids in insects is referred to as knock down resistance (*kdr*) in reference to the reduced knock down time. This resistance to pyrethroids in mosquitoes is due to a single nucleotide substitution at the target site of the insecticide. Pyrethroids and DDT both target the voltage-gated sodium channel, which comprises four domains (I–IV), each consisting of six transmembrane helices (S1–S6). In several insect species, the most common *kdr* mutation is a leucine to phenylalanine at amino residue 1014 (designated L1014F) substitution in the S6 hydrophobic segment of domain II in the sodium channel gene, such as that found in pyrethroid-resistant West African *An. gambiae* and *An. stephensi* (Ranson *et al*., 2002). However, a second substitution at the same position in which a leucine is replaced with a serine (L1014S) has been found in East African *An. gambiae* (Ranson *et al*., 2002). (Ranson *et al*., 2002). Both mutations have been reported in *An. sacharovi* Favre (Lüleyap et al., 2002) in Turkey.

Acetylcholinesterase

Biochemical assays in several mosquito species have identified insecticide resistance due to insensitive acetylcholinesterase, the target site of organophosphate and carbamate insecticides. Acetylcholinesterase has a key role in the nervous system, terminating nerve impulses by catalyzing the hydrolysis of the neurotransmitter acetylcholine. The insecticides inhibit enzyme activity by covalently phosphorylating or carbamylating the serine residue within the active site. Studies on *An. albimanus* from Central America have shown that an altered acetylcholinesterase is the most common organophosphate and carbamate resistance mechanism and the sequences of two AChE genes (*ace*-1 and -*2*) were identified from the *An. gambiae* genome.

GABA

The target site of cyclodiene insecticides such as dieldrin is the type A receptor for the neurotransmitter γ-aminobutyric acid (GABA). Binding of GABA to the receptor elicits rapid gating of an integral chloride-selective ion channel. GABA receptors comprise five subunits arranged around the central ion channel. Resistance to dieldrin appears to be related to amino acid replacement by single point mutations in the GABA receptor subunit genes (Ffrench-Constant et al., 2004).

Metabolic resistance and enzymes involved

Insecticide resistance also occurs when elevated levels or modified activities of oxidases, esterases or glutathione S-transferases prevent the insecticide from reaching its target site of action decreasing it sensitivity (Corbel and N'Guessan, 2013).

Cytochrome P450 role in insects

Cytochrome P450 monooxygenases (CYPs) are Phase 1 metabolic enzymes capable of oxidizing endogenous and exogenous compounds by oxidation or other related reactions (Scott and Wheelock, 1991, Berge et al., 1998). They belong to a superfamily of monooxygenases found in almost all living organisms and are predominantly localized in the endoplasmic reticulum membranes as integral membrane proteins, where they metabolize a variety of endogenous and xenobiotic compounds (Scott and Wheelock, 1991). CYPs also reside in other subcellular compartments, including the plasma membranes and mitochondria. CYP localization in mitochondria is regulated in one of two ways. Proteins of these enzyme families are also involved in synthesis of and break down of endogenous metabolic compounds, protection against oxidative stress, transmission of nerve signals and transportation of compounds through cells (Scott and Wheelock, 1991). The cytochrome P450 monooxygenases of insects have several functional roles including growth, development, feeding, resistance to pesticide, and tolerance to plant toxins. Cytochrome P450 monooxygenases also are intimately involved in the synthesis and degradation of insect hormones and pheromones including 20-hydroxyecysone and juvenile hormone (JH) (Scott and Wheelock, 1991).

Insect P450 monooxygenases can be detected in a wide range of tissues. Highest P450 monooxygenase activities are usually associated with the midgut, fat bodies, and Malpighian tubules. Dramatic variation in the levels of cytochrome P450 and monooxygenase activity are seen during the developments of most insects. In general P450 levels are undetectable in eggs, rise and fall in each larval instar, are undetectable in pupae and are expressed at high levels in adults (Scott and Wheelock, 1991).

Role of P450-monooxygenase in insecticide Resistance

Insects commonly become resistant to insecticides due to increased detoxification mediated by the cytochrome P450 monooxygenase system. This resistance mechanism is very important because it can confer both high levels of resistance and may also confer cross-resistance to unrelated compounds due to the breadth of substrates the P450 monooxygenase can metabolize.

There are multiple forms of cytochrome P450 in insects which are regulated independently of each other. In most cases where a link between insecticide resistance and elevated P450 activity has been shown, the Cyp gene belongs to the Cyp6 family. For example, CYP6D1 is over-produced in pyrethroid-resistant *M. domestica* due to up-regulated transcription and in a pyrethroid-resistant strain of *An. gambiae* from East Africa. Recent studies in *An. funestus* have identified cyp6p3 and cyp6p9 as the prime candidates conferring pyrethroid resistance in the species (Wondji et al., 2009).

Esterase-Mediated Resistance

Esterase is a group of hydrolase enzymes capable of hydrolyzing compounds containing ester bonds. Esterases are frequently implicated in the resistance of insects to OP, carbamates, and pyrethroids through gene amplification, upregulation, coding sequence mutations, or a combination of these mechanisms. The most widely studied mosquito species demonstrating this resistance mechanism are members of the genus Culex, including *Culex pipiens pipiens, C. p. quinquefasciatus* and *C. tritaeniorhynchus*. There are many reports of enhanced esterase activities in other mosquitoes, for example, in permethrin-resistant *An. gambiae*. However, there is as yet no evidence of this mechanism in pyrethroid-resistant mosquitoes. Amplifications of specific esterase genes have been doc-

umented in at least two orders, including Hemiptera (*Myzus persicae*, *Schizaphis graminum*, and *N. lugens*) and Diptera (*Culex pipiens* complex and *C. tritaeniorhynchus*). In *M. persicae*, overproduction of carboxylesterase E4 or its paralog FE4 protein via gene amplification is responsible for enhanced degradation and sequestration of a wide range of insecticides including OPs, carbamates, and pyrethroids (Raymond et al., 1993).

Glutathione

Glutathione S transferases (GST) are found ubiquitously in aerobic organisms. The main function of GST is considered to be detoxification of both endogenous and xenobiotic compounds directly catalyzing the secondary metabolism of a vast array of compounds oxidized by the cytochrome P450 family. Resistance to GST activity was first identified in organophosphate resistance where it can detoxify the active oxon analogue as described in *An. subpictus* (Hemingway *et al.*, 2004).

Secondary mechanism

Behavioral resistance

Behavioral resistance is avoiding contact with the toxicant. Behavioral resistance in vectors in some countries has arisen in response to prolonged spraying walls of huts in Africa with DDT solution as a residual for season long protection programs. This "bite and run" behavior was observed in populations of *An. gambiae s.s.* in the Tanga region of Tanzania (Gerold, 1977). This greatly reduced the survival prospects of indoor resting *Anopheles* mosquitoes sufficiently reducing malaria transmission (MacDonald, 1957). Behavioral resistance is the most subtle and difficult resistance to measure.

Cuticle resistance

Some mosquitoes have also evolved thicker or altered cuticles causing reduced penetration of insecticide. This has been reported in *Culex* (Apperson and Georghiou, 1975) but not in *Anopheles* species. The biochemical basis of reduced penetration is not clear, although lipid and protein composition of the integument have been proposed as potential mechanisms. For some insecticides the time to knockdown is significantly reduced in insects that possess the gene for reduced penetration, but toxicity is the same for both resistant and susceptible strains.

Fitness costs in the absence of insecticide selection pressure

Several studies have shown that in the absence of insecticide selection pressure, resistance genes have a cost in the biology of organisms such as low fertility and fecundity rates (Mandla et al., 2001, Arnaud et al., 2002), reduced survival, prolonged development (Voordouw et al., 2009), reduced body size, altered wing morphology, oviposition behavior, fluctuating asymmetry (Mandla et al., 2001), as well as mating competitiveness (Rowland, 1991). But there have been exceptions where resistance genes have been reported to confer a fitness advantage. In *An. funestus*, the resistant mosquitoes were reported to have both higher fecundity, as well as more viable eggs, when compared with the susceptible strain. In terms of development, no significant differences in pupation and emergence rates, or adult longevity between the resistant and susceptible strains were observed (Okoye et al., 2007). Developmental time and body size are key life history traits which affect fitness of organisms. Similarly, the degree of expression of many genes is influenced by environmental conditions. Higher temperatures can affect aquatic larvae and terrestrial adults.

Effects of Environmental Factors and Link to Fitness

Many environmental factors such as temperature, rainfall, larval density, chemical pollution and nutritional deficiency are known to affect mosquito life history (Mandla et al., 2001). Of these, temperature is perhaps the most important factor shown to affect larval survival and development, rate of emergence to adult, as well as the amount of larval food available in habitats (Teng and Apperson, 2000). All have implication for malaria transmission (Gu et al., 2006).

Development time and adult body size are consequences of growth and developmental processes which can influence components of fitness (Kingsolver and Huey, 2008). In disease vectors this may have implications on adaptation to different environments and as a consequence on disease transmission, yet the effect of temperature on pyrethroid resistant mosquitoes expressing P450 detoxification mechanism is not known.

Effects of glycogen

Insects store energy reserves in the form of glycogen and triglyceride in the adipocytes, the main fat cell body (Arrese and Soulages, 2010). Glycogen is storage in substantial amounts in fibrillar thoracic muscles (Clements, 1992). Glycogen and trehalose are major carbohydrates that play a role in energy metabolism of insects (Candy, 1985). Glycogen is stored within cells and can provide substrates directly without necessity to move into cells (Candy, 1985). Trehalose is circulated in hemolymph and provides immediate energy sources for insects (Candy, 1985). Daily variation in temperature may be associated with variation in other physical and biological factors and these may interact to affect survival strategies. Sugar is the basic food of adult mosquitoes and the only nutrient consumed by males and females although the latter require vertebrate blood meal

for egg production (Foster, 1995). Most female mosquitoes will take a sugar meal before a blood meal, and other studies have shown they a preference for sugar over blood or rarely bite until after a sugar meal (Andersson, 1992). However sugar feeding is strongly periodic and is influenced by weather season and locality (Foster, 1995). Glycogen and sugar are required for flight, maintenance and egg production. Insects must utilize energy, and if they are not feeding must utilize the reserves accumulate at eclosion or in period of food abundance (Arrese and Soulages, 2010).

Statement of the problem

Pyrethroids are the primary insecticides used for control of *An. gambiae* to prevent transmission of malaria in endemic areas. However, overuse has resulted in increasing reports of pyrethroid resistance in *An. gambiae* (Corbel et al., 2007, Adasi and Hemingway, 2008, Protopopoff et al., 2008). The interaction of the resistant phenotype and environmental conditions could affect life history traits such as development time, lifespan. These factors are linked to fitness, and can affect the rate of development of insecticide resistance, yet little is known regarding the life history traits of several resistant strains.

While associations of fitness costs and insecticide resistance has been reported in other Culicidae, there has been less reported regarding life history traits in permethrin-resistant *An. gambiae* and none in *An. gambiae* with co-occurrence of permethrin resistance with metabolic detoxification mechanism.

This dissertation is a compilation of three manuscripts which examine components of life history traits linked to fitness in RSP. The first manuscript titled “Increased mitochondrial reactive oxygen species and reduced adult lifespan in an insecticide re-

sistant strain of *Anopheles gambiae*" compares development time, body size, lifespan between females of ASEMBO1 and RSP strain. In addition it looks at mitochondrial reactive oxygen species between the two strains as well as expression of several antioxidants between females of the two strains. The next manuscript titled "Sex, temperature and genetic effects on developmental time and body size in *Anopheles gambiae"* evaluates the effects of temperature on development time, body size and sex between ASEMBO1 and the RSP strain at 25˚C and 30˚C. The last manuscript titled "Comparisons between laboratory-reared permethrin susceptible *An. gambiae* and permethrin resistant *An. gambiae* with metabolic detoxification enzyme systems: survival and glycogen utilization in relation to temperature" compares glycogen levels in teneral adults and in starved and honey-fed adults to understand the effect of temperature on the physiology of the RSP strain. In addition the effects of temperature on lifespan under the two nutritional states (starvation and honey-fed) are also evaluated. Lastly an overall summary and conclusions of results are presented and suggestions of future studies provided.

INCREASED PRODUCTION OF MITOCHONDRIAL REACTIVE OXYGEN SPECIES AND REDUCED ADULT LIFE SPAN IN AN INSECTICIDE-RESISTANT STRAIN OF *ANOPHELES GAMBIAE*

by

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Submitted to *Bulletin of Entomological Research*

Abstract

Control of the malaria vector *An. gambiae* is still largely obtained through chemical intervention using pyrethroids, such as permethrin. However, strains of *An. gambiae* that are resistant to the toxic effects of pyrethroids have become widespread in several endemic areas over the last decade. The objective of this study was to assess differences in five life-history traits (larval developmental time and the body weight, fecundity, hatch rate, and longevity of adult females) and energy metabolism between a strain of *An. gambiae* that is resistant to permethrin (RSP), due to knockdown resistance and enhanced metabolic detoxification, and a permethrin susceptible strain reared under laboratory conditions. We also quantified the expression levels of five antioxidant enzyme genes: *GSTe3*, *CAT*, *GPXH1*, *SOD1*, and *SOD2*. We found that the RSP strain had a longer developmental time than the susceptible strain. Additionally, RSP adult females had higher wet body weight and increased water and glycogen levels. Compared to permethrin susceptible females, RSP females displayed reduced metabolic rate and mitochondrial coupling efficiency and higher mitochondrial ROS production. Furthermore, despite higher levels of *GSTe3* and *CAT* transcripts, RSP females had a shorter adult life span than susceptible females. Collectively, these results suggest that permethrin resistance alleles might affect energy metabolism, oxidative stress, and adult survival of *An. gambiae*. However, because the strains used in this study differ in their genetic backgrounds, the results need to be interpreted with caution and replicated in other strains in order to have significant implications for malaria transmission and vector control.

Key words: *Anopheles gambiae*; permethrin; ROS; mitochondria; insecticide resistance.

Introduction

Permethrin is an insecticide that belongs to the pyrethroid family of neurotoxic agents designed to kill insects by altering the permeability of the voltage-gated sodium channel (VGSC). Pyrethroids are highly preferred to other insecticides in vector control management practices due to their low toxicity to humans, rapidity of action, and *easy* and fast *degradability* in the environment (Ray, 2001). As such, permethrin-based insecticide treated nets and indoor residual spraying have been extensively used to control *An. gambiae* populations in malaria endemic areas and have been effective in reducing the transmission of the parasites from infective mosquito females to humans (Enayati & Hemingway, 2006; WHO, 2007). However, over the last decade, strains of *An. gambiae* that are resistant to the toxic effects of permethrin and other pyrethroids have become widespread in several endemic areas in Africa (Casimiro et al., 2006; Adasi & Hemingway, 2008). The most established source of permethrin resistance is represented by point-mutations within the gene encoding the VGSC that have been associated with knockdown resistance to different insecticides (Ranson et al., 2000). But, enhanced metabolic *detoxification mechanisms* through up-regulation of cytochrome P450 monooxygenase (P450), glutathione S-transferase (GST), and non-specific esterase genes have also been reported in *Anopheles* mosquitoes (Vulule et al., 1999; Hemingway & Ranson, 2000; Djouaka et al., 2008; Lumjuan et al., 2011). While significant progress has been made in understanding the molecular mechanisms underlying the resistance to permethrin in malaria vectors (Li et al., 2007; Soderlund, 2008), there have been few reports of how alleles that confer resistance to insecticides affect other fitness characteristics of the malarial vectors in insecticide free environments.

Life history theory applied to this problem suggests that we should expect alleles influencing resistance to insecticide to come at the cost of other fitness traits, especially in an insecticide free environment. The life-history theory is based on the idea that physiological traits such as reproduction, storage, somatic maintenance, growth, and development are energetically costly traits. Because resources are limited, differential allocation of energy among these competing demands can produce trade-offs among traits and natural selection is thought to have shaped the way organisms partition their limiting resources to these fitness components, balancing the costs and benefits (Wiley, 1974). Fitness costs associated with resistance mechanisms have been reported in insecticide-free environments reviewed in (Brooke & Koekemoer, 2010; Kliot & Ghanim, 2012). For example, work in *An. gambiae* and *An. stephensi* showed that strains resistant to dieldrin had reduced fecundity compared to susceptible individuals despite similar longevity (Rowland, 1991). Reduced fecundity and shorter reproductive period and lifespan were also observed in carbofuran resistant aphids (Roberto & Omoto, 2006). However, loss of fitness has not been observed in other studies (Okoye et al., 2007), suggesting that a cost of resistance may not always occur (Coustau et al., 2000; Rigby et al., 2002). This is particularly true if different molecular mechanisms of resistance exist and/or ecological factors are involved (Coustau et al., 2000). Because evolutionary fitness costs are the cornerstones of economic optimal models of malaria vector insecticide resistance (Brown et al., 2013); more research in this area is necessary. Such research could elucidate whether new resistance management strategies for insecticide use are needed to maintain or restore its efficacy (Read et al., 2009).

The employment of mechanisms of detoxification in insects can be energetically costly (Coustau et al., 2000). Thus, it is conceivable that increased metabolic detoxification in pyrethroid resistance would result in a resource allocation trade-off between the detoxification mechanisms and other energetically demanding physiological functions, such as growth or reproduction (Rivero et al., 2010). In the present study, we compared life-history traits and energy metabolism between a wild-derived permethrin resistant strain of *An. gambiae,* and a permethrin susceptible strain reared under laboratory conditions. The permethrin resistant strain used in our study was previously characterised for a *knockdown resistance (kdr)* mutation and enhanced levels of P450 and esterase enzyme activities (Vulule et al., 1999). We *focused on this resistant strain* of *An. gambiae* because growing evidence suggests that pyrethroid resistance in the wild is likely due to a combination of target-site insensitivity and metabolic-based mechanisms (Brooke & Koekemoer, 2010).

Materials and Methods

Strains and colony maintenance

A strain of *An. gambiae* with reduced susceptibility to permethrin (RSP) and a permethrin susceptible (ASEMBO1) strain were used. The strains were obtained from the Malaria Research and Reference Reagent Resource Center (MR4) (http://www.mr4.org/). RSP has the East African form of the *kdr* sodium channel mutation allele, L1014S, and increased P450 and beta-esterase activities (Vulule et al., 1999). RSP was originally isolated from a permethrin impregnated bed net study carried out in four adjacent villages in North West of Kisumu, Kenya (Vulule et al., 1999). The strain was established from a colony representative of *An. gambiae* selected for permethrin tolerance. Laboratory selec-

tion for permethrin resistance consists of treating a cohort of fourth-instar larvae with 1ppm permethrin for 24 hours every three generations. Strain identification is performed every 10 generations by PCR (http://www.mr4.org/).

The permethrin susceptible strain ASEMBO1 was originally collected 50 km west of Kisumu in a control area for the United States Agency for International Development cohort bed net project approximately 4.5 km from the study area (http://www.mr4.org/).

Experimental conditions

Mosquitoes were maintained in insectary rooms at 25°C ± 2°C, 80% ± 10% relative humidity, and 16 hr light/8 hr dark cycle. Larvae were provided abundant food consisting of ground Tetramin (fish food). Adult mosquitoes were fed on 10% honey solution soaked in cotton sticks. Unless otherwise stated, experimental groups of each strain were three to five day old adults reared in 30 cm^3 cages. For egg production, young adults of the respective strains were allowed to freely mate and feed on blood from ears of a restrained rabbit. Moist filter paper was placed in cages for oviposition and the eggs collected were incubated overnight in a Petri dish and were hatched in deionised (DI) water. The larvae were reared in plastic pans (29.5 x 23.5 x 15 cm) containing approximately 1000 mL of water in environmental chambers (Thermo Scientific, Dubuque, Iowa, USA). Each pan contained groups of 50 larvae which fed *ad libitum* on Tetramin. Emerging adults were reared in the environmental chamber and fed *ad libitum* on 10% honey solution.

Developmental time

Groups of 16 first-instar (L1) larvae of each strain were randomly selected and transferred into 12 emergence containers (11 cm in diameter) each holding 100 mL DI water and approximately 4.8 mg of Tetramin added daily and maintained in environmental chambers. Larvae were counted daily until they reached the pupa stage when they were transferred into individual test tubes containing 3 ml of DI water.

Body weight and glycogen levels

To determine wet weight, female mosquitoes were anaesthetised with CO_2, transferred into vials in groups of five, and weighed to 0.1 mg accuracy with an analytical balance (OHAUS corp. Pine Brook, NJ). Mosquitoes were then dried in a bath incubator (Fischer Scientific) at 60˚C for 1 hour and the dry weight was measured as a proxy for their size (Siegel et al., 1994). *Water* content was calculated by subtracting the *dry weight* from the wet weight.

Glycogen content was assessed in three-five day old females fed on 10% honey using the protocol described in (Jumbo-Lucioni et al., 2010). Briefly, for each strain, 10 independent replicates, each containing a group of 10 mosquitoes were assayed. Groups of mosquitoes were homogenised on ice using 40µl of homogenization buffer (0.01 M KH_2PO_4 and 1 mM EDTA pH 7.4). The homogenates were centrifuged in a microcentrifuge at 2000 rpm for 2 minutes at 4 °C. Aliquots of 1.67 µl of homogenate were added to 250 µl of a reagent containing 0.1 U/ml of amyloglucosidase. After 30-minute incubation period at 37°C, OD_{540} was measured. The concentration of glycogen was determined from a glycogen standard run with each replicate. An independent set of 10 independent replicates was assayed for triacylglycerol content spectrophotometrically using a com-

mercially available kit (Sigma-Triglyceride kit) following manufactures protocol. Each sample was assayed twice and the mean was used in the analyses.

Fecundity

After blood feeding, 40 mosquitoes of each strain were randomly aspirated and transferred into individual cages with moist filter paper for egg laying. The cages were provided with 10% honey solution and egg production monitored daily. The eggs produced were counted and incubated overnight in Petri dishes to determine hatch rate after which the filter paper was replaced. Eggs were hatched in rearing pans in 1000 mL of DI water. Care was taken to ensure all eggs stuck on the sides of the rearing pan were in contact with DI water. Hatched eggs were counted on the second day.

Adult life span

One hundred and twenty pupae of each strain were randomly collected in pupa cups and transferred to three cages (40 per cage) in an environmental chamber. Pupa cups were removed from the cages after 24 hours so that pupae that failed to emerge were excluded. The emerged adults were fed on 10% honey solution *ad libitum*. The cages were monitored daily and dead individuals were counted and removed until all individuals had died.

Metabolic rate measurement

Metabolic rate was determined as described in (De Luca et al., 2010). Briefly, metabolic rate as CO_2 production was measured using a flow-through respirometry system (Qubit System Research, Kingston, Ontario, Canada). Groups of five females of each strain were anaesthetised with CO_2 and gently transferred to the respirometry chamber.

CO_2 was then measured for 10 min/chamber with a 30 second flush period between measurements at a flow rate of 30ml/min. The amount of CO_2 produced by each group of mosquitoes was calculated using C950 Data Acquisition software (Qubit System Research, Kingston, Ontario, Canada).

Mitochondrial respiration rate assay

Mosquitoes were anaesthetised with CO_2 and thoraces dissected from 30 females per replicate. All mitochondrial isolation steps in the six replicates were performed on ice. Mosquitoes were chilled briefly on ice and thoraces were separated from the heads and abdomens. Dissected thoraces were placed into 200µl of ice-cold isolation buffer [250 mM sucrose, 5 mM Tris-HCl, 2 mM EDTA, 1% (w/v) bovine serum albumin (BSA), pH 7.4 at 4°C; (Miwa et al., 2003) supplemented with protease inhibitors (leupeptin 1mg/ml, aprotinin 1mg/ml and pepstatin 1mg/ml) in a 1.5 ml Eppendorf tube. The samples were pounded gently 126 times over a 2 minute period, using a custom-built, motorised micromortar. Mashed mosquitoes were filtered through a 5 micron nylon mesh, and the volume was raised to 400µl by washing the nylon membrane with additional isolation buffer. A cycle of low-speed centrifugation (1 min centrifugation at 1000g) was followed by centrifugation of the filtered solution for 10 min at 3000 g at 4°C, and the pellet re-suspended in 100µl of isolation buffer. Protein concentrations in the mitochondrial fractions were determined using a Lowry assay.

Using freshly isolated mitochondria, mitochondrial respiration assays were performed using a polarographic oxygen sensor (Oroboros oxygraph, OROBOROS® INSTRUMENTS**,** Innsbruck, Austria) with 0.2 mg/ml of freshly isolated mitochondria incubated in respiration medium (120mM KCl, 5mM KH_2PO_4, 3mM Hepes, 1mM EDTA, 1mM

$MgCl_2$, and 0.2% BSA, pH 7.2; (Ferguson et al., 2005). Oxygen consumption rates were measured at 25° C (Sacktor & Sanborn, 1956). As implemented by (Miwa et al., 2003), we measured state 3 and state 4 respiration rates using the NAD^+-linked substrates pyruvate 5mM/proline 5mM to deliver electrons into mitochondrial complex I predominately. NAD^+-linked substrates were added to the chamber and allowed to equilibrate for 1 min, followed by the addition of ADP at a concentration of 400μM to elicit ADP-dependent state 3. This was followed by the determination of the state 4 respiration rate, once all the added ADP had been exhausted and a steady state is reached (Affourtit et al., 2012). Mitochondrial coupling efficiency (P: O ratio), e.g. the relationship between ATP synthesis and oxygen consumption, was calculated as the amount of ADP consumed per oxygen being reduced during state 3 (Jumbo-Lucioni et al., 2012). All assays were performed within three hours of mitochondrial isolation. Data were analyzed using the software *DatLab* Version 4.1.0.8.

Detection of ROS

Mitochondria for ROS analysis were stored in ice cold conditions and used within 5 hrs after isolation. ROS levels were measured using 10-acetyl -3, 7 dihydroxyphenoazine (Amplex Red, AR; Molecular Probes, Eugene, OR), to detect hydrogen peroxide (H_2O_2) in the presence of horseradish peroxidase, producing the red-fluorescent oxidation product (excitation/emission = 571/585 nm), resorufin. The ROS production of isolated mitochondria was measured as a basal rate (state 2 respiration) initiated by the reaction of saturated levels of substrates (pyruvate and proline), which evaluates ROS produced in a leak-like state without ADP and no oxidative phosphorylation. ROS production was also measured in the presence of rotenone and antimycin A, known inhibitors of complex I

and complex III of the electron transport chain (ETC), respectively. Complexes I and III have been reported as the primary sites of mitochondrial ROS production (Hinkle et al., 1967; Boveris et al., 1972; Cadenas et al., 1977; Raha et al., 2000) and these inhibitors would assess that ROS production capacity of the ETC at these complexes. All these reactions were performed in triplicate for both strains at the same time in the same plate. In addition, a vehicular control was included to provide values for non-mitochondrial ROS. The data in arbitrary units are mean activity of the reaction for 30 minutes measured in a Synergy 2 plate reader at 600 nm.

Quantitative (q) PCR

Groups of 10 permethrin susceptible and RSP females each in six replicates were homogenised using TRI REAGENT (Promega) as described in the manufacturer's instruction. First strand cDNA was synthesised from 500 ng/ μL of total RNA using Super-Script III, RNaseOUT, reverse transcriptase buffer, $MgCl_2$ and an $Oligo(dT)_{20}$ (Invitrogen) as described in the manufacturer's protocol. Thirty five cycles of amplification were performed in PCR machine. The amplification cycle was as follows: 95˚C for 5 min, 95˚C for 30 sec, 53˚C for 30 sec and 72˚C for 45 sec and 72˚C 7 min. We performed quantitative qPCR using a SYBR Green Master mix and 50 ng total of cDNA per reaction and run in a Stratagene Mx3000P® qPCR machine. The primers used for qPCR on the same total RNA are listed in Table 2.

Data analysis

A χ^2 test was used to compare mean longevity between strains. A log-rank test was used to compare median longevity between strains and a Kaplan-Meier curve was provided to illustrate the difference between strains. The data analysis for metabolic rate

and glycogen levels was performed using analysis of covariance, with body weight used as a covariate. A two-sample *t*-test was used to compare differences between means for the other phenotypes. Statistical significance was set at $\alpha < 0.05$ for each test.

Results

Developmental time

We found that the median developmental time from L1 to pupa was significantly longer (4%) in the RSP strain compared to the permethrin susceptible strain, (log-rank test $\chi^2_1 = 8.9$, $p = 0.003$) (Figure 1). Significantly longer (6%) was also the median developmental time from L1 to adult emergence (log-rank test $\chi^2_1 = 13.6$, $p = 0.0002$) (Figure 1).

Body weight, energy storage, and metabolic rate

Evidence exists of a strong trade-off between larval developmental time and adult weight in insects (Santos et al., 1994). Therefore, we measured both wet body weight and dry weight of 3-5 day old females. RSP females had significantly higher wet weight than permethrin susceptible females ($p = 0.044$) (Figure 2A). On the other hand, there were no significant differences in dry body weight between the strains ($p = 0.250$) (Figure 2B). As expected considering the higher wet weight, the RSP females had on average 5% more water content ($p = 0.026$) than permethrin susceptible female (Figure 2C).

The capacity of glycogen to bind water is 3-5 times its own weight (Schimdt-Nielsen, 1997). To explore whether the higher water content of RSP females was accompanied by an increase in glycogen level, we measured the glycogen content of whole-body sugar-fed females. Compared to permethrin susceptible females, RSP females had on average 9.5% higher levels of body weight-adjusted glycogen in ($p = 0.014$) (Figure 2

D). No statistical difference in triacylglycerol levels ($p = 0.27$) was, however, observed between the two strains (Figure 2 E). The mean triacylglycerol content for the RSP and permethrin-susceptible females was 11.36 μl/mg ± 1.71 and 14.51 μl/mg ± 2.17, respectively.

Previous studies of desiccation resistance and water balance in natural populations of *Drosophila* (Gibbs & Matzkin, 2001) have shown a positive correlation between metabolic rates and water-loss rates (Gibbs & Matzkin, 2001). The relationship between metabolic rate and water loss has been observed in a variety of other insects as reviewed by (Chown & Gaston, 1999) and is explained by the fact that reducing metabolic rates can help the insect to conserve water by reducing the need for gas exchange. Thus, we measured CO_2 production in the two strains. There was a statistical difference in metabolic rate between the strains ($p = 0.034$). As predicted, the mean VCO_2 in RSP was lower than in permethrin susceptible females, 20.48 μl/h ± 1.27 and 25.07 μl/h ± 1.48, respectively (Figure 2 F).

Fecundity

The mean number of eggs produced in one gonotrophic cycle between the two strains was not significantly different (Table 1). No differences were also observed in the number of eggs hatched and the time from blood feeding to laying eggs between the two strains (Table 1).

Longevity and mitochondrial bioenergetics

We observed a significantly reduced adult life span of RSP females compared to permethrin susceptible females (log-rank test $\chi^2_1 = 10.49$, $p = 0.0012$) (Figure 3).

Previous work in *Drosophila* reported a positive correlation between inter-individual variability in survival and mitochondrial bioenergetics (Melvin & Ballard, 2006). Thus, we next measured mitochondrial coupling efficiency (P: O ratio) and H_2O_2 production rate using NADH-linked substrates. Notably, mitochondria isolated from thoraces of RSP females showed a significantly lower coupling efficiency than those isolated from thoraces of permethrin susceptible females ($p = 0.050$) (Figure 4A). Furthermore, the RSP females' mitochondria produced significantly more ROS with only substrates present and no ADP (no oxidative phosphorylation or ATP production occurring), reflective of the leakiness of the system to proton re-entry (state 2; $p = 0.010$), and also produced significantly more ROS from complex I (rotenone; $p = 0.017$) than those from susceptible females (Figure 4B). There was no statistically significant difference in ROS production from complex III between the strains (antimycin A; $p = 0.096$) (Figure 4B).

Antioxidant gene expression

To control for the oxidative stress induced by the enhanced production of ROS in mitochondria, cells produce different enzymes that scavenge ROS, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and GST (Hayes & McLellan, 1999). Previous work in *An. gambiae* showed that the expression of *GSTe3* is significantly enhanced following H_2O_2 exposure (Kumar et al., 2003; Ding et al., 2005). Additionally, it has been reported that CAT is the primary antioxidant enzyme involved in oocyte protection by ROS damage in *An. gambiae* (DeJong et al., 2007). To assess whether the increased mitochondrial ROS production in the RSP strain is associated with

the induction of components of the antioxidant system, we measured the expression of *GSTe3, CAT, GPXH1, SOD1, SOD2* genes using mRNA extracted from whole-body of females of each strain. We found a statistically significant difference in *GSTe3* (p = 0.011) and catalase (p = 0.0068) expression levels between the two strains, with RSP females showing on average 80% *GSTe3* (Figure 5A) and 92% *CAT* (Figure 5B) respectively higher transcript levels than permethrin susceptible females. No significant difference was observed in the expression levels of either *GPXH1* (p = 0.650) (Figure 5C), *SOD1* (p = 0.430) (Figure 5D) or *SOD2* (p = 0.480) (Figure 5E).

Discussion

Here, we compared several life history and energy metabolism traits of a permethrin susceptible strain of *An. gambiae* and a strain harboring kdr and metabolic-based mechanisms conferring permethrin resistance (RSP strain). We observed that the RSP strain had lower metabolic rate, slower developmental time, and shorter adult life span than the permethrin susceptible strain. Additionally, consistent with previous evidence in different organisms of a relationship between enhanced levels of ROS and age-associated decline (reviewed in (Marchi et al., 2012), mitochondria isolated from the thoraces of the RSP females were found to have lower coupling efficiency and higher ROS production rates. Collectively, these results suggest that permethrin resistance alleles could affect energy metabolism, oxidative stress, and adult survival of *An. gambiae* and therefore impose strong fitness costs to the malaria vector. However, one major limitation of this study is that the RSP and permethrin susceptible strains have different genetic background and thus we cannot exclude the possibility that different loci may be responsible for the phenotypic differences observed between the strains. Also, although our strains of

An. gambiae were originally isolated from the wild in the same geographic area in Kenya, they were subsequently reared under laboratory conditions for over ten years and may suffer from inbreeding depression. Therefore, our results need to be interpreted with caution and replicated in independent studies in order to have significant implications for malaria transmission and vector control.

In our study, we found that both developmental times from L1 to pupation and from L1 to adult emergence were longer in RSP when compared to the permethrin susceptible strain. However, despite the longer developmental time, we did not observe a difference in dry body weight between females of the two *An. gambiae* strains. This result is interesting considering the strong relationship between molecular and physiological mechanisms that regulate the duration of developmental time and rate of growth and final body size (Shingleton, 2011). Compared to females of the permethrin susceptible strain, adult females of the RSP strain also showed higher water and glycogen levels, which is in agreement with the high energetic investment that is required to support the body's detoxifying apparatus (Coustau et al., 2000). To our knowledge, our study is the first to report an increase in energetic resources in a strain of *An. gambiae* with enhanced metabolic detoxification of permethrin.

P450 monooxygenase-mediated detoxification is a major mechanism of resistance to insecticides due to the wide variety of substrates that P450s can metabolise (Félix & Silveira, 2012). The P450 monooxygenase system consists of two main components: the cytochrome P450, which acts as the substrate binding protein (and terminal oxidase), and the NADPH-cytochrome P450 reductase (P450 reductase), which transfers electrons from NADPH to cytochrome P450 (Kawano et al., 1987; Scott & Wheelock, 1991; Guzov et

al., 1996). As previously described in (Vulule et al., 1999), the RSP strain used in this study is characterised by elevated levels of P450 enzymatic activity. The overproduction of detoxifying enzymes in insects can be energetically costly (Coustau et al., 2000), and investment in resistance can be expected to produce reduced investment in competing organismal functions. Notably, our study did not show evidence of a reproductive fitness cost associated with the presence of both target-site insensitivity and metabolic-based mechanisms of permethrin resistance in *An. gambiae*. However, the lifespan of RSP females was significantly reduced compared to the permethrin susceptible females. A potential explanation for these findings is that the resistant strain might invest more energy in enhancing the antioxidative and detoxification mechanisms and maintaining reproductive functions at the cost of somatic maintenance and repair, allowing faster rates of aging and decreased longevity (Rose & Charlesworth, 1981a; Rose & Charlesworth, 1981b). Consistent with this idea, we found lower mitochondrial coupling efficiency and higher ROS production rates in the mitochondria isolated from the thoraces of the RSP females. ROS, including superoxide and its dismutation product H_2O_2, are essential as signaling molecules in defense against infection and in reproduction (Sanz & Stefanatos, 2008). But, if produced in excess, they can oxidise and damage various cellular components, including mitochondrial proteins, membranes, lipids, and nuclear and mitochondrial genomes, and thus have been implicated in the aging process of a variety of species (Sanz & Stefanatos, 2008), including *Anopheles* (Monaghan et al., 2009). In insects, increased levels of P450 activity has been associated with high levels of ROS production as by-products of the detoxification processes (Murataliev et al., 2008). As such, it is tempting to speculate that the RSP strain may exhibit higher levels of ROS produced by P450 than

the permethrin-susceptible strain. These ROS may in turn oxidise and damage mitochondrial DNA, protein, and lipids, possible leading to mitochondrial dysfunction and excessive production of ROS by redox-coupled reactions within the ETC, as suggested by our mitochondrial bioenergetic results.

Antioxidant enzymes are produced by the cell in response to the oxidative stress induced by the enhanced production of ROS in mitochondria. We observed a 80% and 90% increase in expression of *GSTe3* and *CAT*, respectively, in young RSP females compared to females of the susceptible strain. As mentioned above, previous work reported that the antioxidant enzyme catalase is responsible for oocyte protection by ROS damage in *An. gambiae* (DeJong et al., 2007). Based on this observation, our gene expression data not only suggest that RSP mosquitoes might activate a strong antioxidant response to counterbalance an increased oxidative environment, but also support our hypothesis of an investment of energy resources to maintain fecundity.

In conclusion, our data provide new insights into the impact of insecticide resistance on the quality of the malaria vector and motivate future studies in other laboratory strains and wild mosquito populations.

Acknowledgments

We thank Michelle Moses Chambers and Gin Chuang for helping with metabolic rate and mitochondrial ROS production measurements, respectively. We also thank Jeff Leips for providing insightful comments on the manuscript. We are grateful to Edward W. Hook III and Sally Fried of the Division of Infectious Diseases, William C. Gorgas Center for Geographic Medicine, for granting permission to use the mosquito facility. The RSP strain was obtained through the MR4 as part of the BEI Resources Repository,

NIAID, NIH: *Anopheles gambiae* RSP, MRA-334, deposited by J Vulule, MQ Benedict. The ASEMBO1 strain was obtained through the MR4 as part of the BEI Resources Repository, NIAID, NIH: *Anopheles gambiae* ASEMBO1 [AB], MRA-186, deposited by MQ Benedict. This study was supported by the Department of Biology at the University of Alabama at Birmingham, the Bioanalytical Redox Biology Core (DRTC P60 DK079626), and NIH grant R01 DK084219 to MD.

Abbreviations

ADP: adenosine diphosphate; AR: Amplex red; ATP: adenosine triphosphate; BSA: bovine serum albumen; *CAT*: catalase; DI: deionised; EDTA: ethylene diamine tetraacetic acid; ETC: electron transport chain; *GPX*: glutathione peroxidase; *GST*: glutathione S-transferase; *kdr*: knockdown resistance; MR4: Malaria Research and Reference Reagent Resource Center; NAD: nicotinamide adenine dinucleotide; ROS: reactive oxygen species; RSP: reduced susceptibility to permethrin; *SOD*: super oxide dismutase; VGSC: voltage-gated sodium channel;

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Tables

Table 1: Reproductive characteristics for the permethrin susceptible and RSP strains of *An. gambiae.*

	Susceptible	**RSP**	***p* value**
Eggs laid	66.07±54.46 (24-182)	56.35±63.85 (8-211)	0.46
Eggs hatched	47.92±42.54 (2-129)	50.08±35.95 (2-139)	0.84
Duration (days) to lay eggs	3.66	3.55	0.66

Mean ± SD (range)

Table 2: Nucleotide sequence of primers used for qPCRs.

Gene	Primer	Sequence
GPXH1	GPXH1-FF	CCGGAAGGCGACGGAGAGGA
	GPXH1-RR	TCCACCGGCTGACCGTCCTT
SOD1	SOD1-FF	TCACTAACAATAATGTTGGCCGTA
	SOD1-RR	AACTTGATCGCATTGCCGAG
RPS7	RPS7-FF	TCATCATCTACGTGCCGGTG
	RPS7-RR	TACACGGCGGTGACGTTC
GSTe3	GSTe3-FF	GAGCTGACGGCAAAGATGATCG
	GSTe3-RR	CCTGCTTCACTAGATCCTTCGC
SOD2	SOD2-FF	CATCAACGTTGTGGGGCTGA
	SOD2-RR	ATGTTACCGAGATCGCCGAC
CAT	CAT-FF	AATTCCCGCTCATTCCGGTT
	CAT-RR	TCAGATAGTTGGCACCCACG

Figures

Figure 1: Developmental time from first-instar larva to pupa (Panel A; *n* = 465) and to adult emergence (Panel B, *n* = 337) in permethrin susceptible and RSP strains.

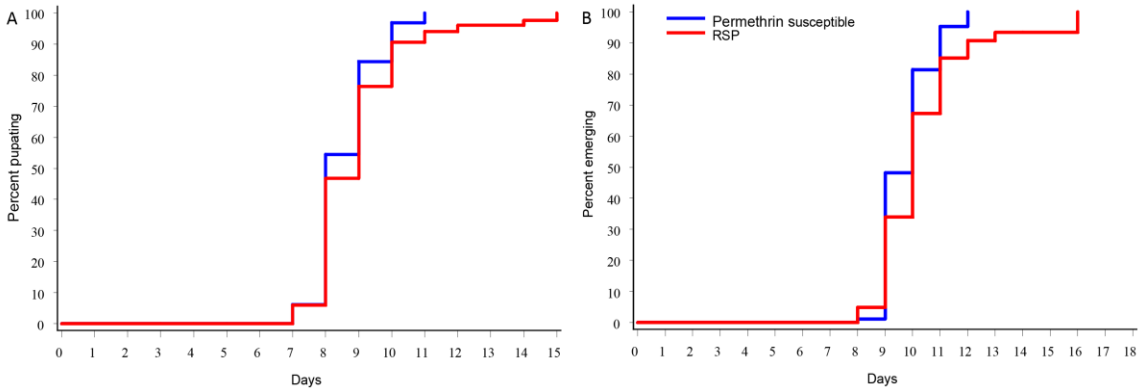


Figure 2: Adult wet weight (Panel A; *n* = 30), dry weight (Panel B; *n* = 30), water content (Panel C; *n* =30), glycogen levels (Panel D; *n* = 20), triacylgycerol storage (Panel E; *n* = 20) and metabolic rate (Panel F; *n* = 10) in permethrin susceptible and RSP females. Glycogen and triacylgycerol levels are normalised for body weight. Values are expressed as mean ± SE. * $p < 0.05$

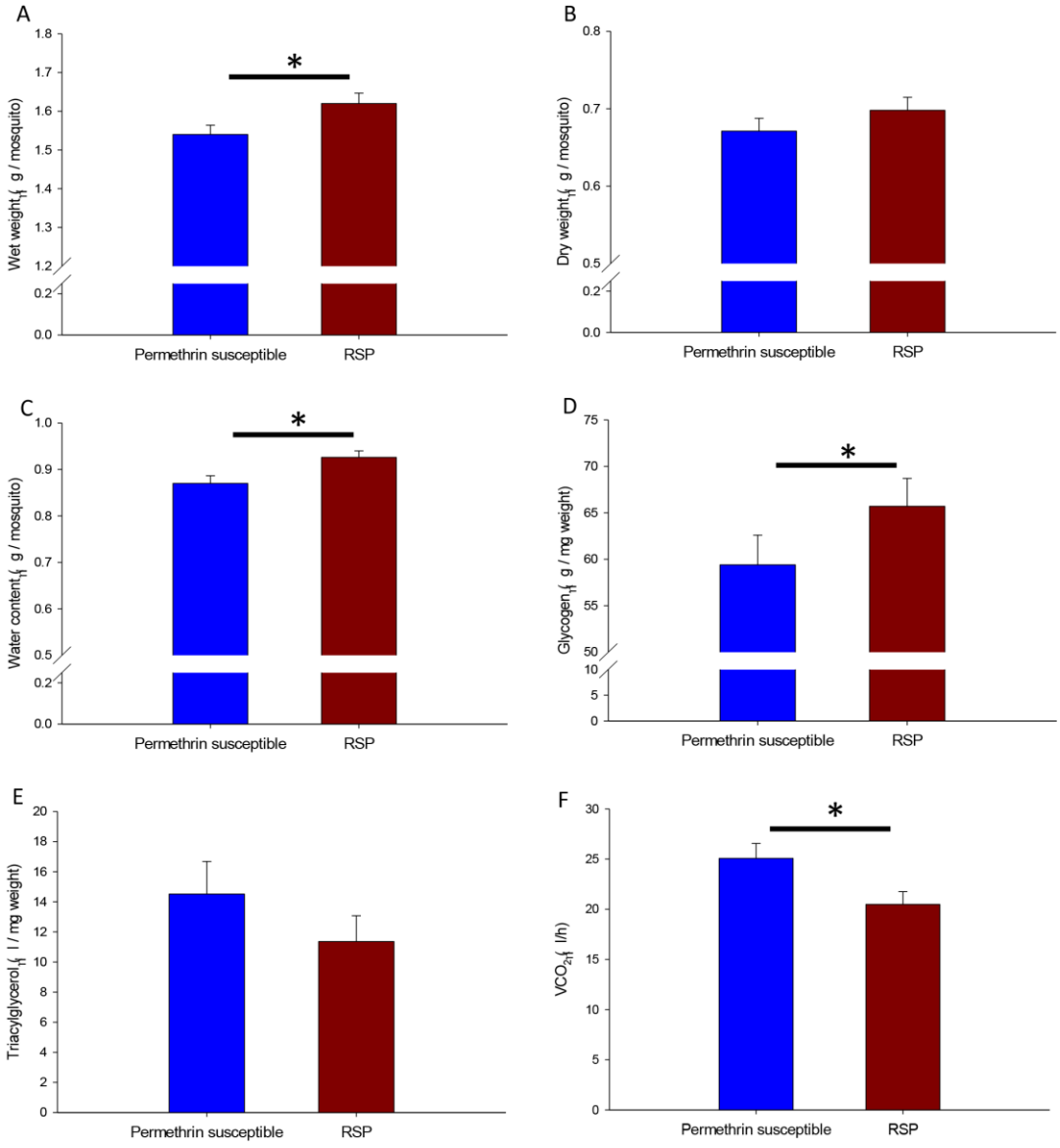


Figure 3: Adult female survivorship curves for the permethrin susceptible and RSP strains of *An. gambiae*. Survival assays were carried out using population cages with initial population sizes of 46-50 individuals.

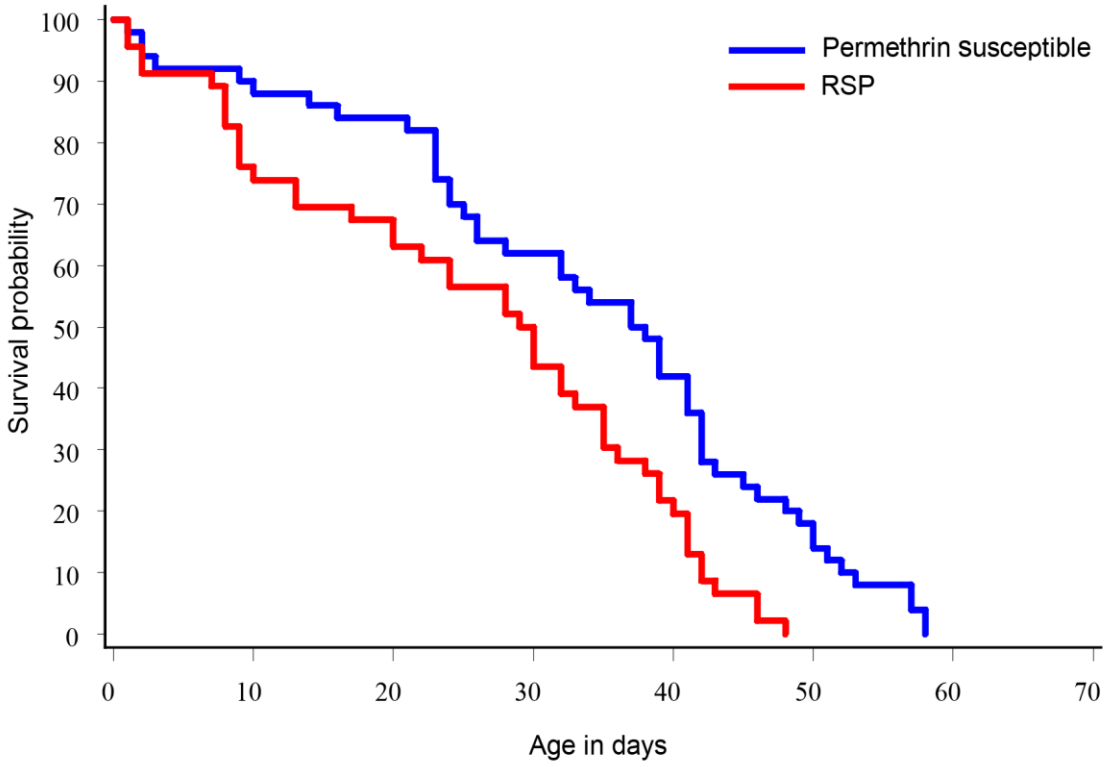


Figure 4: Coupling efficiency (or P:O ratio) (Panel A) and ROS production (Panel B) of mitochondria isolated from thoraces of permethrin susceptible and RSP females using NAD^+-linked substrates (pyruvate and proline). Values are given as means ± SE of six independent replicates.* $p < 0.05$.

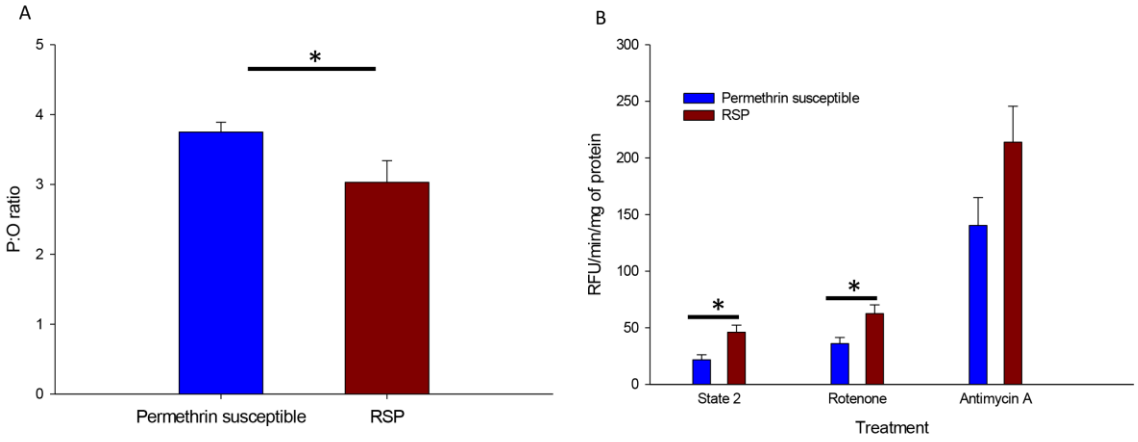
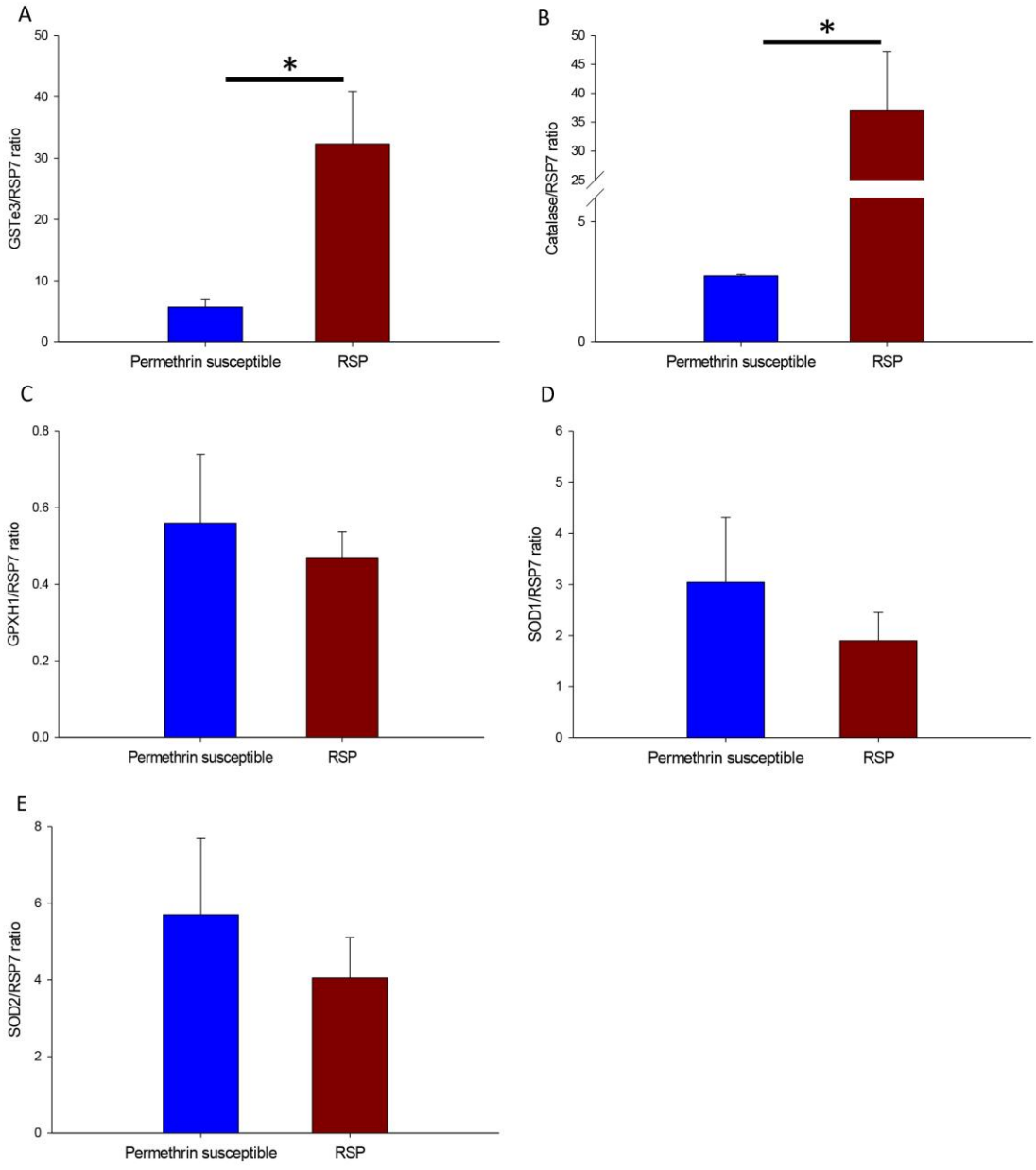


Figure 5: Relative mRNA expression of antioxidant enzymes in whole body extracts of permethrin susceptible and RSP females. (Panel A) *GSTe3: Glutathione S Transferase e3;* (Panel B) *CAT: Catalase;* (Panel C) *GPXH1: Glutathione Peroxidase;* (Panel D) *SOD1: Superoxide Dismutase 1;* and (Panel E) *SOD2: Superoxide Dismutase 2.* Transcript levels of the five genes were normalised to *RSP7 Ribosomal Protein S7*. Values are given as means ± SE of six independent replicates. * $p < 0.05$.



SEX, TEMPERATURE, AND GENETIC EFFECTS ON DEVELOPMENTAL TIME AND BODY SIZE IN *ANOPHELES GAMBIAE*

by

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In preparation for submission to *Journal of American Mosquito Control Association*

Abstract

Prolonged use of permethrin to control the mosquito *Anopheles gambiae* has resulted in the evolution of various resistant phenotypes. These include insecticide metabolic detoxification through elevated levels of mixed function oxidases and target site insensitivity, conferred by a mutation in the voltage-gated sodium channel also known as knock down resistance (*kdr*). In natural populations temperature affects larval survival, development, and rate of emergence of *An. gambiae,* but its effects on resistant strains are not well studied. Given that recent prediction models project warmer climates, shifting population demographics or resistance strategies could increase disease transmission, concomitant with the expected increase in pyrethroid resistance resulting from extensive and prolonged use of this insecticide. In this study we compared developmental time and body size between two *An. gambiae* strains, a reduced susceptibility to permethrin (RSP) strain carrying the *kdr* in addition to metabolic resistance, and a permethrin susceptible (ASEMBO1), both reared at 25˚C and 30˚C. Both strains showed reduced survivorship at 30˚C. RSP had a longer mean developmental time than ASEMBO1 strain. Females had a longer developmental time than males, and rearing at 30˚C resulted in faster development. The mean dry weight was significantly lower in the mosquitoes cultured at 30˚C than at 25˚C. These results show that temperature and insecticide resistance may interact to affect mosquito life-history traits associated with fitness, which could impact insecticide vector management strategies.

KEY WORDS *Anopheles gambiae,* insecticide resistance, temperature, development, body size

Introduction

Anopheles gambiae sensu stricto, a member of the *An. gambiae* species complex, is a major vector of *Plasmodium*, a blood parasite that causes malaria (Coetzee et al. 2000). Population control of *An. gambiae* depends largely on pyrethroids, a class of neurotoxic agent that is low in mammalian toxicity, rapid in action, and will degrade in the environment. However, prolonged exposure to this class of insecticides has led to development of insecticide-resistant phenotypes in *An. gambiae* (Ranson et al. 2011). It is widely recognized that pyrethroid resistance in *An. gambiae* is due in part to two major mechanisms (a) insecticide detoxification through elevated levels of mixed function oxidases, and (b) target site insensitivity, conferred by a mutation in the voltage-gated sodium channel also known as knock down resistance (*k.d.r.*) (Muller et al. 2008). The overproduction of detoxification enzymes in insecticide resistant organisms is thought to exert energetic costs which may contribute to fitness (Raymond et al. 2001, Rivero et al. 2011, Roush and McKenzie 1987). In addition, the biochemical network in which these enzymes function may produce synergistic, or antagonistic, effects on overall physiological performance and, as a consequence, affect fitness (Raymond et al. 1989).

Temperature affects larval survival and development rate to adult emergence in mosquitoes (Teng and Apperson 2000). The effect of temperature in insecticide-resistant organisms has been investigated in several studies and results have varied. In a study on the effect of temperature in insecticide-resistant and susceptible strains of the Diamondback moth, *Plutella xylostella*, hatching and larval survival were relatively constant within a large temperature range, but were lower at 35°C (Fang et al. 2008). The study reported no significant differences in survival rate from neonate to 4th instar, nor differences in

pupation rate and emergence rate between the susceptible and resistant strains (Fang et al. 2008). In aphids, resistant individuals were less able to survive low temperatures (Foster et al. 1996).

In defining the relation of temperature and development or size, Kirby and Lindsay (2009), indicated that higher rearing temperature resulted in smaller size individuals of *An. gambiae.* Lyimo et al. (1992) also reported a similar trend but both sexes were combined in analysis. In many insects including mosquitoes, there is sexual size dimorphism at adult stage resulting from differences in growth, duration of growth period, or a combination of both (Blanckenhorn et al. 2007, Stillwell et al. 2010). Exposure to various temperatures in insecticide-resistant and non-resistant strains of *Culex quinquefasciatus* resulted in sex- and resistance-specific differences in survival, developmental rate, and body size (Mpho et al. 2001).

Diurnal and seasonal variations in temperature are common in tropical regions where *An. gambiae* is endemic (Paaijmans and Thomas 2011), thus it is important to ask what the effects of temperature are in these insecticide-resistant phenotypes. The effect of temperature in *An. gambiae* s.s. has been examined previously in several studies. A study involving *An. gambiae* s.s. larvae reported that low temperatures slowed development, higher temperatures led to increased development, while temperatures of 35°C and above were fatal (Bayoh and Lindsay 2004). The study further reported that larvae developed into adults at temperatures ranging from 16°C to 34°C; however, larval survival was shortest (less than 7 days) at 10°C to 12°C and 38°C to 40°C and longest (more than 30 days) at 14°C to 20°C (Bayoh and Lindsay 2004). The resistance phenotype is dynamic and fluctuates throughout the transmission seasons (Ranson et al. 2009). Given that re-

cent models predict warmer climates, shifting population demographics or resistance strategies could increase disease transmission (Gething et al. 2010) concomitant with the expected increase in pyrethroid resistance resulting from extensive and prolonged use of this insecticide. It is recognized that pyrethroid-resistant phenotypes in *An. gambiae* will increase because population control depends on this class of insecticides. Thus, there is urgent need to understand how temperature affects important life history traits linked to fitness, including survival, development time and body size, in pyrethroid resistant *An. gambiae*.

In this study we examined survival, developmental time, and body size in resistant and susceptible laboratory strains of *An. gambiae* when cultured at two rearing temperatures. We used a pyrethroid-susceptible (ASEMBO1) strain and a strain that is resistant to pyrethroid (RSP) due to a *kdr* mutation and enhanced metabolic detoxification. We were not only interested in the effects of temperature on these strains, but also in determining whether or not temperature interacts with the RSP phenotype to affect life history traits related to fitness. Kingsolver and Huey (2008) have found that in most ectothermic organisms a higher rearing temperature results in smaller sized adults. Thus, under controlled laboratory environment we hypothesize that at elevated rearing temperature we will have smaller individuals and prolonged developmental time in resistant strain compared to the susceptible strain. In addition, there should be an increase in larval developmental time accompanied by a larger body size at the lower rearing temperature (Atkinson 1994, Partridge et al. 1994).

Materials and Methods

Mosquito colony

The *An. gambiae* strains used in this study included a (RSP) (MRA-334, MR4, ATCC Manassas Virginia) strain and a permethrin-susceptible (ASEMBO1) (MRA-186, MR4, ATCC Manassas Virginia) strain. The strains were obtained from the Malaria Research and Reference Reagent Resource Center (MR4) (http://www.mr4.org/).

RSP carries the East African form of the *kdr* sodium channel mutation allele, L1014S, as well as increased P450 and beta-esterase activities (Vulule et al. 1999). RSP was originally isolated from a permethrin impregnated bed net study carried out in four adjacent villages in North West of Kisumu, Kenya (Vulule et al. 1999). The strain was established from a colony representative of *An. gambiae* selected for permethrin tolerance.

The ASEMBO1 strain was originally collected 50 km west of Kisumu, Kenya in a control area for the United States Agency for International Development cohort bed net project approximately 4.5 km from the study area. Dr. Atieli collected blood-fed females from houses from which he obtained eggs to initiate a colony. Preliminary species identification was based on the frequency of *An. gambiae* collected in the same area (http://www.mr4.org/).

For general rearing, both mosquito strains were maintained in an insectary at 25˚C ± 2˚C, 80% ± 10% relative humidity, and 16: 8 hour light and dark cycle. Young adults of the respective strains were allowed to freely mate and fed on blood from ears of a restrained rabbit to produce eggs. Moist filter paper was placed in cages for oviposition and the eggs collected were incubated overnight in a Petri dish and hatched in deionized (DI)

water. Larvae were provided abundant food consisting of ground TetraMin (fish food, Tetra Werke) and adult mosquitoes were fed on 10% honey solution saturated on cotton sticks.

Experimental groups

Unless otherwise stated, the larvae of experimental groups were reared in plastic pans (29.5 x 23.5 x 15) cm containing approximately 1000 mL of DI water. Each pan contained groups of 50 larvae which were fed *ad libitum* on TetraMin in environmental chambers (Thermo Scientific, Dubuque, Iowa, USA) at either 25 or 30˚C for each strain. Newly emerged mosquitos were fed *ad libitum* on 10% honey solution at their respective culture temperature of 25 or 30˚C in 30 cm (length x height x width) cages until 3 to 5 days old (adult). Adults were then collected for analysis.

Developmental time

Groups of 16 first-instar (L1) larvae of each strain were randomly selected and transferred into 12 emergence containers (11 cm in diameter) holding 100 mL DI water and approximately 4.8 mg of TetraMin added daily. The emergence containers were transferred to environmental chambers maintained at a constant temperature of either 25 or 30˚C. Larvae were counted daily until the pupae stage, at which time individual pupae were transferred into a test tube containing 3 ml of DI water, allowed to emerge and were sexed. For the populations, developmental time was quantified as mean days from L1 to adult emergence.

Dry weight

To determine dry weight, mosquitoes were anaesthetized with CO_2 and transferred into vials in groups of five. These groups were collectively dried in a bath incuba-

tor (Fischer Scientific) at 60˚C for 1 hour and weighed to 0.1 mg accuracy with an analytical balance (OHAUS corp. Pine Brook, NJ) as an approximation of size (Siegel et al. 1994). Individual weight was estimated at one fifth of the combined dry weight.

Statistical analyses

The effects of strain and temperature on survival to adult emergence were analyzed in logistic regression (as implemented by Proc Phreg in SAS V9.3).

Development time was calculated as the mean number of days from L1 to adult emergence. Analysis of variance (ANOVA, as implemented by Proc GLM in SAS V9.3) was used to compare mean development time between strains, sexes and temperatures and interactions. For dry body weight, the effects of strains, sexes, temperature and their interactions were also compared in ANOVA using the GLM procedure in SAS.

Results

Survival

Survival during development of L1 to emergence did not vary between strains at 25˚C or 30˚C; however, survivorship decreased in both strains at 30˚C.

Development time

Analysis of variance showed a significant difference for main effect of strain ($F_{1, 7}$, = 23.25, $p < 0.0001$). Generally, mean development time (L1 to emergence, sexes combined) was significantly longer in the RSP strain than in the ASEMBO1 strain (Fig. 1 A).

There was a significant difference for main effect of sex ($F_{1, 7}$, = 9.09, p = 0.0027). Mean development time was significantly longer in females than in males (Fig. 1 B).

There was a significant difference for main effect of temperature ($F_{1, 7}$, = 167.70, $p < 0.0001$). Mean development time was shorter for individuals reared at 30˚C than at 25˚C (Fig. 1 C).

Dry weight

There was a significant effect of temperature on dry weight ($F_{1, 7}$, = 29.40, $p < 0.0001$) and a significant temperature by sex interaction ($F_{1, 7}$, = 68.46, $p < 0.0001$) (Table 2). Generally, dry weight was significantly higher in mosquitoes cultured at 25˚C than at 30˚C (Fig. 2 A). Dry weight was higher in females reared at 25˚C than at 30˚C. A Tukey-Kramer adjustment for multiple comparison test of all means indicated the dry weight in males did not vary with rearing temperature (Fig. 2 B). There was a significant effect of sex on dry weight ($F_{1, 7}$, = 316.23, $p < 0.0001$) and a significant strain by sex interaction ($F_{1, 7}$, = 5.65, $p = 0.0196$) (Table 2). Mean dry weight was significantly higher in females than in males (Fig. 2 C). Females of both strains had a higher dry weight than males at both temperatures (Fig. 2 D).

Discussion

Survivorship in this study was comparable to that reported in other studies. Kirby and Lindsay (2009) reported mortalities of 20% at 25˚C and 50% at 30˚C for *An. gambiae*, while (Jannat and Roitberg 2013) reported 20% in rearing *An. gambiae* at 30˚C. Observed differences among the studies may be due to differences in rearing methods or density of larvae in culture containers. In this study, the amount of food per container remained constant and the water was not changed in the course of the experiment. The trend to reduced survivorship at 30˚C in the current study suggests that stress is induced

at elevated temperatures in both strains. Differences between the strains were not consistent and trends were not observed.

Prolonged development from L1 to adult emergence of RSP females reared at 25˚C when compared to permethrin susceptible strain has been observed (Otali personal communication). Rearing at 30˚C resulted in shorter development time compared to 25˚C in both strains, indicating that temperature has a direct effect on development rate (van der Have and de Jong 1996). Prolonged development may result in increased generation time, leading to decreased rates of population growth (Kingsolver and Huey 2008) and potentially decreased fitness (Roff and Fairbairn 2007). We hypothesize that the prolonged development in the RSP strain may, in part, be due to stress associated with elevated temperatures; however, mechanisms related to stress tolerance are not fully understood. We hypothesize that the increased development time of RSP strain compared to ASEMBO1 reflects the potential role of the sodium channel during normal development. Alternatively, there is an increase metabolic cost associated with altered protein synthesis in the RSP strain (Coustau et al. 2000). Despite differences in developmental times, there were no differences in wet body weight of teneral males compared to females at 25˚C or 30˚C in both RSP and ASEMBO1.

We observed prolonged developmental times of females when compared to males. Our results are in agreement with the observation in mosquitoes that males emerge earlier than females (Clements 1992). Energetic demands may increase at higher temperatures and the potential increase in cost associated with female development may delay (increase) the time of emergence.

In adult females, we found that there was no difference in dry body weight between RSP and ASEMBO1 females. It is possible that mechanisms related to pyrethroid resistance and metabolic detoxification does affect development time but not post-emergent weight gain in females at these temperatures.

In adult males, dry weight did not differ at 25˚C or 30˚C. These data contrast with those of females and suggest that pyrethroid resistance, while affecting development in males, will not affect post-emergent weight gain at 30˚C. This observation is increasingly important if we consider the effects of rising temperatures during global climate change.

Lower adult weights in females in both RSP and ASEMBO1 strains reared at 30˚C as compared to 25˚C occurs concomitant with reduced development time, and suggest an energetic cost associated with high temperature. In addition the evolution of larger body size at a lower rearing temperature accompanied by an increase in larval developmental time (Partridge et al. 1994) suggests that temperature or a causally associated variable can likely influence development in nature (James et al. 1995).

In insects, development time and adult body size are determined by a set of underlying factors including growth rate, critical weight, and hormonal influence (D'Amico et al. 2001). A possible explanation for the lower dry weight at 30˚C is the thermal sensitivity of critical weight – the weight at which larvae commit to metamorphosis and a hormonal cascade is initiated, resulting in cessation of growth prior to metamorphosis (Nijhout and Williams 1974). A recent study in drosophila reported reduced critical weight at elevated temperature, suggesting larvae instigate the signal to stop growth at a smaller size (Ghosh et al. 2013). In contrast, males of the RSP or ASEMBO1 strains reared at 30˚C did not differ in dry weight compared to rearing at 25˚C. These results are

in contrast to that reported in males of *Aedes albopictus* in which dry weight of males reared at 30˚C were higher relative to those reared at 24˚C (Lounibos et al. 2002). In the current study RSP males were similar in size to ASEMBO1 males at 30˚C and at 25˚C. Mechanisms leading to temperature-dependent differences in adult dry weight in RSP are not known. A possible explanation for this finding is males may have a higher temperature threshold above the 30˚C used in this study. (Nijhout et al. 2010) incorporated several factors affecting development time and body size of *Manduca sexta* in a mathematical model and showed that over small ranges of growth rates, the correlation between development time and body size can be positive, zero or negative depending on environmental or genetic influence. The lack of a pattern between development time and body size in males reared at 30˚C is not known and requires further investigation.

In conclusion, this study indicates that development time is affected by pyrethroid resistance, with the RSP strain, in general, showing increased time to adult development. These differences in development time are obvious in males reared at 30˚C, suggesting that a compensatory mechanism, possibly sex specific, is involved in the control of development time.

Observed differences in development time were larger, with increased development time to emergence in the RSP strain and in females of both strains. Since high temperature decreased survival, we suggest that this secondary stressor (elevated temperature) exacerbates the strain and sex specific effects related in part to pyrethroid resistance. This study suggests further there is an increased energetic cost associated with females in both strains at 30˚C (decreased body size), but not in males. However, food intake was

not measured with this study so conclusions concerning energetic costs are speculative and require further study.

Many current models suggest temperature will be elevated during global climate change. Elevated temperatures will affect development and energetics, and various strains of mosquitos may respond differently to altered global climates. Additional information on elevated temperatures is required to develop integrated vector management strategies.

Acknowledgements

We are grateful to Edward W. Hook III and Sally Fried of the Division of Infectious Diseases, William C. Gorgas Center for Geographic Medicine, for granting permission to use the mosquito facility. We are also grateful to Dr. Bud Fischer for support. The RSP strain was obtained through the MR4 as part of the BEI Resources Repository, NIAID, NIH: *An. gambiae* RSP, MRA-334, deposited by J Vulule, MQ Benedict. The ASEMBO1 strain was obtained through the MR4 as part of the BEI Resources Repository, NIAID, NIH: *An.gambiae* ASEMBO1 [AB], MRA-186, deposited by MQ Benedict.

This study was supported by the Department of Biology at the University of Alabama at Birmingham, and the National Institutes of Health, National Institute of Diabetic and Digestive and Kidney diseases, through grant [R01DK084219 to MD].

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TABLES

Table A1: ANOVA of development time to adult emergence of RSP and ASEMBO1 strains.

Source	df	SS	F value	p value
Temp	1	154.58	167.70	< 0.0001*
Strain	1	21.43	23.25	< 0.0001*
Sex	1	8.37	9.09	0.0027*
Temp X Sex	1	1.53	1.66	0.197
Strain X Temp	1	2.13	2.32	0.128
Strain X Sex	1	1.21	1.31	0.252
Strain X Temp X Sex	1	2.60	2.82	0.093

Table A2: ANOVA of dry body weight of RSP and ASEMBO1 strains.

Source	df	SS	F value	p value
Temp	1	0.0880	29.40	< 0.0001*
Strain	1	0.0020	0.67	0.414
Sex	1	0.9474	316.23	< 0.0001*
Temp X Sex	1	0.2015	68.46	< 0.0001*
Strain X Temp	1	0.0053	1.78	0.185
Strain X Sex	1	0.0169	5.56	0.019*
Strain X Temp X Sex	1	0.0006	0.22	0.640

FIGURES

Figure 1: Mean development time from L1 to adult emergence (Panel A *n* = 588) in permethrin susceptible and RSP strain (Panel B *n* = 588) in males and females and (Panel C) when cultured 25˚C and 30˚C.

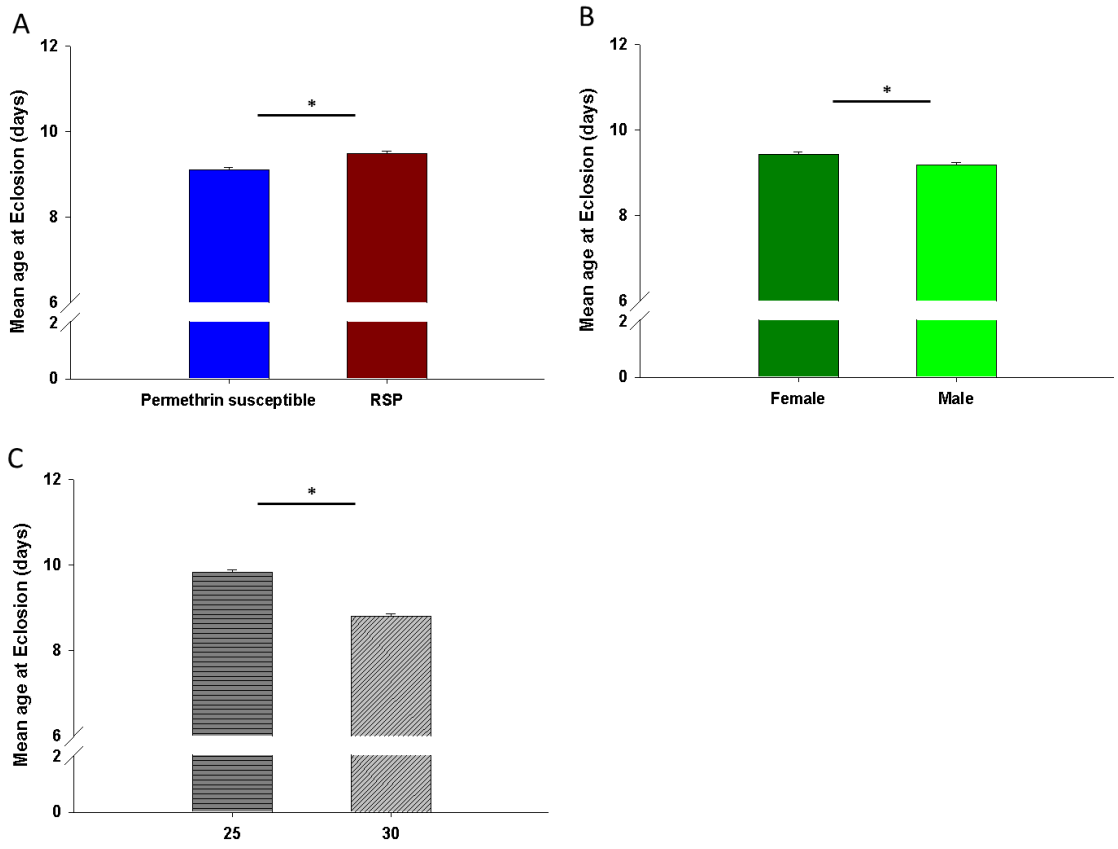
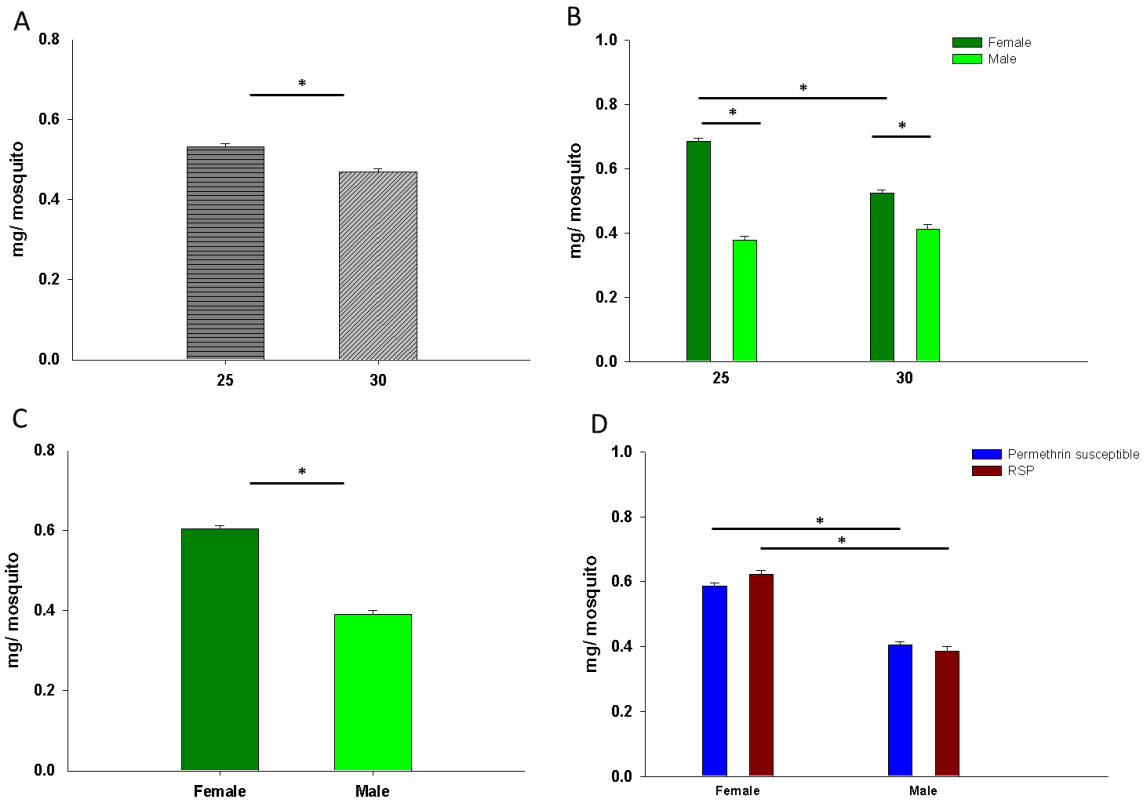


Figure 2: Mean dry weight of RSP and permethrin susceptible strains (Panel A *n* = 97) when reared at 25˚C and 30˚C, (Panel B *n* = 97) interaction between temperature and sex, (Panel C *n* = 97), effect of sex and (Panel D *n* = 97) interaction between strain and sex.



COMPARISONS BETWEEN A LABORATORY-REARED PERMETHRIN SUSCEPTIBLE STRAIN AND A PERMETHRIN RESISTANT STRAIN OF *ANOPHELES GAMBIAE* (DIPTERA: CULICIDAE): SURVIVAL UNDER STARVATION AND GLYCOGEN UTILIZATION IN RELATION TO TEMPERATURE

by

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In preparation for submission to the *Journal of Medical Entomology*

Abstract

In this study the storage and utilization of glycogen reserves were evaluated in two strains of *Anopheles gambiae.* A reduced susceptibility to permethrin (RSP) strain, carrying the knock down resistance (*kdr*) mutation in addition to metabolic resistance, and a permethrin susceptible (ASEMBO1) were assessed to understand effects of rearing temperature on survival strategies between the two strains. Glycogen content was measured in teneral adults and in adults reared at 25˚C or 30˚C and exposed to two nutritional states (honey -fed or starved). Glycogen levels were low in teneral adults, increasing significantly in fed adults. Glycogen content in teneral adults, fed and starved adults was significantly higher in the RSP strain than in the ASEMBO1 strain. Glycogen content in teneral adults and in fed adults was significantly higher in mosquitoes reared at 30˚C than at 25˚C. However, under starvation there was no effect of temperature on glycogen content between strains. Under starvation conditions, the RSP strain had longer lifespan than the ASEMBO1 strains at both 25˚C and 30˚C. We suggest that genomic alterations induced by permethrin resistance alter the metabolic profile related to energy production, conferring a selective advantage to the RSP strain. The consequence of this alteration is not only increased resistance to the stressor permethrin, but to other stressors including nutrient limitation. Results using our laboratory reared strains show that differences in genetic background may affect glycogen content and survivorship, both of which can be influenced by the thermal and nutritional history of the mosquitos.

Key words: *Anopheles gambiae*, insecticide resistance, temperature, energy storage, energy utilization, glycogen, starvation

Introduction

As a result of intensive selection pressure from prolonged use of pyrethroids in malaria control programs, selection of insecticide resistant phenotypes has occurred in *An. gambiae* (Corbel et al. 2007, Adasi and Hemingway 2008, Protopopoff et al. 2008) . In tropical regions where resistant strains are prevalent, these populations can experience substantial temperature variations (Paaijmans and Thomas 2011). Temperature is an important environmental factor affecting the distribution, colonization, abundance, behavior and lifespan of arthropods (Howe 1967). In most studies of insects, an elevation of rearing temperature has resulted in a decrease in lifespan (Swain et al. 2008) and an increase in energetic demands on ectotherms by increasing the rate of biochemical reactions (Schoolfield et al. 1981). Resistance alleles have often been associated with negative pleotropic effects that lead to fitness disadvantage in the absence of insecticide selection pressure (Brown 1958, Bourguet et al. 2004, Sakyi et al. 2005), but may confer an adaptive advantage in response to specific challenges. Thus the interaction between temperature stress with insecticide resistance phenotype can affect fitness but its interaction with insecticide resistance has not been well explored in *An. gambiae*. For example, a comparative study between susceptible houseflies with those carrying *kdr* mutation found the susceptible strain had a preference to warmer temperatures while none was exhibited by resistant strains (Foster et al. 2003). But a study of spatial temporal patterns of mosquitoes in Uganda reported that in dry seasons the frequency of mosquitoes with the L1014S *kdr* mutation was higher infected with *Plasmodium falciparum* the parasite that causes malaria than in non-infected in the population (Verhaeghen et al. 2010). This indicates temperature may influence insecticide resistance genes, vectorial capacity, and vector dis-

tribution dynamics with potential epidemiological consequences. Other factors, including nutritional status, may interact with temperature to affect lifespan and fitness. For example, during dry seasons, sources of sugar (adult mosquito food) are likely to be highly variable. Thus, variation in environmental temperature and differences in nutritional state may lead to variable effects on longevity in adult stage of insects.

Glycogen and trehalose are major carbohydrates that play a role in energy metabolism of insects (Candy 1985). Glycogen is stored within cells and can provide substrates directly without necessity to move into cells (Candy 1985). Trehalose is circulated in hemolymph and provides immediate energy sources for insects (Candy 1985). Glycogen utilization of mosquitoes has been studied in several genera including *Aedes, Culex* and *Anopheles*. (Briegel 1990a) examined four *Anopheles* species: *An. albimanus, An. gambiae, An. stephensi, and An. quadrimaculatus* and found that these caloric reserves are important for reproductive potential and other metabolic parameters. Caloric reserves are accumulated during larval development and/ or from blood feeding or from sugar feeding as adults, and are critical determinants of adult reproduction and survival (Foster 1995, Briegel 2003). Glycogen reserves peaked at 1-2 days post emergence and then gradually declined (Briegel 1990b). Glycogen content of permethrin resistant *Culex pipiens quinquefasciatus* expressing cytochrome P450 detoxification mechanisms strain (ISOP450) have been reported in a comparative study of a permethrin susceptible (SLAB) strain (Hardstone et al. 2010). They found glycogen content of both teneral and four day old females in the resistant strain was significantly lower than in the SLAB strain (Hardstone et al. 2010). However under starvation there was no difference in glycogen content between the two strains. The study reported ISOP450 females had significantly longer life

span compared to the SLAB strain, but there was no difference in males of the two strains (Hardstone et al. 2010). Despite these studies, currently there is no information to our knowledge regarding glycogen reserves and utilization in permethrin resistant *An. gambiae* expressing cytochrome P450 and esterase detoxification mechanism.

In *Culex pipiens quinquefasciatus* mosquitoes, longevity with respect to different nutritional states has previously been evaluated in permethrin resistance strains due to elevation cytochrome P450 detoxification enzymes (Hardstone et al. 2010). Hardstone et al. (2010) reported that females of the resistant strain (ISOP450) provided with 20% sugar solution lived longer than females of the permethrin susceptible strain (SLAB), under standard rearing conditions at 27˚C but did not observe sugar-fed males or starved males and females. In *Culex* strains the resistance to permethrin is solely due to cytochrome P450-mediated detoxification (Hardstone et al. 2007).

In this study we used two laboratory-reared *An. gambiae* strains: a permethrin susceptible (ASEMBO1) and a permethrin resistant strain carrying the *kdr* mutation with metabolic detoxification mechanism (RSP), both originally from the same geographical location, to assess the impact of temperature on nutritional status of the population. Specifically, we evaluated the effects of temperature and food availability on glycogen content of resistant and susceptible adults including unfed teneral adults. Lifespan under starvation for each strain was determined at 25˚C and 30˚C. Because temperature changes are common within the tropics, there is need to understand the biological or physiological effects of these changes in energy utilization in these strains of *An. gambiae*. Evaluating the effects of temperature and glycogen utilization on adult lifespan under different nutritional conditions should improve our understanding of the biology of permethrin resistant

An. gambiae expressing cytochrome P450 and esterase detoxification mechanism and provide potential information for integrated vector management.

Materials and Methods

Mosquito colony

The two *An. gambiae* strains used in this study were a reduced susceptibility to permethrin (RSP) (MRA-334, MR4, ATCC Manassas Virginia) strain and an ASEMBO1 permethrin-susceptible (MRA-186, MR4, ATCC Manassas Virginia) strain. The strains were obtained from the Malaria Research and Reference Reagent Resource Center (MR4) (http://www.mr4.org/).

RSP carries the East African form of the *kdr* sodium channel mutation allele, L1014S, as well as increased P450 and beta-esterase activities (Vulule et al. 1999). RSP was originally isolated from a permethrin impregnated bed net study carried out in four adjacent villages in North West of Kisumu, Kenya (Vulule et al. 1999). The strain was established from a colony representative of *An. gambiae* selected for permethrin tolerance. Laboratory selection for permethrin resistance consists of treating a cohort of fourth-instar larvae with 1ppm permethrin for 24 hours every three generations. Strain identification is performed every 10 generations by polymerase chain reaction (http://www.mr4.org/).

The permethrin susceptible strain ASEMBO1 was originally collected 50 km west of Kisumu in a control area for the United States Agency for International Development cohort bed net project approximately 4.5 km from the study area. Dr. Atieli collected blood-fed females from houses from which he obtained eggs to initiate a colony. Prelimi-

nary species identification was based on the frequency of *An. gambiae* collected in the same area (http://www.mr4.org/).

For general rearing, both mosquito strains were maintained in insectary at 27˚C ± 2˚C, 80% ± 10% relative humidity, and 16 hour light/8 hour dark cycle. For egg production, young adults of the respective strains were allowed to freely mate and blood fed on ears of a restrained rabbit. Moist filter paper was placed in cages for oviposition and the eggs collected were incubated overnight in a Petri dish and were hatched in deionized (DI) water. Larvae were provided abundant food consisting of ground TetraMin (fish food) and adult mosquitoes were fed on 10% honey solution soaked in cotton sticks.

Experimental groups

Unless otherwise stated, the larvae of experimental groups were reared in plastic pans (29.5 x 23.5 x 15) cm containing approximately 1000 mL of DI water. Each pan contained groups of 50 larvae which fed *ad libitum* on TetraMin in environmental chambers (Thermo Scientific, Dubuque, Iowa, USA) at 25 or 30˚C of each strain. Adults were three to five days old reared in 30 cm^3 cages and fed *ad libitum* on 10% honey solution also in environmental chamber 25 or 30˚C.

Glycogen content

Glycogen content was assessed in mosquitoes using the protocol described in (Jumbo-Lucioni et al. 2010). Briefly, mosquitoes were homogenized on ice using 40µl of homogenization buffer (0.01 M KH_2PO_4 and 1 mM EDTA pH 7.4). The homogenates were centrifuged in a microcentrifuge at 2000 rpm for 2 minutes at 4˚C. Aliquots of 1.67 µl of homogenate were added to 250 µl of a reagent containing 0.1 U/ml of amylogluco-

sidase. After 30-minute incubation period at 37˚C, OD_{540} was measured. The concentration of glycogen was determined from a glycogen standard run with each replicate.

Teneral adults

Upon emergence, adults were anaesthetized with CO_2 and glycogen content. For each strain reared at 25˚C or 30˚C, 6 to 10 independent replicates each containing a group of 10 mosquitoes were assayed and treated as described above.

Starvation conditions

One hundred and twenty pupae of each strain were randomly collected in pupae cups and transferred to three cages (40 per cage) in an environmental chamber. The emerged adults were fed on DI water. The cages were monitored every 8 hours and dead individuals were counted and removed until the last one.

Data analysis

A Kaplan-Meier curve was provided to illustrate the difference between strains and sexes at different temperature and a log-rank test was used to test for each corresponding difference. The Fisher's exact test was used to compare the two strains in the number of deaths. For amount of glycogen, the effects of strains, sexes, temperature and their interactions were also compared in ANOVA using the GLM procedure in SAS. Statistical significance was set at 0.05 for each test. SAS 9.3 were used for all statistical analyses.

Results

Effects of temperature on glycogen content between teneral RSP and ASEMBO1 strains.

Analysis of variance showed a significant difference in glycogen content for main effect of strain (F = 10.19; df = 1, 3; P = 0.0037) and temperature (F = 5.46; df = 1, 3; P = 0.0274) (Table 1). The amount of glycogen per individual in teneral adults was significantly higher in the RSP strain than in the permethrin susceptible strain (Fig. 1 A). The amount of glycogen per individual in was significantly higher in teneral adults reared at 30˚C than at 25˚C (Fig. 1 B).

Effects of temperature on glycogen content in fed RSP and ASEMBO1 strains.

There were significant effects for main effects of strain (F = 23.50; df = 1, 3; P = < 0.0001). The amount of glycogen was significantly higher in the RSP strain than in permethrin susceptible strain (Fig. 2 A). There were significant effects for main effect of temperature (F = 10.76; df = 1, 3; P = 0.0016) (Table 2).The amount of glycogen was significantly higher in the population reared at 30˚C than at 25˚C (Fig. 2 B). There was no significant strain by temperature interaction (F, = 2.03; df = 1, 3; P = 0.158).

Effects of temperature on glycogen content in starved RSP and ASEMBO1 strains.

At the time of their death, glycogen levels were significantly lower in starved adults (Fig. 3) than in fed adults. There was a significant main effect of strain (F = 7.26, P = 0.0093) but no significant effects for main effects of temperature or strain by temperature interactions; (F = 0.35; df = 1, 3; P = 0.55) and (F = 0.18; df = 1, 3; P = 0.669), respectively (Table 3). The amount of glycogen was significantly higher in the RSP strain than in ASEMBO1 strain (Fig. 3).

Strain and sex specific differences in survival time under starvation conditions at 25˚C and 30˚C.

The mean survival time of starved RSP strain was significantly longer than in ASEMBO1 strain reared at 25˚C (χ^2 = 21.43, df = 1, *P* = < 0.0001). The mean survival time across sexes was 106.5 + SE 1.45 hours in the RSP strain and 96.3 + SE 1.56 hours in the ASEMBO1 strain (Fig. 4). The mean survival time of females and males was significantly longer in RSP strain than in ASEMBO1 strain (χ^2 = 11.76, df = 1, *P* = 0.0006 and χ^2 = 14.69, df = 1, *P* = 0.0001, respectively). The mean survival time of females was 109.5 + SE 1.95 hours in RSP strain and 99.7 + SE 1.65 hours in ASEMBO1 strain (Fig. 5). The mean survival time of males was 104.9 + SE 1.97 hours in RSP strain and 91.8 + SE 2.81 hours ASEMBO1 strains (Fig. 5).

The mean survival time under starvation was shorter for individuals reared at 30˚C than at 25˚C in both the RSP and ASEMBO1 strains (Fig. 4). The mean survival time (sexes combined) was significantly longer in the RSP strain than in the ASEMBO1 strain reared at 30˚C (χ^2 = 80.49, df = 1, *P* = < 0.0001). The mean survival time was 75.5 + SE 1.04 hours in the RSP strain and 62.3 + SE 0.86 hours in the ASEMBO1 strain.

The mean survival time (females and males) was significantly longer in RSP than in ASEMBO1 strain (χ^2 = 20.30, df = 1, *P* = < 0.0001 and χ^2 = 51.14, df = 1, *P* = < 0.0001, respectively). The mean survival time of females was 75.0 + SE 1.57 hours in the RSP strain and 62.6 + SE 1.50 hours in the ASEMBO1 strain (Fig. 5). The mean survival time of males was 75.8 + SE 1.36 hours in RSP strain and 62.1 + SE 1.04 hours in ASEMBO1 strain (Fig. 5).

The mean survival time did not vary between sexes in RSP (χ^2 = 1.13, df = 1, *P* = 0.286) but was significantly longer in females than in males of ASEMBO1 strain reared at 25˚C (χ^2 = 5.60, df = 1, *P* = 0.0179). The mean survival time was 109.5 + SE 1.95

hours in females and 104.9 + SE 1.97 hours in males of the RSP strain. The mean survival time was 99.7 + SE 1.65 hours in females and 91.8 + SE 2.81 hours in males of ASEMBO1 (Fig. 5).

The mean survival time did not vary between sexes within the RSP strain (χ^2 = 0.225, df = 1, *P* = 0.634) or the ASEMBO1 strain reared at 30˚C (χ^2 = 0.32, df = 1, *P* = 0.57) (Fig. 9). The mean survival time was 75.0 hours + SE 1.57 in the females and 75.8 hours + SE 1.36 in the males of the RSP strain. The mean survival time was 62.6 hours + SE 1.50 in the females and 62.1 hours + SE 1.04 in the males of ASEMBO1 strain.

Discussion

This study evaluated the effects of temperature on glycogen content in teneral and adult ASEMBO1 and RSP strains of *An. gambiae.* Adults were exposed to two nutritional states (fed and starved) to understand the effect of genotype in survival strategies between the two strains.

Higher glycogen content was found in the teneral RSP strain. Larger amounts of teneral reserves can ensure self-maintenance during adverse weather conditions or scarcity of sugar early in life. Higher glycogen content is also associated with increase in flight activity. The RSP strain with higher glycogen could benefit from increased capacity to fly in search of nutrition and mates. The mechanism leading to the accumulation of glycogen in the RSP variant at this life stage is not known, but suggests glycogen is conserved during development. However, this is not the case with all permethrin resistant with metabolic detoxification systems. For example newly emerged *Culex pipiens* reared at a comparable temperature are reported to have lower glycogen reserves compared to susceptible strains (Hardstone et al. 2010, Rivero et al. 2011). This observation supports the idea

that mosquito genera are different and results from one species or strain cannot be used to make generalizations.

We found no significant differences in teneral adult glycogen content between sexes in either strain. This suggests that while temperature may influence development parameters at eclosion (manuscript 2), it may not be a significant factor affecting glycogen content.

Higher glycogen content was observed in fed adults of the RSP strain compared to the ASEMBO1 strain. Although the overall content of glycogen increased in fed adults, the pattern between strains is consistent with that observed in the teneral life stage. These data suggest that 1) glycogen storage capacity is enhanced in the RSP strain, and/or 2) the RSP strain is more efficient in glycogen utilization. Alternatively, there may be a difference in the overall activity of the RSP strain (less active) compared to the ASEMBO1 strain, but activity was not recorded in this study. Glycogen utilization is catalyzed by glycogen phosphorylase, which provides glucose residues for the production of trehalose, which itself may affect desiccation and lifespan. Our results suggest that the interaction of elevated temperature and the RSP phenotype would affect glycogen mobilization and utilization.

Glycogen content was differentially affected by strain and temperature but with no significant interactions between temperature and strain. These results indicate a complex interaction of strain and temperature, and starvation conditions, acting as a stressor, may contribute significantly to the variation in glycogen content.

Temperature appears to stimulate or promote the use of all glycogen reserves in RSP strain during starvation. This glycogen utilization strategy has important implication

for vector behavior. In *Aedes aegyti* and *Culex nigripalpus*, resource deprivation, which is directly correlated with low energetic reserves, renders the mosquitoes more responsive to sugar-rich odors like honey and less responsive to host odors. Under starvation there is rapid weight loss during which glycogen stores are depleted and water is lost. Tolerance to high temperature would suggest these strains can be spread over a wider geographical area even when food is not available.

Although only glycogen was measured in the current study, it must be noted the fat body of insects metabolize a combination of fuels including lipids, carbohydrates and amino acids during starvation (Arrese and Soulages 2010). Female *Anopheles* mobilize 75% of their lipid reserves and up to 53% of their proteins during the 24 to 48 hours of starvation (Briegel 1990b). In *Aedes aegypti*, 80% of the teneral reserve and 15% of protein have been reported lost under starvation (Briegel 1990c).

The finding that RSP strain has decreased survival time under optimal feeding conditions (10% honey) at 25˚C and 30˚C (Chapter 2, Appendix 1), but increased survival time under starvation, suggests that under low carbohydrate availability the RSP strain may have a better competitive ability to survive than ASEMBO1 strain. Glycogen and lipid reserves can increase starvation resistance by providing food reserves and may also play a role by reducing the amount of water lost from flies (Rose et al. 1992). The mechanism leading to enhanced carbohydrate storage/utilization is not known. Consequently, we hypothesize that this biochemical and physiological adaptation may enable the RSP strain to survive warmer temperatures than the ASEMBO1 strains when nutrition is limited. This advantage would be lost when food is available.

Higher glycogen reserves, together with rearing at elevated temperature, favored the RSP strain during starvation. RSP strain also may be more resistant to starvation than ASEMBO1 because they have lower metabolic rates as we found in females (manuscript 1). This higher efficiency in glycogen utilization at elevated temperature in addition to the longer survival under starvation would suggest drier and hotter months may favor adult RSP populations. Prolonged survival when nutrients are limiting could result in higher numbers of the resistant strain. Our results contrast somewhat with those of (Hardstone et al. 2010) who reported significantly lower glycogen content in the ISOP450 strain of females but found no difference in survival under starvation, but are in agreement with (Briegel 1990b) who reported no sexual dimorphism in energetic reserves of *Anopheles*. We suggest that the genomic alterations induced by permethrin resistance alter the metabolic profile related to energy production, conferring a selective advantage to the RSP strain. The consequence of this alteration is not only increased resistance to the stressor permethrin, but to other stressors including nutrient limitation (McNamara and Buchanan 2005).

Energetic reserves can have impact on vectors of diseases with significant consequences in disease transmission and have been used as indirect measurements of fitness.

The fact that the patterns of survival time under starvation are clearly different between RSP and ASEMBO1 shows that genes with major effects can be differentially expressed under varying environmental conditions. This conclusion is important for vector control since it suggest in nature we should expect to encounter similar trends generated by differences in genotype.

Acknowledgements

We are grateful to Edward W. Hook III and Sally Fried of the Division of Infectious Diseases, William C. Gorgas Center for Geographic Medicine, for granting permission to use the mosquito facility. We are also grateful to Bud Fischer for support. The RSP strain was obtained through the MR4 as part of the BEI Resources Repository, NIAID, NIH: *An. gambiae* RSP, MRA-334, deposited by J Vulule, MQ Benedict. The ASEMBO1 strain was obtained through the MR4 as part of the BEI Resources Repository, NIAID, NIH: *An.gambiae* ASEMBO1 [AB], MRA-186, deposited by MQ Benedict. This study was supported by the Department of Biology at the University of Alabama at Birmingham, and the National Institutes of Health, National Institute of Diabetic and Digestive and Kidney diseases, through grant [R01DK084219 to MD]

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TABLES

Table 1. ANOVA of amount of glycogen per individual of teneral RSP and ASEMBO1 strains

Source	df	SS	F value	*p* value
Strain	1	478.01	10.19	0.0037*
Temp	1	256.29	5.46	0.0274*
Strain X Temp	1	33.26	0.71	0.407

Table 2. ANOVA of amount of glycogen per individual of honey fed RSP and ASEMBO1 strains

Source	df	SS	F value	*p* value
Strain	1	24836.06	23.50	< 0.0001*
Temp	1	11374.55	10.76	0.0016*
Strain X Temp	1	2143.84	2.03	0.158

Table 3. ANOVA of amount of glycogen per individual of starved RSP and ASEMBO1 strains

Source	df	SS	F value	*p* value
Strain	1	0.59	0.18	0.0093*
Temp	1	17.35	5.25	0.554
Strain X Temp	1	74.91	22.64	0.669

FIGURES

Figure 1. Glycogen content (mean ± SE) (Panel A *n* = 6- 10) of teneral RSP and permethrin susceptible strain, (Panel B *n* = 6- 10) when cultured 25˚C and 30˚C.

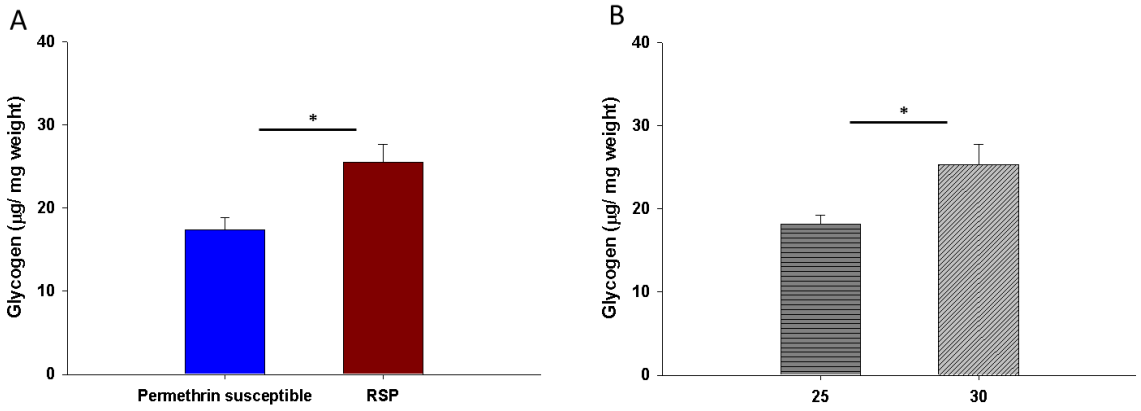


Figure 2. Glycogen content (mean ± SE) (Panel A *n* = 10) of fed RSP and permethrin susceptible strain, (Panel B *n* = 10) glycogen content of fed adults when cultured 25˚C and 30˚C.

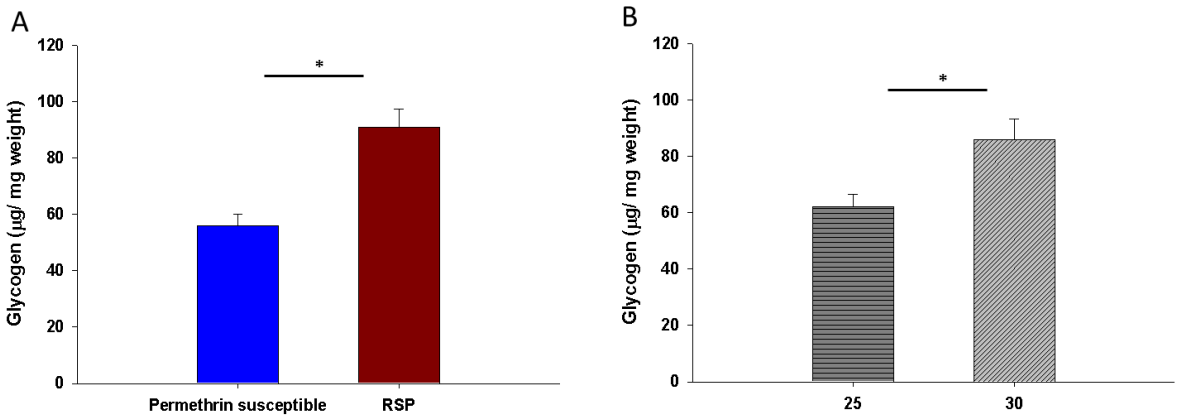


Figure 3. Glycogen content (mean ± SE) of starved RSP and permethrin susceptible strains (*n* = 10)

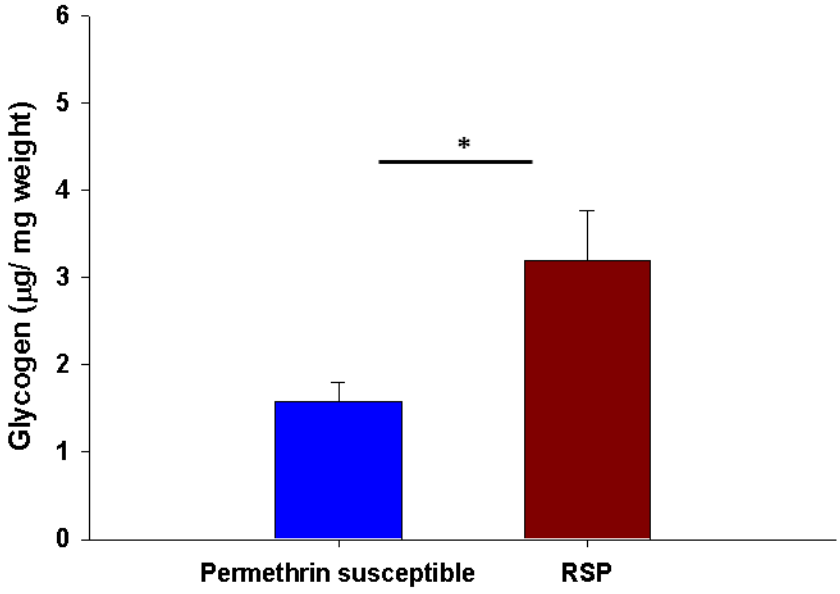


Figure 4. Survivorship of adult combined males and females from starved RSP and ASEMBO1 strains at 25˚C and 30˚C**.** Survival assays were carried out in triplicate using population cages with initial population sizes of 40 individuals

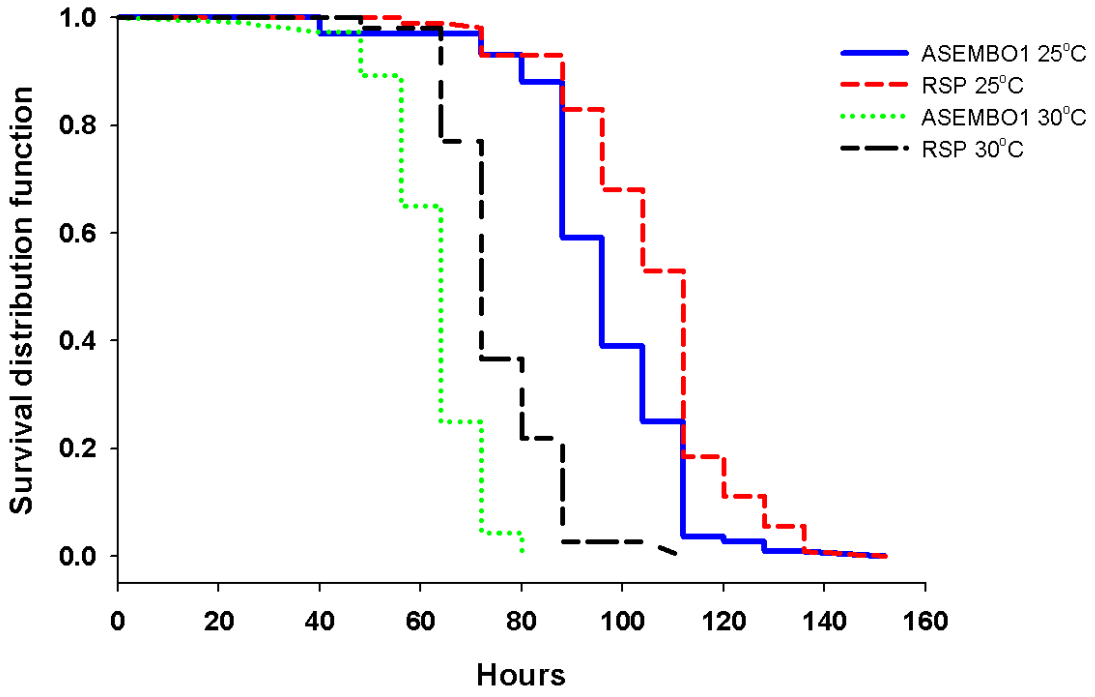
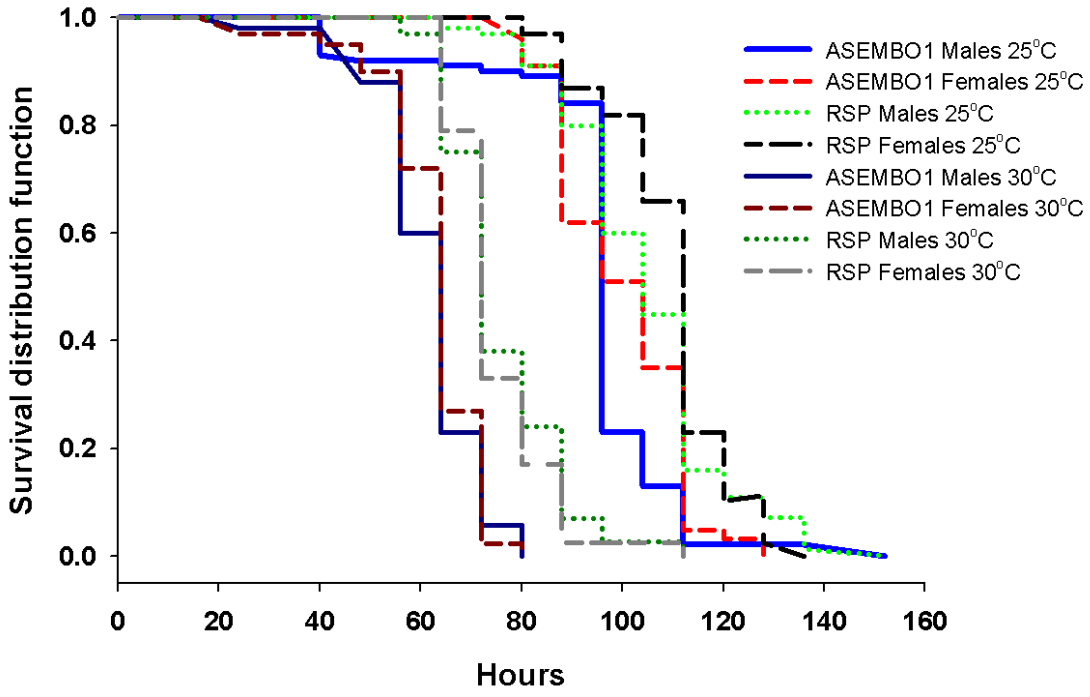


Figure 5. Survivorship of adult separate males and females from starved RSP and ASEMBO1 strains at 25˚C and 30˚C. Survival assays were carried out in triplicate using population cages with initial population sizes of 40 individuals



SUMMARY AND CONCLUSIONS

Understanding the biology of vectors of diseases is a valuable component in integrated vector management strategies (WHO, 2007, 2008). By comparing life-history and energy metabolism traits between the permethrin susceptible and the RSP strain this dissertation expands our knowledge on the effects of co-occurrence of the *kdr* (L1014S) mutation and enhanced cytochrome P450 and esterase detoxification is the malaria vector *An. gambiae* (Vulule et al., 1999). In the manuscript titled "Increased production of mitochondrial reactive oxygen species and reduced adult lifespan in an insecticide resistant strain of *Anopheles gambiae*" we investigated life history and energy metabolism traits between females of the permethrin susceptible and the RSP strain. We found the RSP strain had prolonged developmental time, higher wet body wet and higher glycogen levels compared with the permethrin susceptible strain. In addition, the RSP strain lived shorter than the permethrin susceptible strain. The RSP strain displayed reduced metabolic rate mitochondrial coupling efficiency and increased mitochondrial ROS production.

To better understand the impact of resistance phenotype in the RSP strain verifying our finding with field strains would be of interest (Raymond et al., 1993) . This is a frequent criticism of laboratory studies of resistance (Bourguet et al., 2004). Another approach would be to repeatedly backcross the RSP strain with the permethrin susceptible strain to that the genetic background of the two strains (Hardstone et al., 2007). Additionally, the differences in development time between the two strains need further investigation. These differences may have been due to differences in feeding rates. It is conceivable that the RSP phenotype may affect feeding or alternatively it may affect feeding or metabolic efficiency. Female mosquitoes are the vectors of the Plasmodium parasite thus

are of obvious interest. Determining life history traits and energy metabolism of both sexes, including mitochondrial ROS and oxidative enzymes, would be warranted for accurate in integration vector management strategies.

There has been a link between elevated ROS and vector competence (Dimopoulos, 2003). The successful invasion and subsequent development of a parasite in a vector depends on several physiological and immunological factors that determine the insect's internal environment (Rivero et al., 2010). The effects of physiological modifications accompany elevated detoxification mechanisms in *An. gambiae* are not known. Studies have shown that the development of *W. bancrofti* larvae was arrested in insecticide resistant *Culex quinquefasciatus* mosquitoes and hypothesized that it was due to the over production of carboxylesterases (Hemingway et al., 2000, McCarroll and Hemingway, 2002). The combined complexity of the mode of action and the multiple substrate specificities of enzymes involved in metabolic insecticide resistance is that these enzymes may have pleiotropic effects on one of the many steps of the immune cascade from the recognition of the parasite as foreign, to the transduction of the signal the and the deployment of the killing mechanism (Dimopoulos, 2003). Alternatively, as a result of the production of large amounts of detoxification enzymes depleting the resource pool, the lack of resources could compromise the vectors ability to mount an immune response, thereby favoring parasite development. Vector behavior, host choice and biting rates have key effects on parasite transmission (Rivero et al., 2010).

Temperature fluctuates in the tropics and can therefore interact with the insecticide resistance phenotype to affect life history traits (Mpho et al., 2002). These effects on temperature directly affect vector development, distribution, thus can shift population

demographics of resistant individuals with consequences in disease transmission. Yet there are no studies that have investigated the effects of temperature on RSP phenotype. The second manuscript title "Sex, temperature, and genetic effects on developmental time and body size in *Anopheles gambiae*". This study in which experiments were conducted at a constant rearing temperature of 25˚C and 30˚C, found that there was reduced survival time in both strains reared at 30˚C than at 25˚C. This will affect generation time of the mosquitos, with implication to population structures when temperature is elevated. Temperature was also a significant factor affecting development time to adult and body size. The temperatures used in this study were within the range the mosquitoes may be exposed to in the nature within the current climate conditions. Another area of further study would be to increase the temperature ranges (Bayoh and Lindsay, 2004) as predicted by climate change models.

In the third manuscript, the effects of temperature on glycogen utilization and survival between the ASEMBO1 strain and the RSP strain was investigated. This is linked to the previous paper on the effects of temperature. The third manuscript titled "Comparisons between a laboratory-reared permethrin susceptible strain and a permethrin resistant strain of *An. gambiae*: survival under starvation and glycogen utilization in relation to temperature'' found glycogen content in teneral adults in fed and starved adults was significantly higher in the RSP strain than in the ASEMBO1 strain. Under starvation conditions, both males and females of the RSP strain had longer lifespan than the ASEMBO1. It is likely the higher levels of glycogen observed in the RSP strain gives this strain the increased tolerance to starvation.

The findings in this dissertation give an insight into the biology of co-occurrence of *kdr* mutation and metabolic detoxification. This information at strain level is a useful tool to include in integrated vector management strategies.

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APPENDIX

A ADULT LIFE SPAN OF FED RSP AND ASEMBO1 STRAINS AT 25˚C AND 30˚C

Methods of Lifespan

One hundred and twenty pupae of each strain were randomly collected in pupae cups and transferred to three cages (40 per cage) in an environmental chamber. Pupae cups were removed from the cages after 24 hours so that pupae that failed to emerge were excluded. The emerged adults were fed on 10% honey solution *ad libitum*. The cages were monitored daily and dead individuals were counted and removed until the last one.

Between-strain differences in lifespan of fed RSP and ASEMBO1 strains at 25˚C and 30˚C

Mean lifespan (both sexes combined) in RSP was shorter than in ASEMBO1 strain reared at 25˚C ($\chi^2 = 5.65$, df = 1, $p = 0.0174$). The mean lifespan was 29.1 + SE 1.97 days in RSP and 34.8 + SE 2.14 days in ASEMBO1 strain (Fig. 4).

Mean lifespan (females and males) was significantly shorter in RSP than ASEMBO1 strain reared at 25˚C ($\chi^2 = 10.49$, df = 1, $p = 0.0012$ and $\chi^2 = 4.33$, df = 1, $p = 0.0372$, respectively). The mean lifespan of females was 25.7 + SE 2.12 days in RSP and 33.8 + SE 2.22 days in ASEMBO1. The mean lifespan of males was 29.8 + SE 2.53 days in RSP and 36.9 + SE 2.74 days in ASEMBO1 (Fig. 5).

Mean lifespan was shorter for individuals reared at 30˚C than at 25˚C in both strains (Fig 4). Mean lifespan (both sexes combined) was significantly shorter in RSP than in ASEMBO1 strains reared at 30˚C ($\chi^2 = 8.39$, df = 1, $p = 0.0038$). The mean lifespan was 15.6 + SE 1.16 days in RSP strain and 20.09 + SE 1.30 days in ASEMBO1 strain.

Mean life span of (females and males) was significantly shorter in the RSP strain than ASEMBO1 reared at 30˚C (χ^2 = 10.95, df = 1, p = 0.0009 and χ^2 = 9.75, df = 1, p = 0.0018, respectively). The mean lifespan of females was 15.6 + SE 0.98 days in the RSP strain and 20.7 + SE 1.16 days in ASEMBO1 strain (Fig. 6 A). The mean lifespan of males was 14.2 + SE 1.28 days in RSP strain and 20.2 + SE 1.85 days in ASEMBO1 strain (Fig. 6).

Between-sex differences in lifespan of fed RSP and ASEMBO1 strains at 25˚C and 30˚C

Mean lifespan was significantly shorter in RSP females than in males reared at 25˚C (χ^2 = 6.61, df = 1, p = 0.0101) (Fig. 5). The mean lifespan was 25.7 + SE 2.12 days in females and 29.8 + SE 2.53 days in males. The mean life span did not vary between sexes within ASEMBO1 strain (χ^2 = 1.99, df = 1, p = 0.158). The mean lifespan was 33.8 + SE 2.22 days in the females and 36.9 + SE 2.74 days in males.

The mean lifespan did not vary between sexes within the RSP strain (χ^2 = 0.19, df = 1, p = 0.66) or the ASEMBO1 strain reared at 30˚C (χ^2 = 1.06, df = 1, p = 0.302). The mean lifespan was 15.6 + SE 0.98 days in females and 14.2 + SE 1.28 days in males of the RSP strain. The mean lifespan was 20.7 + SE 1.16 days in females and 20.2 + SE 1.85 days in males of ASEMBO1 strain.

Between-strain differences in lifespan at 25˚C and 30˚C

Life span for RSP was significantly reduced than for ASEMBO1 at both culture temperatures. Our results appear to be in agreement with reports which suggest that in the absence of insecticide selection pressure there is a cost in lifespan of the RSP strain. These findings are the similar to those reported in other studies of resistant organisms.

Metabolic rate and mitochondrial ROS are important factors to be considered in the context of lifespan. Several studies in different species have shown there is an inverse correlation between ROS and lifespan (Sanz and Stefanatos, 2008). Previously we have shown that RSP females reared at 25˚C have a lower metabolic rate compared to ASEMBO1 (chapter 2) in addition a higher mitochondrial ROS. Combinations of these factors are probably the major reason for the reduced lifespan in the RSP strain. Whether these observation in females of the RSP strain are identical in males remains to be verified.

We observed that rearing at 30˚C compared to rearing at 25˚C resulted in significantly reduced lifespan in both strains. This is in agreement with the general observation that insects survive longer when reared under lower temperatures (Chen et al., 2005). (Miquel et al., 1976) have observed that manipulations such as temperature which increase metabolic rate tend to decrease longevity.

Not only does elevated temperature promote the utilization of glycogen, it also lowers lifespan in both strains. Presumably, elevated temperature increases enzymatic reactions which enhance utilization at higher temperatures more efficiently in RSP strain.

Between-sex differences in lifespan at 25˚C and 30˚C

Under similar conditions of temperature it is expect that the sexes of the same strain should have no differences in longevity. In this study however, there was variation in longevity between males and females of the RSP strain reared at 25˚C.

The reduced lifespan in females of at elevated temperature in both strains will have an impact on malaria transmission. Plasmodium parasite has a long extrinsic period (10-21 days) depending on species and temperature.

B IACUC FORM

Human Subjects
The University of Alabama at Birmingham defines a human subject as not only a living human being, but also human tissue, blood samples, pathology or diagnostic specimens, study of medical records, observation of public behavior, and all questionnaires or surveys.

Does the research proposed by the student involve human subjects? ☐ Yes (continue below) ☒ No
This research is:
Approved _____ Date _____

IRB Protocol No. _____

Attach a copy of your IRB approval. Your own name must appear on the original approval or on an attached amendment.

Animal Subjects
The University of Alabama at Birmingham defines a laboratory animal as any vertebrate animal (e.g., traditional laboratory animals, farm animals, wildlife, and aquatic animals) and certain higher invertebrate animals used in research, teaching, or testing at UAB or sponsored through UAB but conducted off-site (i.e., field research or at collaborating institutions, etc.).

Does the research proposed by the student involve animal subjects? ☒ Yes (continue below) ☐ No

This research is:
Approved Yes Date 1/6/2011

IACUC Protocol No. 091008286

Attach a copy of your IACUC Notice of Approval, showing your research subject and the animal project number. If your own name does not appear on the Notice of Approval, take this form to the IACUC office for verification of approval.

The IACUC office verifies that Dennis Otali (name of candidate) is covered under the attached approval.

Signature of IACUC representative David Cannon Date: 7/15/2011

NOTE: The student's advisor, the student, and the Graduate Program Director agree that no research will be initiated until an application is submitted for review and approved by the appropriate review boards (IRB and/or IACUC) if the proposed thesis or dissertation project requires approval. If approval already exists, this student's name must be added to the existing protocol before candidacy will be approved by the Graduate School. It is the responsibility of the student's advisor and the student to comply with federal and UAB regulations associated with this research. Documentation of continuous, appropriate approval will be required before degree conferral; all required IRB and/or IACUC approvals must be current at the time final versions of theses or dissertations are submitted to the Graduate School.

Student's Signature — Biology, Dept. — July 11th 2011, Date

Infectious Diseases — 7-11-2011