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POTENTIAL FITNESS TRADE-OFFS OF ELEVATED ESTERASE ACTIVITY ASSOCIATED WITH INSECTICIDE RESISTANCE IN THE MOSQUITO CULEX QUINQUEFASCIATUS

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A Thesis

Presented to the

Faculty of

California State University,

San Bernardino

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

in

Biology

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by

Timothy Reinhard Schulte

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ABSTRACT

One of the current major issues in the control of vector-borne disease is resistance to insecticides. Resistance to one or more insecticides has appeared in over 580 different arthropod species. Widespread resistance has led to the field of Insecticide Resistance Management (IRM). The goal of IRM is to develop insecticide-use strategies that prevent or slow down the evolution of resistance. Computer models have shown that more effective IRM strategies rely on the assumption that resistance carries a fitness cost in the absence of insecticide use.

In the mosquito Culex quinquefasciatus resistance to certain organophosphate insecticides is caused by an increased production of esterase enzymes due to the amplification of certain esterase genes. Individuals in field populations can vary in esterase activity over a continuous range. The potential effects that different levels of esterase activity have on phenotypic traits with potential effects on fitness was investigated in a lab study of a California strain derived from a field collection. Development times (egg hatch to adult) were measured on samples of 500 individuals reared under four combinations of two temperatures: 25 and 30 degrees C; and two diets: a High food diet providing ample food to developing larvae, and a Low food diet that used about 25% of the amount of food provided in the High food diet. Individuals were frozen as adults after emergence for further analysis. The temperature and diet combinations were designed to create different levels of stress on the developing larvae. For

each experiment esterase activity and protein content were measured on samples of males and females across the observed range of development times. The effects of temperature and diet on development time, protein content and esterase activity were determined using ANOVA. The relationships among these variables were determined using regression and correlation analysis. Temperature and diet had consistent significant effects on development time and protein content, but not on esterase activity. Coefficients of variation for development time and protein content were substantially higher in experiments using the Low food diet, suggesting increased stress on developing larvae.

Consistent evidence for a fitness trade-off between esterase activity and protein content (i.e. body size) was found in female mosquitoes. Individuals with higher esterase activity tended to have smaller body size in all four experiments. This trade-off was most apparent in the experiment with the most stressful rearing conditions (Low food diet – 30°C; regression $p < 0.001$), however regression p values for two of the other experiments were < 0.1, and < 0.2 in the final experiment. Surprisingly, in the experiment with the least stressful conditions (High food diet - 25°C), there were highly significant negative relationships between esterase activity and development time in both males and females (regression p values < 0.001). This study represents the first attempt to relate quantitative variation in esterase activity among individuals within a strain with variation in phenotypic traits that have the potential to affect fitness. The

implications of these results for IRM models of the evolution of resistance is discussed.

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CHAPTER ONE

INTRODUCTION

General Background of Insecticide Resistance

One of the current major worldwide issues in the control of vector borne disease is resistance to insecticides. Resistant populations are appearing in a wide range of insect species including pests in agriculture and vectors of disease. Mosquitoes are one of the most important disease vectors and are responsible for disease outbreaks involving malaria, dengue, West Nile virus, and the recent Zika virus. (Caraballo & King, 2014; Islam et al. 2017). Up to 700 million people are infected and more than a million die each year due to mosquito-borne illness (Caraballo & King, 2014). According to the World Health Organization (WHO, 2019), among vector species 68 countries have reported resistance to at least one class of insecticide, with 57 of those countries reporting resistance to 2 or more classes. Dichlorodiphenyltrichloroethane (DDT) was one of the more popular options in the early 1940s because it caused high mortality in mosquitoes and had relatively low toxicity to humans. However, in 1947 mosquitoes showed resistance to DDT in Florida and it quickly became a worldwide phenomenon. Subsequently resistance appeared in several mosquito species (Brown, 1986). Since then, new classes of insecticides have been developed, but resistance to almost all classes of insecticides has evolved in one or more vector species (WHO, 2019).

Mechanisms of Resistance

There are four main mechanisms that can contribute to an insect's resistance to insecticides: 1) a change in the behavior of an individual that either reduces the time it spends in treated areas or completely avoids them, effectively reducing their exposure to the insecticide; 2) a change to the cuticle of an individual that reduces the penetration of the insecticide; 3) a mutation that causes the target site of the insecticide to become insensitive to that insecticide; 4) increased expression or alterations to the structure of enzymes that increase their ability to detoxify the insecticide (Ferrari, 1996). It is possible for an insect to use only one mechanism, however in nature there are usually combinations of two or more. Changes in behavior and reduced penetration of the insecticide are limited in their effectiveness by themselves but coupled with other mechanisms can amplify their effectiveness. It is important to note that all these mechanisms for resistance have a genetic basis (Ferrari, 1996).

Background on Active Esterases Associated with Organophosphate Resistance

In mosquitoes (Diptera: Culicidae) one of the main mechanisms of organophosphate insecticide (OP) resistance involves detoxifying enzymes from the carboxylesterase family. OP insecticides work by inhibiting acetylcholinesterase (AChE) which results in insect paralysis or death. The function of AChE is to hydrolyze acetylcholine (a neurotransmitter). Most OPs are esters of phosphoric acid and can be sequestered by a carboxylesterase (Esterase) (Hemingway & Karunaratne, 1998).

One of the earliest studies to associate high esterase activity and OP resistance was done by Kazuo Yasutomi (1970). He used thin layer electrophoresis to investigate the relationship between esterase activity and organophosphate resistance. Gel electrophoreses was carried out on four colonies derived from two populations of *Culex pipiens pallens*. He used a susceptible colony that was collected in 1956 from a rain-water tub in Tokyo, Japan and was maintained in the lab. The resistant colonies were collected in 1969 from an open sewer in Amagasaki City. Colony 1 was susceptible to OPs and colonies 2-4 were resistant. The esterase electrophoretic bands of colonies 2-4 showed greater intensity than colony 1 meaning they had higher esterase activity. Colonies 3 and 4 underwent 2 and 5 generations of further insecticide selection pressure respectively. Colony 4 showed the greatest band intensity of the four colonies. In addition to showing an association between esterase activity and OP resistance, this study demonstrated the usefulness of gel electrophoresis for identifying resistant individuals.

In the 1970's native protein electrophoresis was used widely by population geneticists to examine genetically based protein variation in populations. Allelic forms of a protein coded by a gene were termed allozymes. Different allozymes differed in amino acid sequence and charge and could be distinguished by their different electrophoretic mobilities on a gel. (Hartl & Clark, 2007). Electrophoresis quickly became a major tool used to study several different aspects of resistance in mosquitoes and was instrumental in the identification of active esterases

associated with OP resistance. The first major application of native protein electrophoresis to the study of resistance was done by Georghiou and Pasteur (1978). They studied 8 California *Culex pipiens fatigans* (= *Cx. quinquefasciatus*) strains. There was one susceptible strain (F1) and one parental OP resistant strain (F5) that five of the strains were derived from (F3, F4, F6, F7, F8). These five strains were reared in laboratory conditions and each selected for resistance to a different insecticide. Strains F3 and F4 were selected with d-trans or d-cis permethrin (a pyrethroid insecticide). Strain F6 was selected with only temephos (an OP insecticide). Strain F7 was selected with temephos and DEF (S,S,S-tributyl phosphorotrithioate), an insecticide synergist that inhibits esterases. Strain F8 was selected with temephos and piperonyl butoxide, an insecticide synergist that inhibits the mixed-function oxidase system. Using protein electrophoresis on the eight strains they identified both a low and high esterase activity group for esterase B (esterases that use β-naphthylacetate and stain red) based on their staining intensity. The high activity esterase electromorph is now known as EstB1 (or simply B1). Eighty-nine percent of individuals from the unselected parental strain F5 expressed EstB1. All the individuals sampled from the OP selected F6 and F8 strains expressed EstB1. When the F3, F4, and F7 strains were analyzed after a number of generations of selection no individuals with the active EstB1 electromorph were observed. It was apparent that EstB1 was actively selected against in these strains since it would be unlikely to have disappeared by chance in all three daughter strains while still appearing at high

frequency in the parental F5 strain. Strains F3 and F4 were subjected to selection with pyrethroid insecticides and strain F7 did not respond to OP selection by increasing the frequency of EstB1 possibly due to the presence of DEF. When DEF was used on strains F6 and F8 in OP insecticide bioassays it completely suppressed insecticide resistance. When it was used on strains F1, F2, F3, F4, and F7 it showed little to no effect on resistance. This indicated that OP resistance in these strains was associated with the active EstB1 electromorph phenotype, and that active esterases did not provide resistance to pyrethroids.

In a follow-up experiment Georghiou and Pasteur and Hawley (1980) conducted a series of crosses between resistant and susceptible *Culex quinquefasciatus* strains and compared the electrophoretic mobilities of the EstB1 variants. S-Lab is a susceptible laboratory strain which lacks high esterase activity and was originally collected in 1950 from the San Joaquin Valley, California. Tem-R is a resistant strain that was developed from a 1974 field collection from Hanford, California by selection with the organophosphate temephos. It was maintained in the lab for many years under temephos selection at an approximate 50% mortality level each generation.

Assuming the two variants were alleles of the same gene they labeled low activity as Est-B^{O} and high activity as Est-B^{A}. They carried out reciprocal mass crosses with the susceptible (S-Lab) strain and the Temephos resistant (Tem-R) strain and then backcrossed the offspring with the S-Lab strain. They also carried

out a S-Lab x S-Lab cross and a Tem-R x Tem-R cross. After the crosses they ran gel electrophoresis on mosquitoes from each strain to determine their phenotypes. The EstB1 electromorph appeared in S-Lab as faint bands on the gel. In Tem-R, EstB1 had a strongly stained band as in their earlier experiment. When crossing S-Lab with S-Lab all the offspring expressed a phenotype of Est-B^O and when crossing Tem-R with Tem-R all the offspring expressed a phenotype of Est-B^A. All the F_1 offspring from the S-Lab x Tem-R cross had a phenotype of Est-B^A, and when the F_1 generation was backcrossed with S-Lab they observed about a 1:1 phenotypic ratio of Est-B^O and Est-B^A. This could be explained if Est-B^O and Est-B^A were alleles of the same gene. For the Est-B^O phenotype the genotype would be B^OB^O and for the Est-B^A phenotype the genotypes would be either B^AB^O or B^AB^A. The B^A allele is dominant because the F_1 generation all expressed the Est-B^A phenotype. This demonstrated that the pattern of inheritance of the high and low activity phenotypes was consistent with the hypothesis that the phenotypes were associated with alleles of the same gene, which is now known as *Estβ1*. These two studies also clearly demonstrated that the B1 high esterase activity electromorph was associated with OP resistance. The next question was to determine the molecular genetic basis for the high activity esterase.

Two explanations for high esterase activity associated with a particular electromorph are possible, gene regulation and gene amplification. Gene regulation could result in enhanced transcription of the esterase gene in resistant

individuals. Gene amplification could increase the number of copies of the esterase gene in resistant individuals. It had been previously demonstrated that gene amplification was the basis for the elevated esterase activity observed in the aphid *Myzus persicae* (Field et al. 1988). Mouchès et al. (1986) set out to determine if gene amplification was the basis of elevated esterase activity in *Culex pipiens*. Their approach was to produce a probe that could be used to hybridize to the *Estβ1* genes in resistant and susceptible individuals to determine if it had been amplified in the resistant strain. RNA was extracted from Tem-R larvae and complementary DNA (cDNA) was produced. The cDNA's were introduced into a restriction site of the expression phage λgt11 and plated onto *E. coli*. One recombinant phage, λgt-est, contained a cDNA insert that coded for an amino acid sequence that was immunoreactive with an antiserum to esterase B1. They used radioactively labelled λgt-est DNA as a probe in order to detect the esterase B1 gene in the DNA of Tem-R and S-Lab individuals. Their results showed a much stronger hybridization to Tem-R DNA than to S-Lab DNA and estimated that Tem-R had at least a 250-fold gene amplification factor. This indicated that resistant *Culex quinquefasciatus mosquitoes* contained 250 times more copies of the *Estβ1* gene than did susceptible mosquitoes. In a simultaneous study, Mouchès et al. (1987) purified Esterase B1 from Tem-R and produced an antiserum. The antiserum contained antibodies that specifically bind to Esterase B1. They compared the amount of Esterase B1 detected between S-Lab and Tem-R. Tem-R individuals had about a 500-fold greater amount of

esterase B1 than did S-Lab individuals. These two studies concluded that the mechanism producing elevated esterase activity in resistant *Culex quinquefasciatus* mosquitoes was gene amplification. Gene amplification complicated the genetic analysis of resistance. Prior to the discovery of gene amplification, resistance to OPs was viewed qualitatively as being due to single gene alleles that conferred susceptibility or resistance. Gene amplification introduces the possibility that the amount of esterase B1 produced could vary with the copy number of *Estβ1* genes an individual carried.

With the knowledge that OP-resistance was associated with high levels of esterase activity, Dary et al. (1990) developed an esterase activity assay that quantifies esterase activity in an individual mosquito. The procedure begins by allowing esterases in either a resistant or susceptible mosquito homogenate to hydrolyze α-napthyl acetate into α-naphthol. The amount of α-naphthol product is detected colorimetrically with Fast Garnet GBC salt which also halts the reaction. The influence of body size is corrected by determining total protein content of the individual. This procedure is accurate enough to determine esterase activity in 1/30 portion of an individual mosquito that does not exhibit elevated esterase activity. Since the portions are so small, the procedure can be done on microplates with up to 80 samples simultaneously.

Using this esterase activity assay, Ferrari & Georghiou (1990) determined esterase activity in individuals from the susceptible S-lab strain, the highly resistant Tem-R strain, and in F_1 individuals from their reciprocal crosses. These

esterase activity measures would be used to produce a standard against which to compare esterase B1 activity variation in field populations and possibly a way to relate esterase activity and resistance levels. Using S-lab and Tem-R they produced four strains: 1) S-Lab females with S-Lab males (S-Lab); 2) Tem-R females with Tem-R males (Tem-R); 3) S-Lab females with Tem-R males (F_1SL); 4) Tem-R females with S-Lab males (F_1TR). The reciprocal crosses were made to determine if there was a maternal effect on esterase activity and resistance. Tem-R had between 115-125 times the esterase activity of S-Lab and both F_1 strains had around 60 times the esterase activity of S-Lab. The esterase activity of the F_1 strains was greater than the average esterase activity between S-Lab and Tem-R indicating the *Estβ1* gene from Tem-R showed a form of incomplete dominance. Within all strains there was considerable variation in esterase activity. For both F_1 strains, Tem-R males and females had about equal mean esterase activities but in S-Lab the mean esterase activity of females was significantly higher than that of the males. They also found no significant maternal affect when comparing the two different F_1 strains.

Since the discovery of EstB1, several additional *Estβ* electromorphs have been identified that have elevated activity in resistant mosquitoes. In addition, a number of active esterase electromorphs associated with alleles of another esterase locus, *Estα*, have been discovered. The current nomenclature for these two esterases is based on whether they preferentially hydrolyze the artificial substrates α-naphthyl or β-naphthyl acetate. (Hemingway & Karunaratne, 1998).

In some cases, only an Estα (A1) electromorph was identified and in others only an Estβ (B1, B6, B7) was shown to have elevated activity. In several other instances, a pair of Estα and Estβ electromorphs (A2-B2, A4-B4, A5-B5, A8-B8, A9-B9) both exhibited elevated activity and were always found in association with one another (Hemingway & Karunaratne, 1998; Chevillon et al. 1999; Raymond et al. 2001) (as cited in Ferrari, 2015). Several studies have been done do determine if overproduction of these other electromorphs was due to gene amplification or gene regulation.

The high activity of the A1 esterase was due to gene regulation (Rooker et al. 1996), while the genetic basis of the elevated activity of all of the other esterases examined to date was gene amplification (Poirié et al. 1992, Vaughn & Hemingway 1995, Rooker et al. 1996, Severini et al. 1997). It was also demonstrated that the *Estα2* and *Estβ2* alleles are co-amplified, with the two-gene pair being the unit of amplification (Rooker et al. 1996, Vaughan et al. 1997). This renders the two genes inseparable by recombination, forming a sort of “supergene” (Guillemaud et al. 1998).

Gene amplification or duplication “is the reiteration of a segment of DNA to generate one or more… additional copies in the genome of the organism” (Bass & Field, 2011). A gene can duplicate through several mechanisms for example a replication error during meiosis, or through unequal crossing over (Klug et. al. 2012). Unequal crossing over can happen when homologous chromosomes or sister chromatids do not lineup precisely and a crossover event occurs. This

results in one DNA strand having the gene deleted while the other has two gene copies. Gene duplications can be advantageous because they reduce the chance of a subsequent mutation being harmful. If a mutation event occurs in one of the gene copies, the individual will still have an unaltered copy of that gene. Another possible advantage of this duplication is an increase in the production of the protein coded by the gene. Gene amplification is a common mechanism for resistance in a wide range of organisms. Once a gene duplication becomes established in a population, unequal crossing over can be repeated and the gene copy number can continue to increase, particularly if the resulting increase in protein production confers a fitness advantage. Heavy metal resistance in the yeast *Saccharomyces cerevisiae* has been linked to amplification of the CUP1 gene on chromosome VIII (Fogel et al.,1988). In the weed *Amaranthus palmeri* gene amplification of the EPSPS gene is the cause for resistance to the herbicide glyphosate. Genomes of resistant plants contained 5-fold to 160-fold more copies of the EPSPS gene. Interestingly these genes were present on every chromosome, so it is believed that unequal crossing over is not the only potential cause of this pattern of gene amplification (Gaines et al., 2010). Gene amplification has been a common mechanism for insecticide resistance in both orders Hemiptera and Diptera.

In the OP-resistant housefly (*Musca domestica*), resistance has been shown to be caused by the amplification of the structural glutathione S-transferase (GST) gene (Zhou & Syvanen, 1997). Overexpression due to gene

amplification of the cytochrome P450 genes is the mechanism for resistance to several insecticides. Examples include the amplification of the cyp6g1 gene in *Drosophila melanogaster* (Daborn et al. 2002) and the CYP9M10 gene in *Culex quinquefasciatus* which has been shown to have about a 260-fold overexpression caused by both a duplication and a single mutation. The gene has a single duplication in a region that is 100kb long and the two duplicated regions are completely identical indicating a recent duplication event (Itokawa et al. 2010). In *Anopheles funestus*, the CYP6P4 and CYP6P9 genes are duplicated in tandem in a pyrethroid-resistant strain resulting in a 51- and 25-fold overexpression compared to susceptible strains. The copies of these genes show significant sequence variation indicating relatively ancient duplications (Wondji et al. 2009).

Several field surveys have been done throughout the world to get a better understanding on the forces influencing the dynamics of amplified esterase genes. The most common approach in these surveys was to perform electrophoretic analysis on samples from field populations. This allowed them to describe the electromorph frequencies and the levels of resistance found throughout the populations and how it changes over generations. This approach gives valuable information as well as a “big picture” on how widespread resistance is and the kinds of esterase electromorphs which are present in a population. However, these studies ignore an additional level of genetic variation associated with amplified esterase genes. When gene amplification is in

question, individuals that appear to have the same electromorph can have very different levels of esterase activity due to differences in the number of esterase gene copies they carry. During genetic crosses, tandemly repeated genes will segregate as a block. This can result in a wide range of gene copy number variants among individuals in a population. Esterase activity variation due to copy number differences is analogous to variation caused by different alleles at a locus, with each different copy-number class representing a different allele (Ferrari & Georghiou 1991). Looking at it this way, because resistance increases with the amount of esterase activity, each copy-number allele results in different levels of resistance. Esterase activity assays done on field populations often show a continuous distribution of esterase activities over a wide range (Milligan 1995; Pietrantonio et al. 2000).

Ferrari and Georghiou (1991) did a quantitative genetic analysis of esterase activity variation in the Tem-R strain. They produced a set of “backcross families” by first crossing S-lab females with Tem-R males and then backcrossing F_1 females with S-Lab males. The offspring of each female were reared together as a family. Within each backcross family half of the individuals would be expected to carry a Tem-R strain chromosome with an amplified block of *Estβ1* genes and a homologous chromosome from the S-Lab strain with no gene amplification. The other half of the individuals in a family should be carry two S-Lab chromosomes. The analysis of esterase activity variation within and between backcross families indicated that the amplified esterase genes were inherited as

a block and that there was genetic variation in esterase activity among families. This suggested that Tem-R strain chromosomes varied in the number of amplified esterase genes they carried.

Ferrari (2015) used family analysis to examine esterase activity variation in a strain derived from a collection of individuals from the Sutter Yuba region of California. The only active esterase electromorph observed in the Sutter Yuba (SY) strain was B1. S-Lab (SL) females were crossed with SY males, and F_1 females were backcrossed with SL males. Individual egg rafts from F_1 females from the backcross were isolated to establish different families. For the chromosome carrying *Estβ1*, within each family half of the individuals would be expected to be "heterozygous" for an SY strain chromosome an SL strain chromosome and half would be expected to be "homozygous" for two SL strain chromosomes. Heterozygous siblings from a single family should each carry the same chromosome with the *Estβ1* gene. If the SY strain chromosome isolated in a family did not carry an *Estβ1* gene amplification then all individuals in the family should exhibit low esterase activity. If the SY strain chromosome carried an *Estβ1* gene amplification then within a family two groups of individuals should be observed, a Low esterase activity group and a High activity group with elevated esterase activity. Variation in the mean esterase activities of High activity groups in different families should reflect variation in the relative sizes of the *Estβ1* gene amplifications on different SY strain chromosomes. Of the 45 families examined, 19 only had a low activity group and 26 included both a low and high activity

group. Mean esterase activities of the high activity groups ranged from 1.7 to 27.5 times the mean esterase activity of the low activity groups. Comparisons of mean esterase activity of the 26 high activity groups indicated at least 11 different esterase activity variants. High activity groups on the lower end of the esterase activity spectrum exhibited lower variation in esterase activity than families on the higher end of the spectrum.

By treating resistance as a qualitative trait, the field surveys mentioned earlier are effectively taking the average esterase activity of all individuals in the population that have the EstB1 electromorph. This is excluding important information about esterase activity variation that could be useful when developing a resistance management strategy. Fitness studies that only look at the cost of having a particular high activity esterase electromorph are treating resistant individuals that fall on opposite ends of the esterase activity distribution as equals. These individuals are likely to spend different amounts of energy depending on how much esterase they produce, which could result in different fitness costs.

Resistance Management Strategies

The continuous use of an insecticide to control a pest population effectively selects for resistance in that population, resulting in the need for a new class of insecticide. It has quickly become evident however that resistance evolves faster than the development of new insecticides (Onstad, 2008; Sparks & Nauen, 2015). Resistance to one or more insecticides has appeared in over 580

different arthropod species (Sparks & Nauen, 2015). Such widespread resistance led to the field of Insecticide Resistance Management (IRM). The goals of resistance management are to prevent or slow down the development of resistance and potentially revert resistant populations to more susceptible levels (Croft, 1990). There are several parameters that need to be considered when designing an IRM strategy.

The Rex Consortium (2010) investigated which parameters were the most and least important in 187 articles on models of the evolution of resistance. All models clearly identified selection as the most important evolutionary force because “pesticides exert a very strong selection pressure, decreasing the impact of migration, mutations and genetic drift on the evolution of resistance.” (p. 385). Since the phenotype of an organism is determined by various selection pressures that suit it to its environment, any strong phenotypic change such as resistance may be expected to be deleterious in its old environment that did not include insecticides (Coustau et al. 2000). Selection is acting on fitness differences among individuals in a population. IRM programs that incorporate one or more insecticides rely on the assumption that resistance carries a cost to fitness in the absence of that insecticide (Ffrench-Constant & Bass, 2017). This fitness cost is also known as a trade-off, “… a decline in nonselected fitness characters that accompanies adaptation to new selective conditions.” (Garland & Rose, 2009). Several IRM programs have been developed with the use of computer models and simulations (see discussion in Ferrari, 1996). The majority

of these models assume that resistance is due to a single gene with two alleles (S & R) and that the initial frequency of the resistant (R) allele in the population is low.

The High Dose Strategy is to maximize selection against heterozygous (RS) individuals by using an insecticidal dose that kills all SS and RS individuals, making resistance functionally recessive (Tabashnik, 1990). At low R allele frequency majority of the R allele copies in the population are found in heterozygous individuals. The frequency of RR survivors is assumed to be low enough that they will be overwhelmed and mate with SS immigrants from adjacent untreated areas (Ferrari, 1996). The issues with this strategy is that the insecticide might not reach all individuals in a population and over time its effectiveness will decay. This could result in the opposite of the desired outcome and increase the frequency of resistant individuals. Another assumption of this strategy is that all resistant phenotypes are equal in fitness, but in the case of gene amplification there can be a quantitative distribution of resistance phenotypes. As seen from the work done by Ferrari (2015), RS individuals can express a wide range of esterase activities. If the insecticidal dose is only strong enough to eliminate individuals on the lower end of that spectrum, individuals on the higher end could still survive.

The Low Dose strategy uses a single insecticide on a population in a way that minimizes selection pressure on susceptible individuals. This is achieved by creating artificial refugia by leaving some areas untreated. Insecticide is applied

to areas around the refugia resulting in the survival of only resistant individuals. The survivors will likely mate with individuals from the refugia. This would keep the frequency of the susceptible (S) allele from getting too low and delay the onset of resistance especially when the R allele is still at low frequency. If there is a fitness cost to resistant individuals when no insecticide is present, the susceptible individuals from the refugia should outcompete them and keep the R allele frequency low. However, this strategy is not effective in vector control since susceptible individuals can carry the disease.

Models that involve two or more insecticides in Mixtures or Rotation incorporates the idea that the only individuals that could survive need to be resistant for all present insecticides (Tabashnik, 1990). These models have suggested that using a mixture of insecticides, rotations in time, or a spatial pattern of application could be useful in managing resistance. Models suggest that the most effective strategy using mixtures or rotations is to use insecticides that have different modes of action (Ferrari, 1996). Resistance to different insecticides with different modes of action is usually influenced by different genetic loci. If a mixture of two different insecticides is used, an individual needs to be at least heterozygous at both loci to survive. In the initial stages of use, when the frequencies of R alleles at a locus are low a double heterozygote in a population would be very rare. Additionally, if resistance to each insecticide carries a fitness cost it could make double heterozygote less fit then single heterozygotes or susceptible individuals. In the Rotation strategy different

insecticides are applied to a population at different periods in time. If the frequency of the R allele for resistance to one insecticide increases, the individuals would be killed when a switch is made to the second insecticide.

In the Rotation strategy, timing the switch between insecticides is important. The switch must be made before double heterozygotes occur at a significant level (Ferrari, 1996). Again, a fitness cost associated with resistance to the insecticides used in the rotation would, in theory, enhance the effectiveness of this strategy in delaying the evolution of resistance.

Finally, in the Spatial strategy multiple insecticides are applied in a mosaic pattern throughout a population. Models using this strategy indicate it is not useful in resistance management (Ferrari, 1996). This could be because a population would separate into smaller subpopulations of resistant individuals for the local insecticide. If these populations were to breed together it would increase the likelihood of producing offspring resistant to both insecticides.

Insecticides are our main tool to control insect pests and disease vectors, and improper implementations of IRM strategies can take that tool away. It is evident that a deep understanding on how resistance can affect an individual's fitness is needed to decide which IRM strategy, or combination of strategies, would be best. If a fitness cost does not exist in a population, then many of these strategies are likely to be ineffective in delaying or reducing resistance. If fitness costs do exist, certain strategies might work better than others and they could

possibly reduce the frequency of resistant individuals in a highly resistant population.

Fitness

According to Ffrench-Constant & Bass (2017) an ideal experimental design to determine if there are fitness costs associated with resistance should include the following components: 1) the study should involve resistant strains derived from the field, and should be studied in the field; 2) the resistant and susceptible genotypes of the collected strains should be known and compared in a similar genetic background; 3) the resistant and susceptible genotypes should compete directly against each other. Such an ideal study would be quite difficult to perform, which is why the vast amount of research done on fitness costs do not meet all of these criteria (Ffrench-Constant & Bass, 2017). However, the results obtained from laboratory cage-based experiments are still informative and can be used in conjunction with field-based experiments. Lab experiments can be used to gain a general knowledge of where trade-offs arise. This knowledge can be tested in the field to devise the best strategy to be used in an IRM program.

Knowing the mechanism of resistance in an insect can help predict where a fitness cost might occur. For resistance mechanisms caused by a single mutation affecting protein structure, a likely cost to fitness could be the result of a change in protein function (Coustau et al. 2000). In *Drosophila melanogaster*, a single amino acid substitution in the GABA receptor subunit encoded by the *Rdl*

gene results in cyclodiene resistance. This same mutation causes that protein to change conformation under high temperatures resulting in paralysis at high temperatures for the fly (Ffrench-Constant et al. 1993). For resistance caused by an overexpression of a gene, it is believed to affect fitness due to a "…resource and energy reallocation at the expense of metabolic and developmental processes." (Kliot & Ghanim, 2012, p. 1431). In cases of resistance due to elevated enzyme activity, resistant individuals need to allocate a greater portion of their energy to produce large amounts of detoxifying enzymes, while susceptible individuals can use that energy for other processes. A study done by Rivero et al. (2011) tested this assumption by quantifying energetic resources (lipids and sugars) in resistant and susceptible *Culex pipiens* mosquitoes. Resistant larvae showed no significant reduction in energy resources but adult resistant mosquitoes on average contained 30% less energetic reserves. The Rivero group believed the amount of food given to the larvae was enough to compensate for the additional cost of resistance, but as soon as the larvae stopped eating and entered metamorphosis the cost of resistance was paid. If there is a clear cost to fitness due to overproduction of esterase, that doesn't necessarily mean we can expect an immediate reduction in the resistant alleles when insecticide is not present (National Research Council, 1986, p.150). The intensity of selection for resistance in the presence of insecticides will be greater than selection for susceptibility in the absence of insecticides. Depending on the cost, it could take several generations before there is a significant reduction of

the resistant alleles. At the same time there could be selection for modifier genes that improve the fitness of the resistant genotypes.

A cost to fitness is often more apparent during extreme changes in environment, such as a rapid change in seasonal temperature. Gazave et al. (2001) determined the fitness costs to overwintering *Culex pipiens* mosquitoes in France. Over the breeding season they collected mosquitoes in a cave every other day for about the first 2 months, every 4 days for the next month, and every 6 days for the last 2 months. There were two main periods in this experiment they dubbed P1 and P2. P1 represented the first arrivers during the winter while P2 represented a second group of arrivers that they believe had to leave their original overwintering cave due to poor conditions. Based on their samples, there was nothing to indicate any fitness costs to resistant individuals in P1. However, in P2 there was a far lower frequency of resistant arrivals, which could indicate a fitness cost. They believe that resistant individuals were more likely to die in the original overwintering cave with poor conditions, or they were not able to survive the migration to the new cave.

It is also important to consider the effect on fitness in both sexes. In *Drosophila melanogaster*, DDT resistant females appeared to have a fitness advantage to susceptible females in the absence of DDT (McCart et al., 2005). Resistant females laid more eggs and a greater proportion of those eggs were viable. Resistant offspring had higher larval and pupal viability as well as shorter development times. Interestingly, a study on pinned museum specimens

indicated the DDT resistant (DDT-R) allele had already existed prior to the use of DDT, however it did not spread until the use of DDT (Ffrench-Constant & Bass, 2017). One possible reason for this could be sexual antagonism (Smith et al., 2011). Prior to the use of DDT, the fitness advantage the susceptible allele had in males could have outcompeted the advantage the resistant allele had in females. To test this, they measured possible fitness costs in DDT-R males. They had a DDT-R Canton-S (CS) strain, a wild caught (WC) strain, and a susceptible strain. CS DDT-R males achieved only 22% of matings when competing with susceptible males. Resistant males were also smaller than the susceptible males. In WC DDT-R males there was no difference in mating success compared to susceptible males and they were larger than the susceptible males. WC DDT-R males were more successful in mating in a non-competitive environment. They are not sure why there was such dramatic size differences between DDT-R and susceptible males. It is known however that male size is an important factor in *Drosophila melanogaster* mating success. In the CS strain, male body size is a trait that females can actively select for while greater egg count and viability is something only natural selection acts on. Prior to the use of DDT, selection for larger males outweighed the advantage resistant females had. But this may be due to one genetic background. This is evidence that supports the sexual antagonism hypothesis, however this may be due to the CS strain having only one genetic background.

Predictions of where a cost might appear can be made, and studies have shown some of these predictions to be accurate. But there is still a level of guess work when choosing for traits. What seems to be consistent across all these studies is that costs are amplified under stressful conditions.

Summary

1. Resistance to organophosphate (OP) insecticides in local populations of *Cx. quinquefasciatus* is associated with overproduced esterase electromorphs coded by two loci, *Estα* and *Estβ*. The molecular mechanism for esterase overproduction is gene amplification.

2. Individuals in a population that exhibit the same esterase electromorph can differ in esterase activity, presumably because they differ in the number of esterase gene copies they carry.

3. An important question in understanding the evolution of resistance to a particular class of insecticide is whether the resistance phenotype carries a fitness cost when that insecticide is not present in the environment. To date this question has not been addressed in the context of quantitative genetic variation affecting esterase activity and resistance where a number of phenotypes that vary in resistance exist in a population.

The overall goal of this study is to determine if there are trade-offs between elevated esterase activity associated with insecticide resistance and phenotypic traits likely to be associated with fitness in a strain of *Culex quinquefasciatus* established from a local field collection. This will be done by

comparing body size and development time in individuals that exhibit different levels of esterase activity. Body size will be estimated by measuring an individual's protein content, which is highly correlated with body size in mosquitoes (Handel & Day, 1989). Development time will be measured in mosquitoes reared under different dietary and temperature regimens designed to simulate either optimal or stressful conditions.

CHAPTER TWO

MATERIALS AND METHODS

Mosquitoes

Canyon Crest is a strain of *Culex quinquefasciatus* established from about 2400 individuals collected during the summer of 2015 from a property about a mile from the UC Riverside campus in Riverside, California. S-Lab is a standard susceptible strain with low esterase activity obtained from the laboratory of G.P. Georghiou at UC Riverside in the 1990s. Early esterase activity analysis revealed the presence of elevated esterase activity levels in the Canyon Crest strain. Prior to the experiments described below, these strains were maintained in the lab at about 25°C under uncrowded conditions with ample food.

Esterase Activity Variation Analysis of the Canyon Crest Strain

To characterize the esterase activity variation present in the Canyon Crest strain, two pans with ~300 larvae each were set up at 25°C and reared on ample food. When pupae appeared, they were collected each day and transferred to a 32oz cup for adult emergence. Adults were provided with wicks soaked in a 10% sucrose solution each day, frozen at between 1-2 days old, and stored in liquid nitrogen. Males from this group were used to analyze the esterase activity variation present in the Canyon Crest Strain. A total of 174 males underwent esterase and protein assays, as well as gel electrophoresis.

Development Time Studies

To determine an appropriate Low food diet for the Development Time study, a pilot experiment was run using different diets (amounts of food). Groups of 50 larvae from the Canyon Crest strain were reared on a standard 100% diet (see Table 1), and modified diets that used 125%, 75%, 50% or 25% of the amounts of food each day as provided on the standard diet. The goal was to identify a diet that would induce stress (in the form of competition for food) among the larvae. The 25% diet had a noticeable effect on development time and survivorship and was chosen as the Low diet in the development time studies.

Table 1. Normal feeding protocol

Day of egg hatch*	30µl
Days 1-2	250µl
Days 3-4	375µl
Days 5-6	500µl
Day 7**	1500µl

** On the day of egg hatch food was added to the scintillation vial. Amount of food was added to the 32oz cup corresponding to the number of days after the hatch. Food was added using a Gilson micro-pipetter while food was stirred in a flask on a stir-plate. **On the 7th day water was changed by pouring cup contents onto a screen, 200ml of water was added to the cup and the screen was placed upside down over cup. Larvae were knocked off the screen into the cup by tapping the other end of the screen. After day 7 no additional food was added to the cups.*

Four development time experiments were done under the four combinations of dietary level (High and Low) and temperature (25° and 30° C):

Experiment 1: High Diet; 25°C

Experiment 2: Low Diet; 25°C

Experiment 3: High Diet; 30°C

Experiment 4: Low Diet; 30°C

As described above in pilot Development Time study, the Low Diet protocol provided larvae with 25% of the amount of food provided on the High Diet. The feeding protocol is shown in Table 2. To set up an experiment, 141 Canyon Crest strain egg rafts were collected. Egg rafts were collected three times separated by 12-hour intervals. In the 30°C experiment there were 100 additional individuals reared from egg rafts collected 48 hours after the first group. The egg rafts in each group were kept together in an 8oz cup to hatch. As soon as eggs in a group hatched, groups of 50 first instar larvae were pipetted into 32oz cups. Ten cups were set up for each experiment (500 larvae). Once pupae began to emerge they were collected at 8am, 2pm, and 8pm every day and transferred to a separate containers based on egg group and time they were collected. Development times were calculated from the time that larvae were added to the rearing cups. Once all pupae emerged as adults in a container, they were frozen and stored in a -80℃ freezer and later transferred to a liquid Nitrogen storage dewar.

Table 2. Modified feeding protocol

Day	High Diet	Low Diet
	100%	25%
1-2	250µl	62.5µl
3-4	375µl	93.75µl
5-6	500µl	125µl
7	1500µl	375µl
Until first pupae*	200µl	50µl

*The procedure is the same up to the water change. *The same amount of food was given each day after*

Protein Assay

Individual mosquitoes were placed in 100µl sodium phosphate buffer (pH 6.5) containing 0.5% Triton X-100 and homogenized using a Dremel tool. 10µl of mosquito homogenate was diluted with 490µl ultrapure distilled water. 100µl of the diluted sample were added to the wells of a microtiter plate on ice. Plates were removed from ice and allowed to incubate at 25℃ for 10 minutes. 200µl of premade BioRad Quickstart Brandford reagent was added to each well. Plates incubated at 25℃ for an additional 10 minutes. Absorbance was measured at 600nm using a microtiter plate reader (BioTek). A standard curve (2-8µg protein/100µl) was run with each protein assay. All samples and standards were run on two replicate plates.

Esterase Assay

20µl of mosquito homogenate was diluted with 380µl sodium phosphate buffer (pH 6.5) containing 0.5% Triton X-100. 50µl of the diluted sample were added to the wells of a microtiter plate on ice. Plates were removed from ice and allowed to incubate at 25℃ for 10 minutes. 100µl of developing solution (2.5ml α-naphthyl acetate in ethanol, 35ml distilled water, 12.5ml sodium phosphate buffer (pH 6.5) containing 0.5% Triton X-100) was added to each well and allowed to incubate at 25℃ for exactly 15 minutes. 100µl of Fast Garnet solution (0.04g Fast Garnet salt in 50ml distilled water) was added to each well. Plates were covered with a paper towel and allowed to incubate at 25℃ for exactly 10 minutes. Absorbance was measured at 550nm with a microtiter plate reader. A standard curve (5-50 nmoles α-naphthol/50 µl) was run with each esterase assay. All samples and standards were run on two replicate plates.

Polyacrylamide Gel Electrophoresis

Electrophoresis of the mosquito homogenate was performed on acrylamide gels in 0.08 M boric acid, 0.09 M Tris, 0.002 M EDTA running buffer, pH 8.3, at 5℃. A standard run time of one hour at a constant current of 20 mA per gel was used. Gels were stained for esterase activity with 1ml of 2% α-naphthyl acetate in ethanol and 40 mg Fast Garnet GBC salt in 50 ml of sodium phosphate buffer (pH 6.5) containing 0.5% Triton X-100. Gels were rinsed and stored with distilled water and kept in a 4℃ fridge.

Each gel contained one sample of Tem-R homogenate to be used as a standard for the B1 allozyme. Tem-R is a strain homozygous for the esterase B1 allozyme and its resistance to OP is due to esterase B1 gene amplification (Mouchès et al. 1986).

Statistical Analysis

The SAS statistical analysis system (SAS Institute 2013) was used to perform calculations to determine protein content and esterase activity. R version 4.2.1 (R Core Team 2022) was used to perform regression, partial correlation, type III two-way analysis of variance, Welch's ANOVA, and produce figures. The Coefficient of variation (C.V.) was calculated by dividing the standard deviation by the mean and used to compare the variability of each set of data.

The data was checked for normality by examining residuals on a histogram and a normal probability plot as well as doing a Shapiro-Wilk normality test. The histogram was observed for its general shape and the normal probability plot was compared to a normal distribution. If the Shapiro-Wilk test p-value is greater than 0.05, then normality is assumed. Equality of variances was checked using the Bartlett test of homogeneity of variances. If the Bartlett test p-value is greater than 0.05, then equal variances is assumed. If no evidence was found, then a type III two-way ANOVA with interaction was performed. If evidence for non-normality and unequal variance was detected a non-parametric test such as Welch's ANOVA was performed. While normality is still an assumption of Welch's ANOVA it is still robust when sample sizes are large (Zar,

1996). An individual Welch's ANOVA would be performed for each possible combination of variables being checked. The cutoff for all significant p-values is less than 0.05.

Linear regression was used to test for significant relationships between the variables: esterase activity, protein content, and development time. A follow up partial correlation analysis was done using the 'ppcor' package and used to determine if the relationship seen between two variables was affected by the third. The cutoff for significance of p-values of the statistical tests was 0.05.

CHAPTER THREE

RESULTS

Esterase Activity Variation in the Canyon Crest Strain

A total of 174 males from the Canyon Crest strain as well as 42 males from the S-Lab strain were analyzed for their esterase activity. The mean esterase activity (nmoles α-naphthyl acetate hydrolyzed per minute per mg mosquito protein) was 144.57 for the Canyon Crest strain and 49.91 for the S-Lab strain. The coefficient of variation of esterase activity was 72.5% for the Canyon Crest strain and 15.4% for the S-Lab strain. The esterase activities of 38% of the Canyon Crest individuals fell within the range of S-Lab (Figure 1). In an electrophoretic analysis of the 174 Canyon Crest individuals 101 A2-B2, 37 B1, and 16 A2-B2/B1 individuals were observed. There were 20 individuals that either had an unfamiliar electromorph or the bands were too faint to determine the electromorph phenotype.

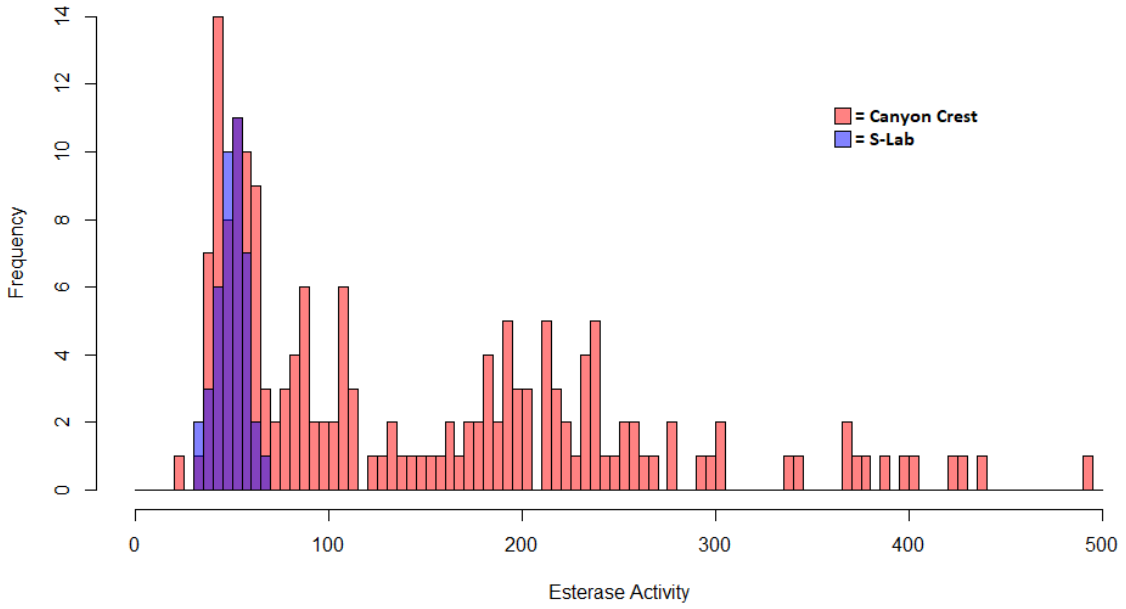

Figure 1. Esterase activity frequency histogram of 174 Canyon Crest males and 42 S-Lab males.

Development Time

A total of 686 male mosquitoes and 633 female mosquitoes from the Canyon Crest strain were collected from the four development time experiments. The full frequency distributions of development time for the four experiments are shown in Figure 2 for males and in Figure 3 for females. Table 3 and Figure 4 summarize the development time data for males. Table 4 and Figure 5 summarize the development time data for females.

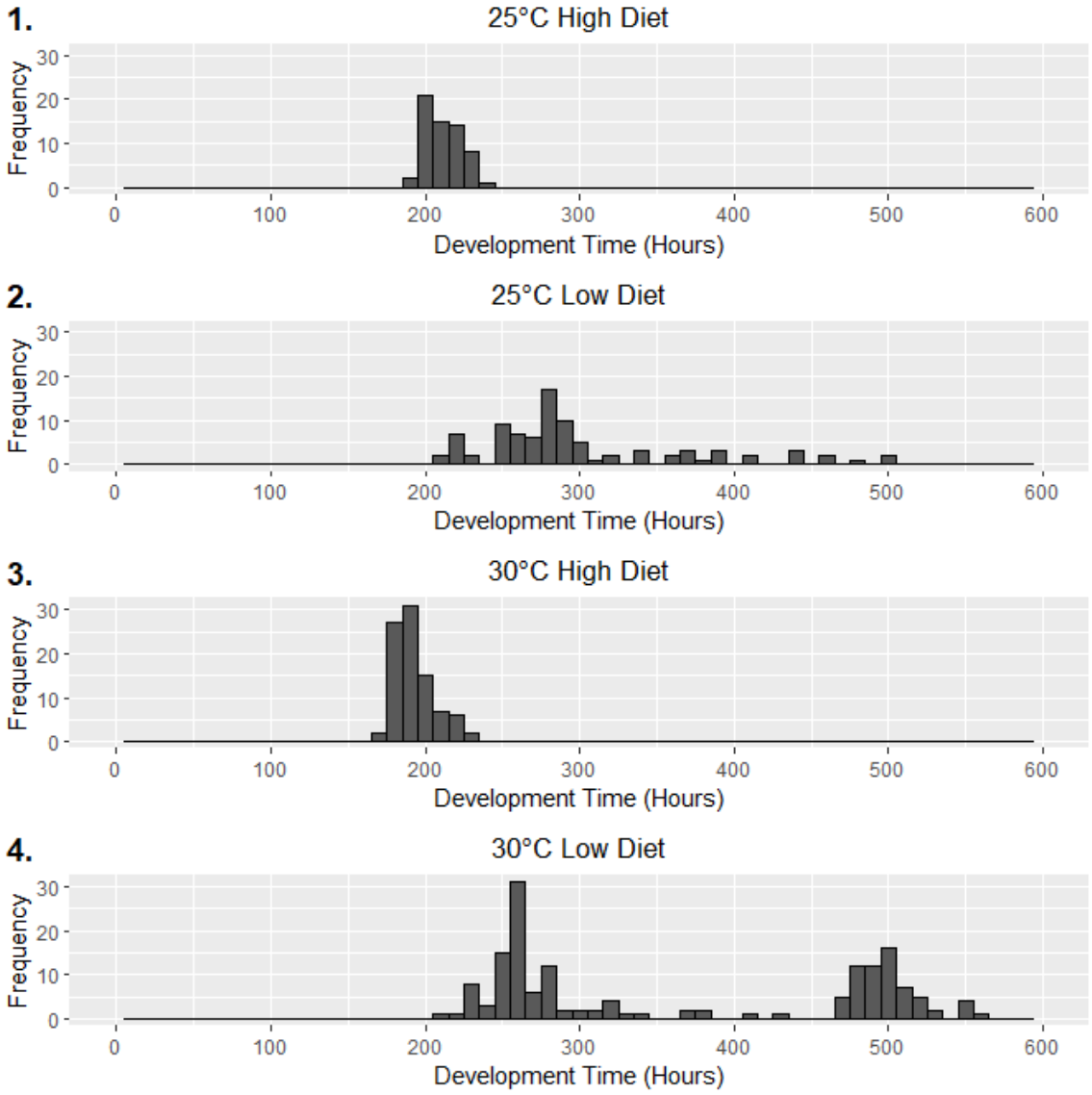

Figure 2. Total development time frequency distributions for male mosquitoes from 4 development time experiments.

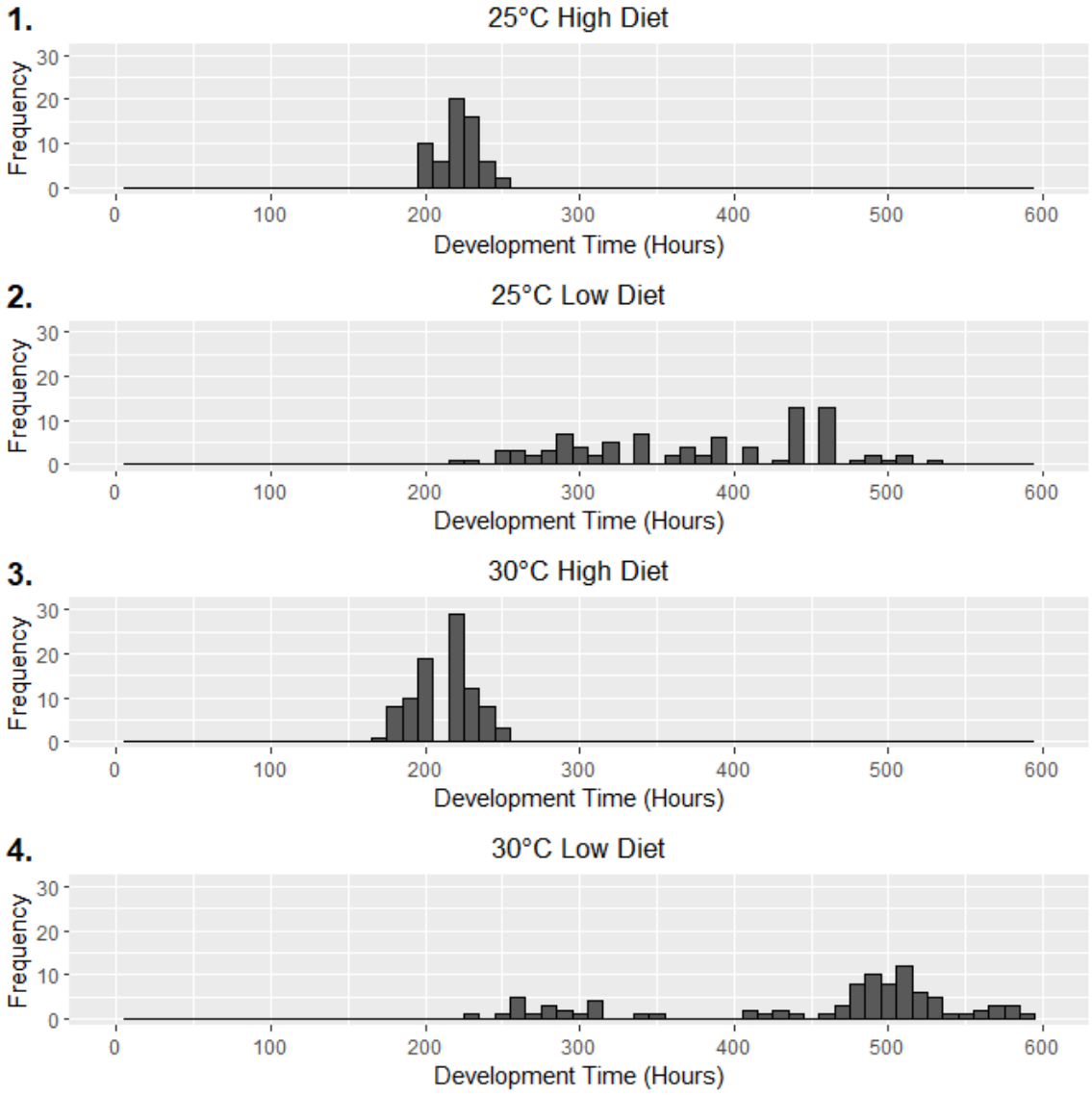

Figure 3. Total development time frequency distributions for female mosquitoes from 4 development time experiments.

Table 3. Summary of development time in hours for male mosquitoes from 4 development time experiments.

Experiment	N	Mean	S.E.	Minimum	Maximum	C.V.
25°C High Diet	130	211	1.46	192	240	5.40%
25°C Low Diet	217	299	7.32	215	504	23.2%
30°C High Diet	180	193	1.43	167	229	7.05%
30°C Low Diet	159	364	9.25	210	561	32.1%

Table 4. Summary of development time in hours for female mosquitoes from 4 development time experiments.

Experiment	N	Mean	S.E.	Minimum	Maximum	C.V.
25°C High Diet	108	222	1.74	198	252	6.08%
25°C Low Diet	240	379	8.37	222	534	20.9%
30°C High Diet	139	214	1.96	167	255	8.69%
30°C Low Diet	146	455	10.4	229	594	21.8%

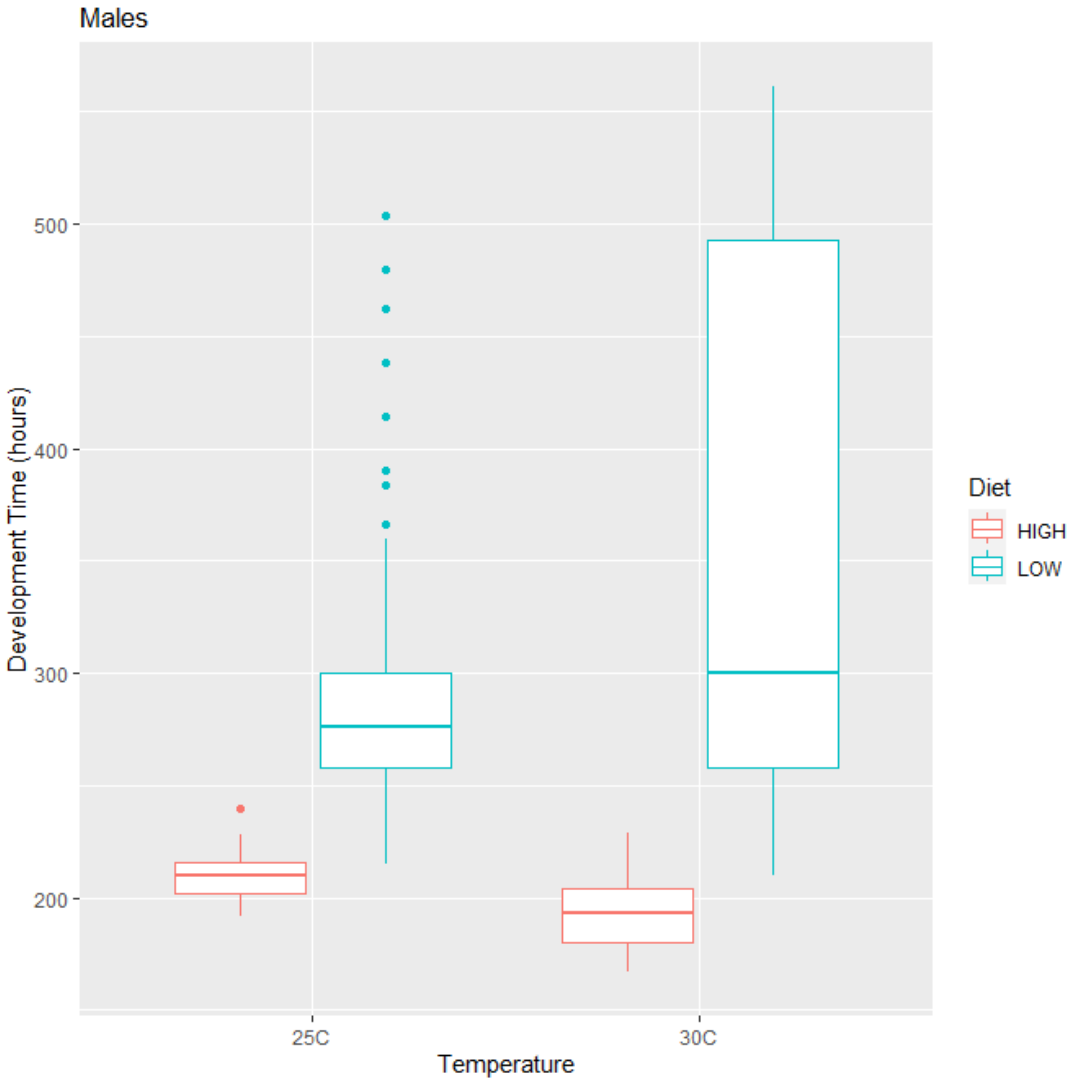

Figure 4. Distribution of male mosquito development time in each experiment. Each box represents the Interquartile range with the horizontal line representing the median and each dot represents an outlier.

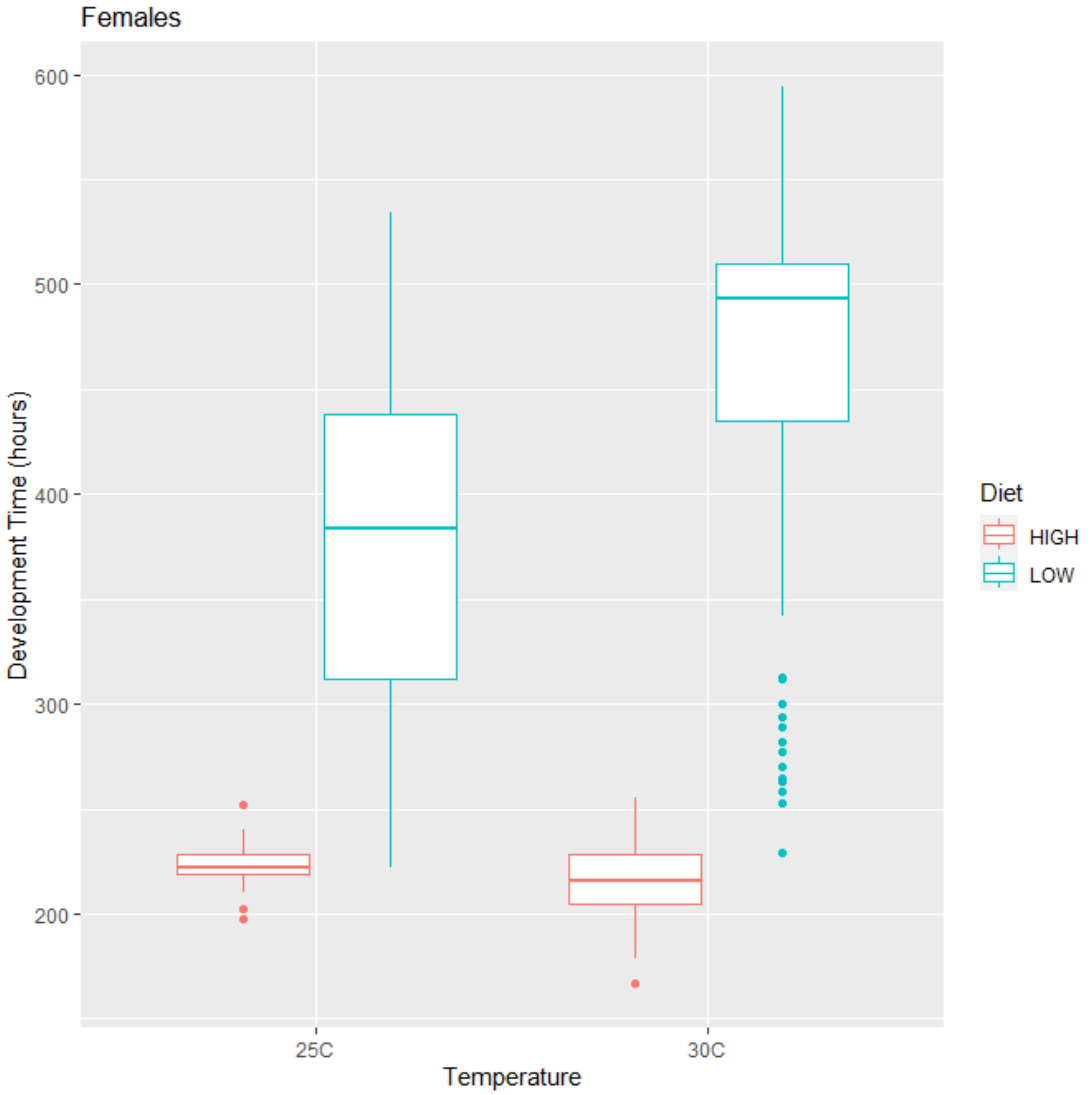

Figure 5. Distribution of female mosquito development time in each experiment. Each box represents the Interquartile range with the horizontal line representing the median and each dot represents an outlier.

The effects of temperature and diet on development times were similar for males and females. Mean development times were considerably shorter on the High diet than on the Low diet and the variation in development times among individuals was substantially higher on the Low diet at both temperatures. A residual histogram and normal probability plot were examined as well as a Shapiro-Wilk normality test was done to check for normality. Bartlett's test of homogeneity of variances was performed to check for equality of variances. There was evidence of non-normality and unequal variances among the distributions of development times in males from the four experiments. Because of these statistical conditions non-parametric Welch's ANOVAs were performed to determine if diet or temperature had a significant effect on development time. Four individual Welch's ANOVAs were performed. Temperature had a significant effect on development time under both the High diet regime ($F_{1,307} = 217$, $P < 0.001$, Figure 6a) and the Low diet regime ($F_{1,230} = 40.5$, $P < 0.001$, Figure 6a). Diet had a significant effect on development time at 25°C ($F_{1,231} = 383$, $P < 0.001$, Figure 6b) and at 30°C ($F_{1,161} = 341$, $P < 0.001$, Figure 6b).

Interaction plots suggested that there was an interaction between the effects of temperature and diet on development time in males (Figure 6). Recognizing the potential effects of non-normality and unequal variances described above a two-way ANOVA was performed to analyze the potential interaction. There was a significant interaction between the effects of diet and temperature (interaction: $F_{1,682} = 63.47$, $P < 0.001$).

A residual histogram and normal probability plot were examined as well as a Shapiro-Wilk normality test was done to check for normality of residuals. The bartlett test of homogeneity of variances was performed to check for equality of variances. There was evidence of non-normality and unequal variances among the distributions of development times in females from the four experiments. Because of these statistical conditions non-parametric Welch's ANOVAs were performed to determine if diet or temperature had a significant effect on development time. Four individual Welch's ANOVAs were performed. Temperature had a significant effect on development time under both the High diet regime ($F_{1,235} = 22.6$, $P < 0.001$, Figure 7a), and the Low diet regime ($F_{1,252} = 66$, $P < 0.001$, Figure 7a). Diet had a significant effect on development time at 25°C ($F_{1,260} = 998$, $P < 0.001$, Figure 7b) and at 30°C ($F_{1,155} = 890$, $P < 0.001$, Figure 7b).

Interaction plots suggested that there was an interaction between the effects of temperature and diet on development time in females (Figure 7). Recognizing the potential effects of non-normality and unequal variances described above a two-way ANOVA was performed to analyze the potential interaction. There was a significant interaction between the effects of diet and temperature (interaction: $F_{1,629} = 59.16$, $P < 0.001$).

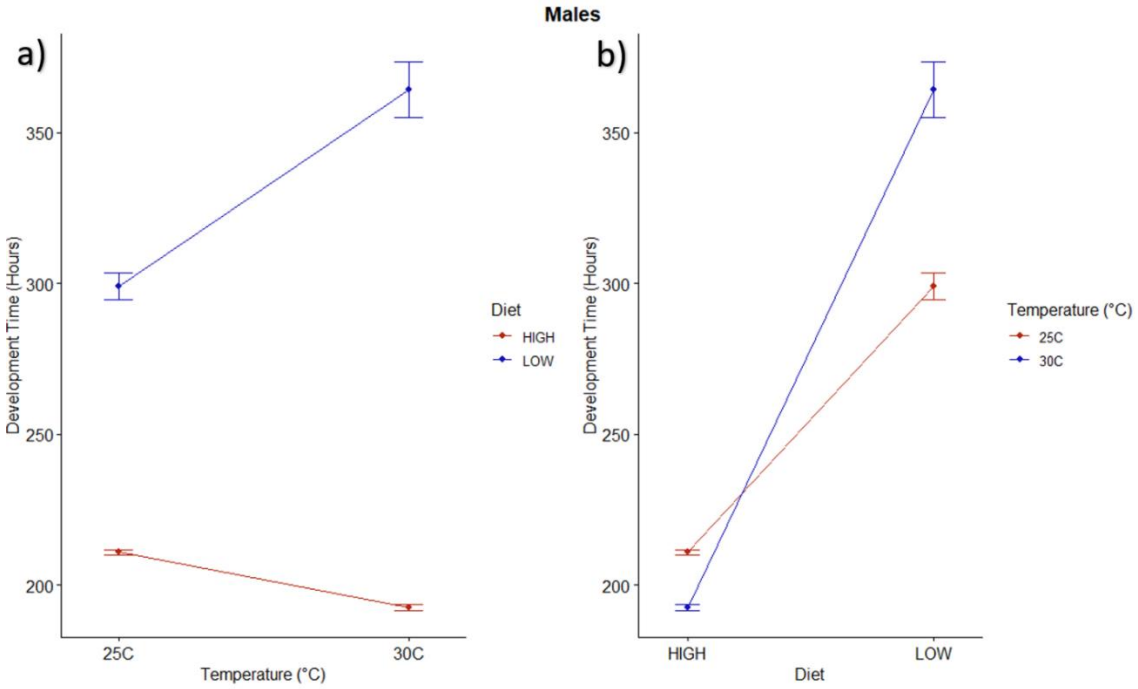

Figure 6. Interaction plot comparing mean development time in male mosquitoes for each experiment. Error bars represent mean +/- S.E.

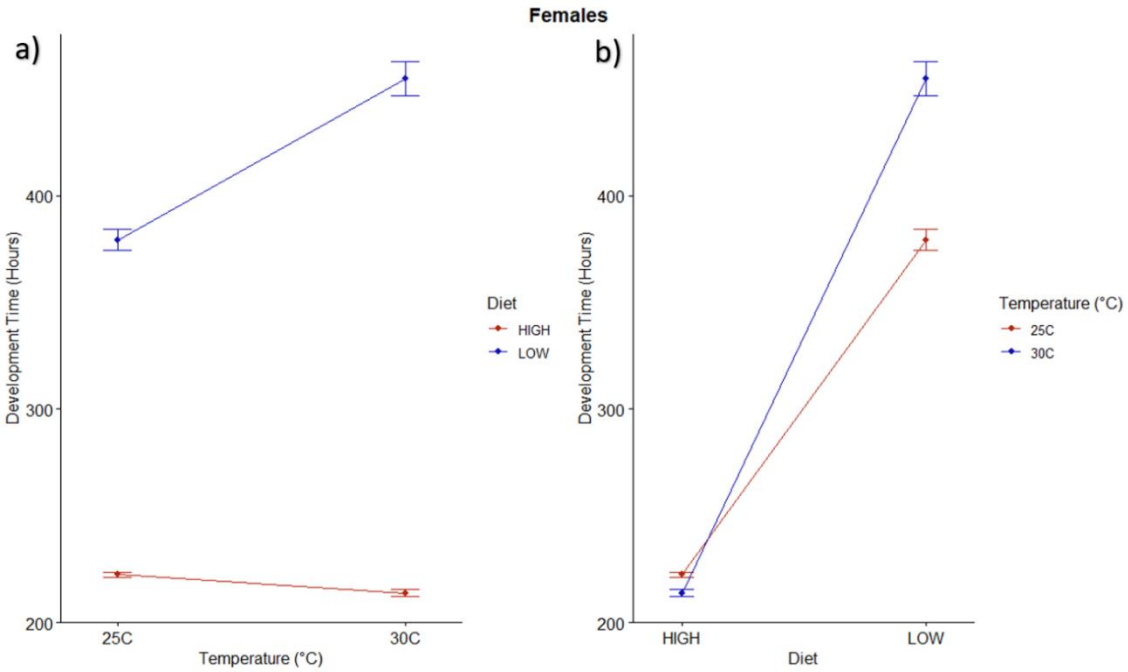

Figure 7. Interaction plot comparing mean development time in female mosquitoes for each experiment. Error bars represent mean +/- S.E.

Esterase Activity

Data from the protein and esterase assays were used to calculate the esterase activity of individual mosquitoes. Esterase activity is expressed as nmoles of alpha-naphthyl acetate hydrolyzed per minute per mg of mosquito protein.

A total of 400 male and 330 female mosquitoes from the Canyon Crest strain from four development time experiments were analyzed. The full frequency distributions of esterase activity for the four experiments are shown in Figure 8 for males and in Figure 9 for females. Table 5 and Figure 10 summarize the

esterase activity data for males. Table 6 and Figure 11 summarize the esterase activity data for females.

Table 5 summarizes the esterase activity results of males. The frequency distributions of total esterase activities of males for the four experiments are shown in Figure 8. Mean esterase activities for males in the four experiments ranged from 82 to 130. The variation of esterase activities among males were similar in all four experiments with coefficients of variation ranging from about 69% to 80%. Mean esterase activities for females in the four experiments ranged from about 93 to 130. The variation of esterase activities among females were similar in all four experiments with coefficients of variation ranging from about 72% to 79%.

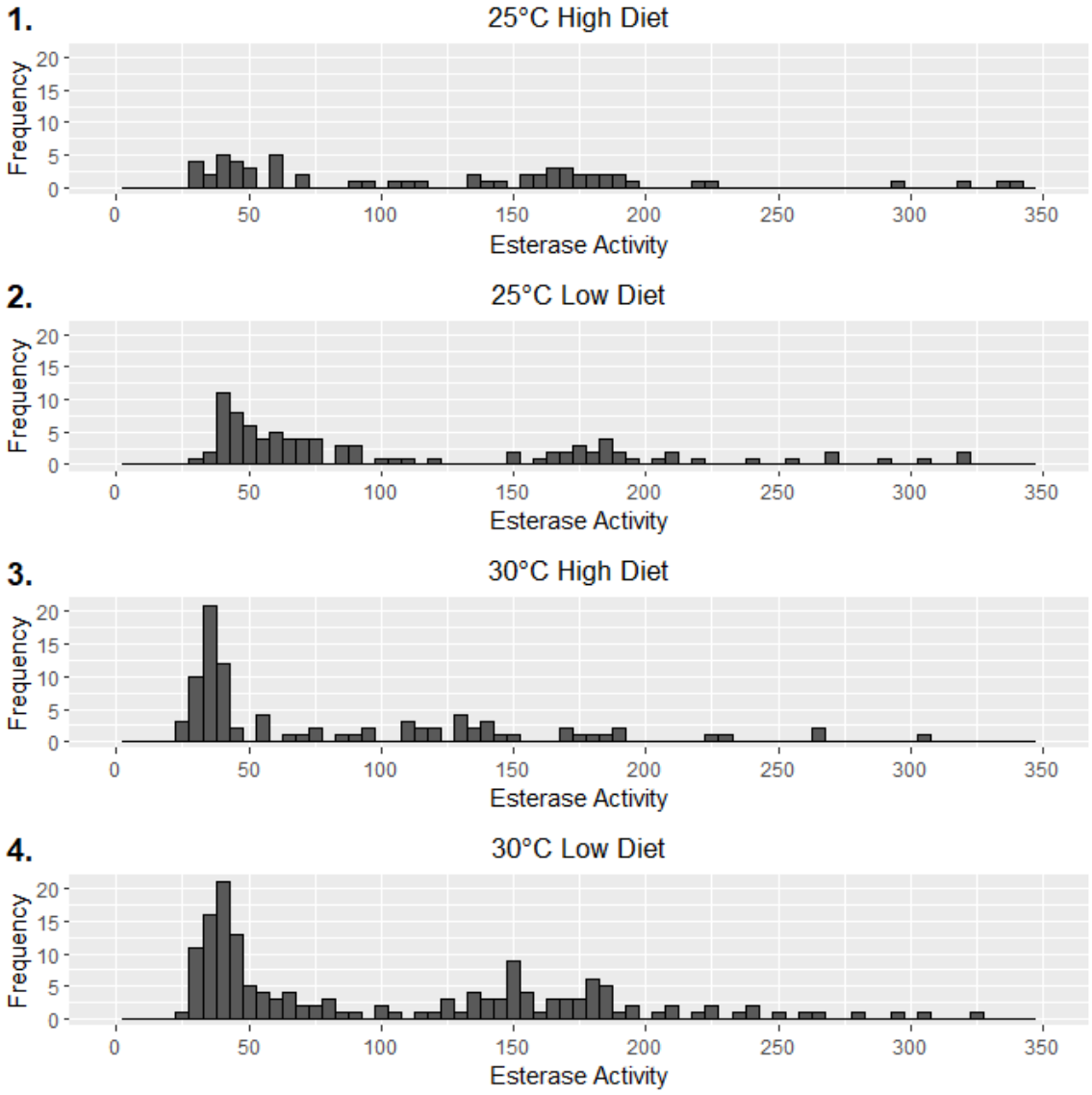

Figure 8. Total esterase activity frequency distributions for male mosquitoes from 4 development time experiments.

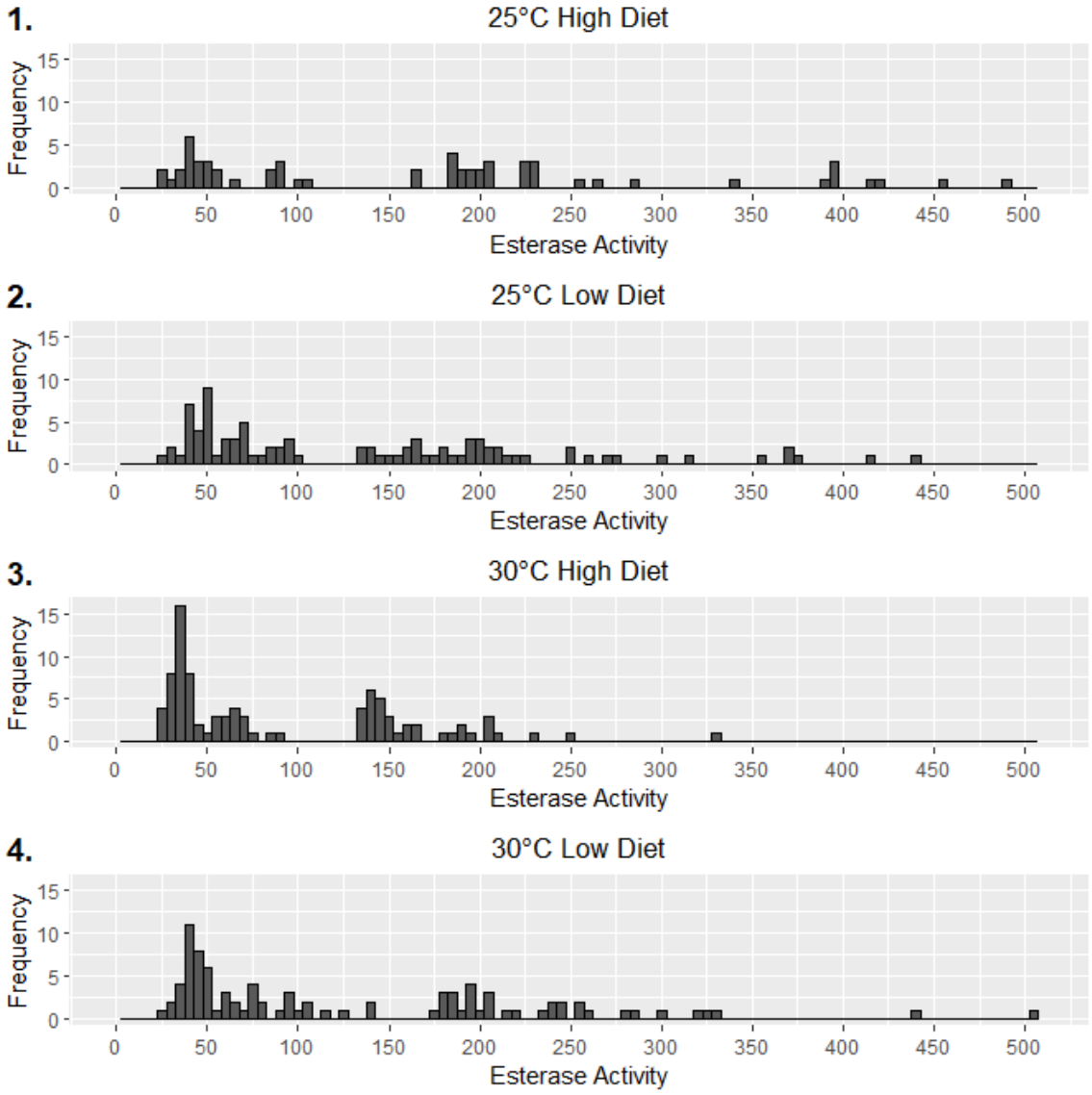

Figure 9. Total esterase activity frequency distributions for female mosquitoes from 4 development time experiments.

Table 5. Summary of esterase activity data for male mosquitoes from 4 development time experiments.

Experiment	N	Mean	S.E.	Minimum	Maximum	C.V.
25°C High Diet	61	130	11.5	27.6	357	69.4%
25°C Low Diet	90	111	8.26	27.7	321	70.5%
30°C High Diet	90	81.9	6.89	23.1	305	79.9%
30°C Low Diet	159	105	5.82	25.4	327	69.8%

Esterase activity units are nmoles α-naphthyl acetate hydrolyzed per minute per mg of mosquito protein.

Table 6. Summary of esterase activity data for female mosquitoes from 4 development time experiments.

Experiment	N	Mean	S.E.	Minimum	Maximum	C.V.
25°C High Diet	60	170	16.5	25.7	489	75.3%
25°C Low Diet	90	139	10.6	26.9	442	71.9%
30°C High Diet	90	92.5	7.15	23.1	329	73.3%
30°C Low Diet	90	130	10.7	24.1	506	78.5%

Esterase activity units are nmoles α-naphthyl acetate hydrolyzed per minute per mg of mosquito protein.

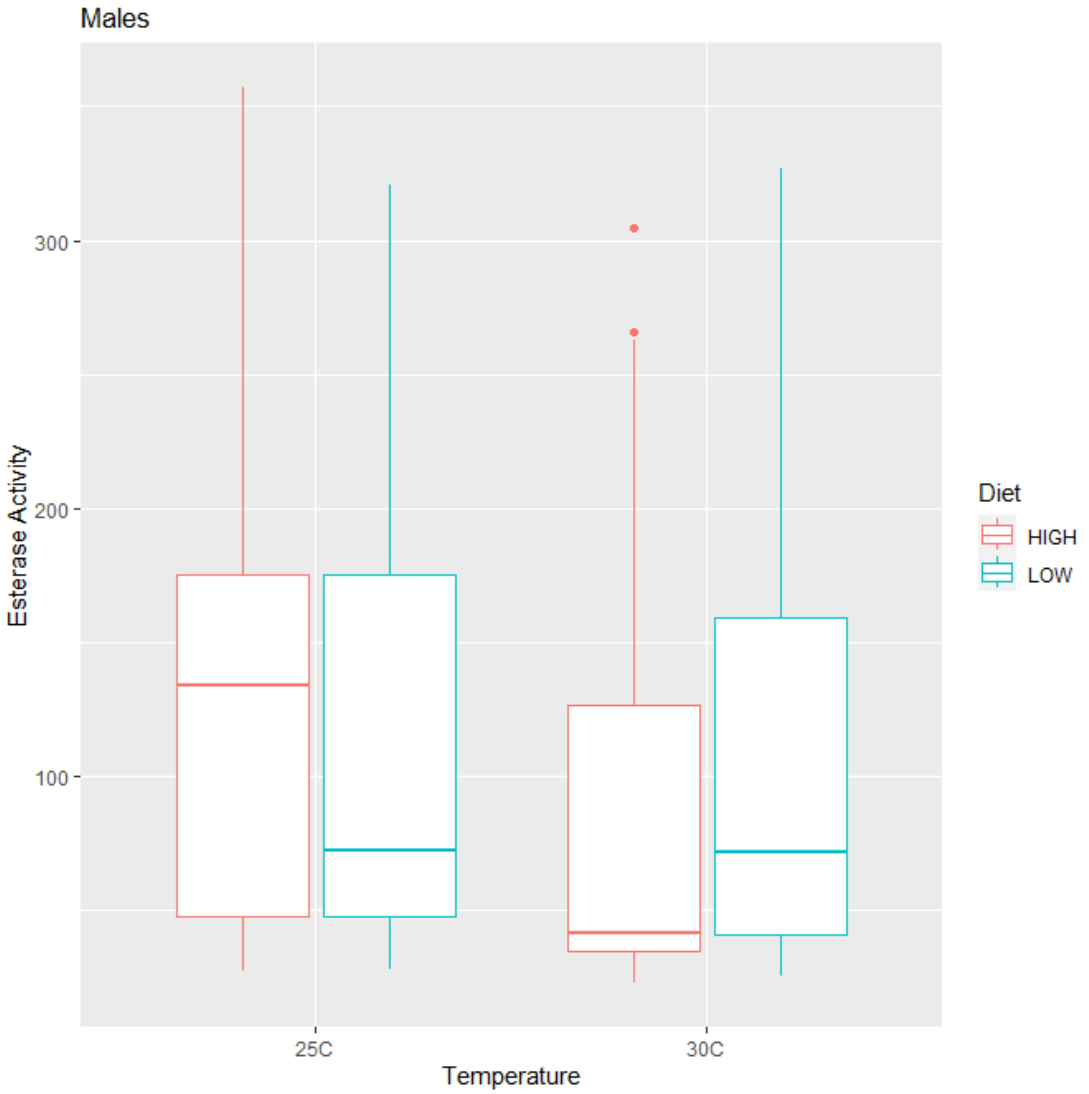

Figure 10. Distribution of male mosquito esterase activity in each experiment. Each box represents the Interquartile range with the horizontal line representing the median and each dot represents an outlier.

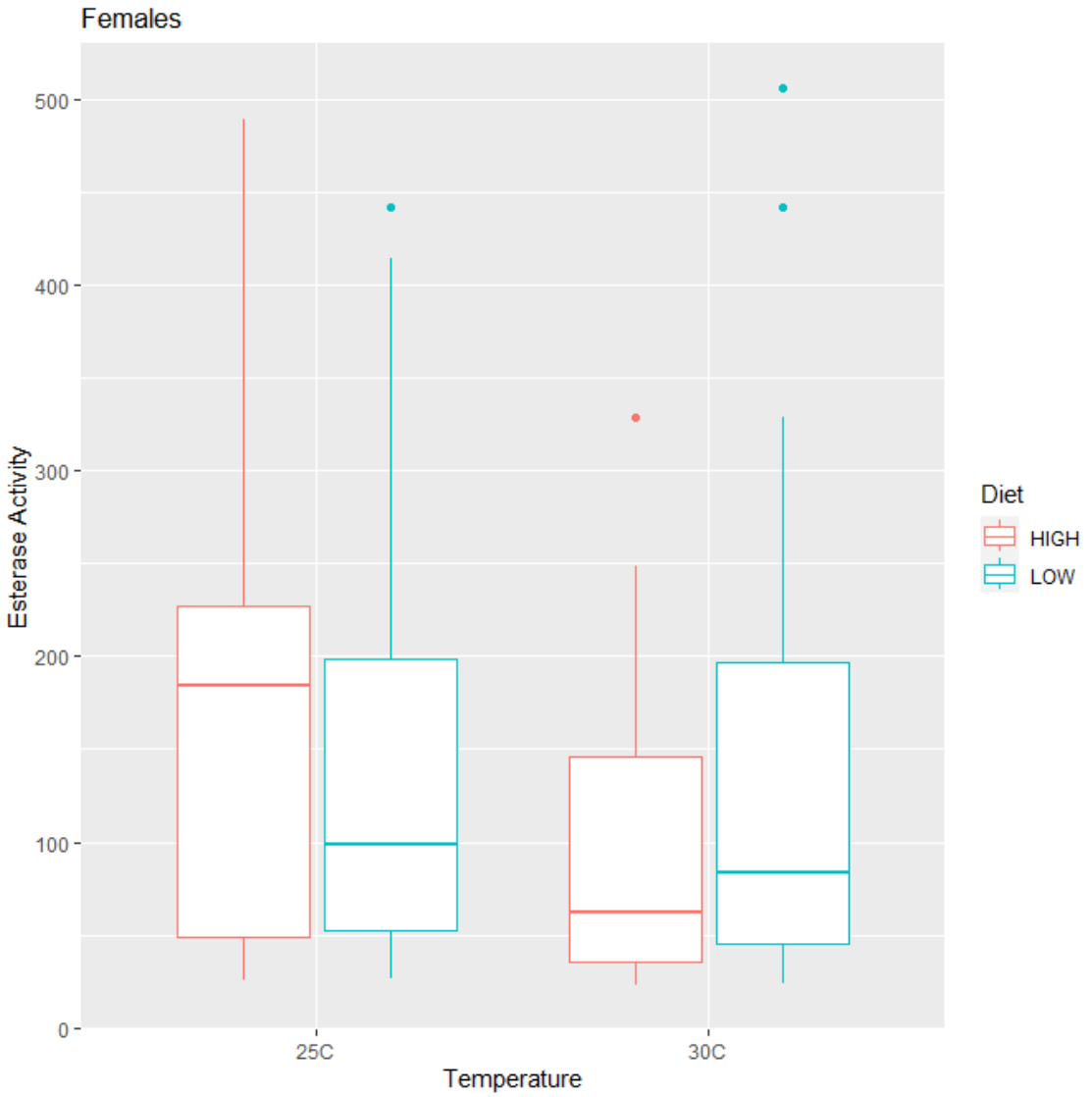

Figure 11. Distribution of female mosquito esterase activity in each experiment. Each box represents the Interquartile range with the horizontal line representing the median and each dot represents an outlier.

There was evidence of non-normality in the distributions of male esterase activity but no evidence for unequal variance. A two-way ANOVA was performed to analyze the effect of temperature and diet on esterase activity. There was a significant interaction between the effects of diet and temperature (interaction: $F_{1,396} = 7.008$, $P = 0.008$, Figure 12). Pairwise comparisons of mean esterase activity between each experiment revealed a significant difference in male mean esterase activity between individuals reared at 25°C and 30°C in the two High diet experiments (Table 7, Figure 12). All other pairwise comparisons were not significant.

There was evidence of non-normality in the distributions of female esterase activity but no evidence for unequal variances. A two-way ANOVA was performed to analyze the effect of temperature and diet on esterase activity. There was a significant interaction between the effects of diet and temperature (interaction: $F_{1,326} = 9.548$, $P = 0.002$, Figure 13). While two pairwise comparisons of female mean esterase activity between experiments were significant, and two comparisons bordered on significant, no clear effects of temperature or diet were apparent across experiments (Table 8). As was observed for males, there was a highly significant difference in mean female esterase activity between individuals reared at 25°C and 30°C on the High diet, but not when reared on the Low diet. The difference in mean esterase activity between females reared on the High diet and the Low diet bordered on significance when reared at 30°C, but showed no difference when reared at

25°C. The other significant difference was between the 30°C High diet and 25°C Low diet experiments (Table 8, Figure 13).

Table 7. Results of the Tukey's test, comparing male esterase activity of each diet and temperature combination.

Pairwise Comparison	p-adjusted
25°C High Diet - 30°C High Diet	< 0.001
25°C High Diet - 25°C Low Diet	0.396
25°C High Diet - 30°C Low Diet	0.109
30°C High Diet – 25°C Low Diet	0.054
30°C High Diet - 30°C Low Diet	0.105
25°C Low Diet - 30°C Low Diet	0.930

Table 8. Results of the Tukey's test, comparing female esterase activity of each diet and temperature combination.

Pairwise Comparison	p-adjusted
25°C High Diet - 30°C High Diet	< 0.001
25°C High Diet - 25°C Low Diet	0.232
25°C High Diet - 30°C Low Diet	0.067
30°C High Diet – 25°C Low Diet	0.009
30°C High Diet - 30°C Low Diet	0.059
25°C Low Diet - 30°C Low Diet	0.918

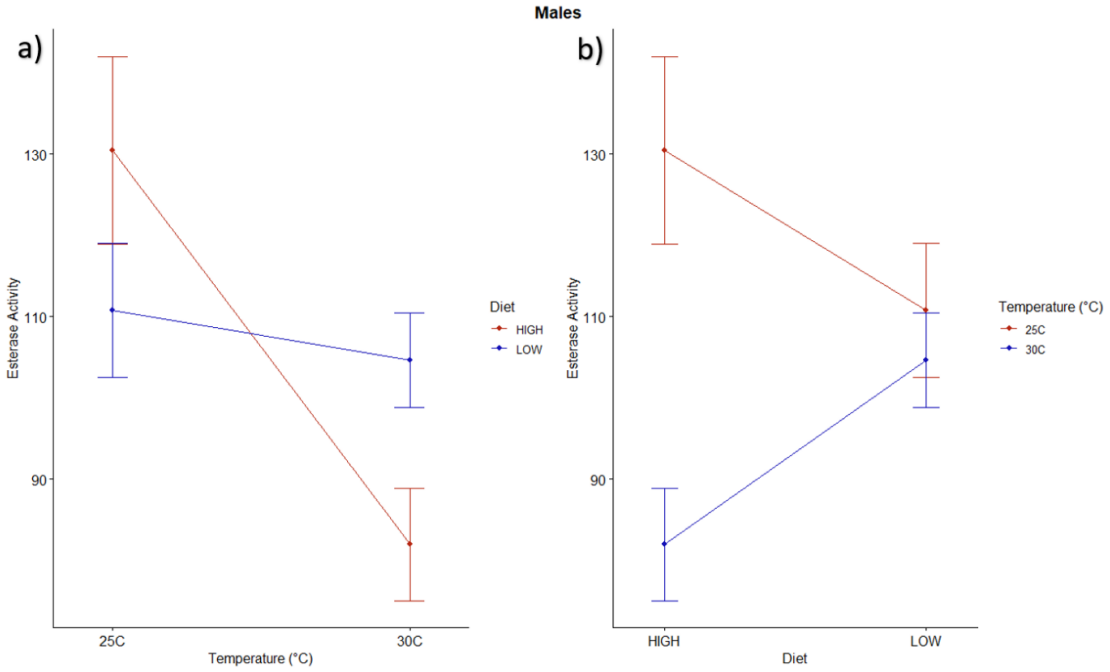

Figure 12. Interaction plot comparing mean esterase activity in male mosquitoes for each experiment. Error bars represent mean +/- S.E.

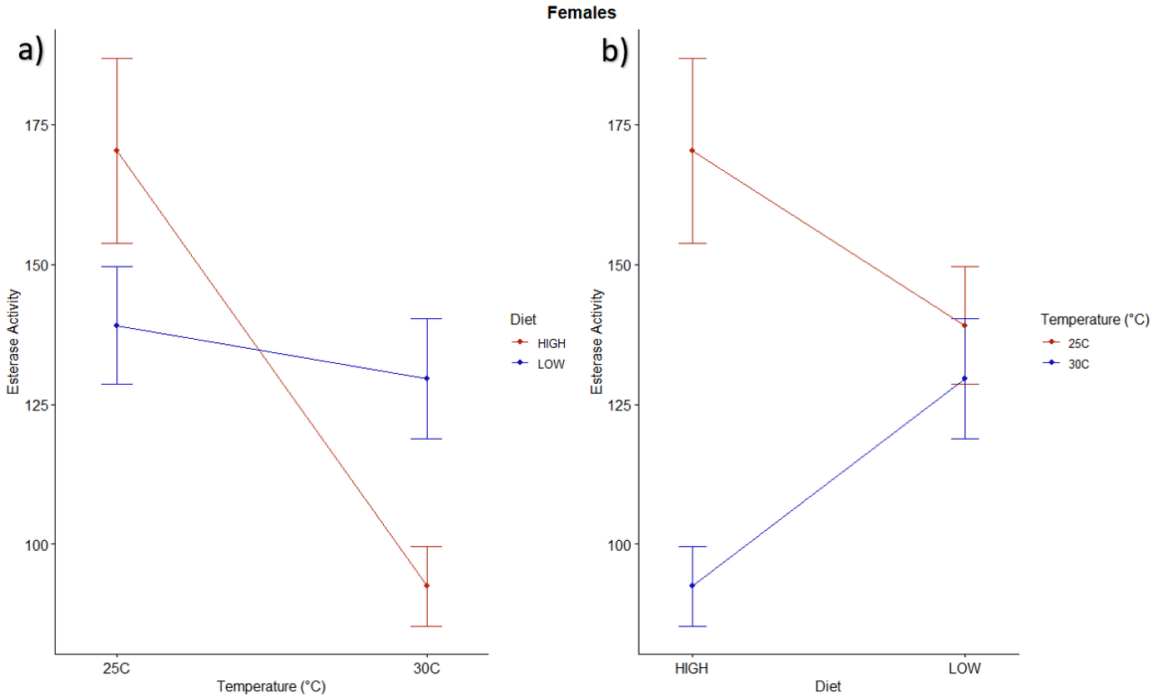

Figure 13. Interaction plot comparing mean esterase activity in female mosquitoes for each experiment. Error bars represent mean +/- S.E.

Protein Content

The full frequency distributions of esterase activity for the four experiments are shown in Figure 14 for males and in Figure 15 for females. Table 9 and Figure 16 summarize the protein content data for males. Table 10 and Figure 17 summarize the protein content data for females. In general, mean protein content in both sexes was substantially higher under High diet conditions at both rearing temperatures. The variation in protein content among individuals in both sexes was substantially higher under Low diet conditions than under High diet

conditions at both rearing temperatures. Coefficients of variation for male protein content were 2.8 times higher under Low diet conditions at 25°C and 2.2 times higher at 30°C. Coefficients of variation for female protein content were 3.1 times higher under Low diet conditions at 25°C and 2.4 times higher at 30°C.

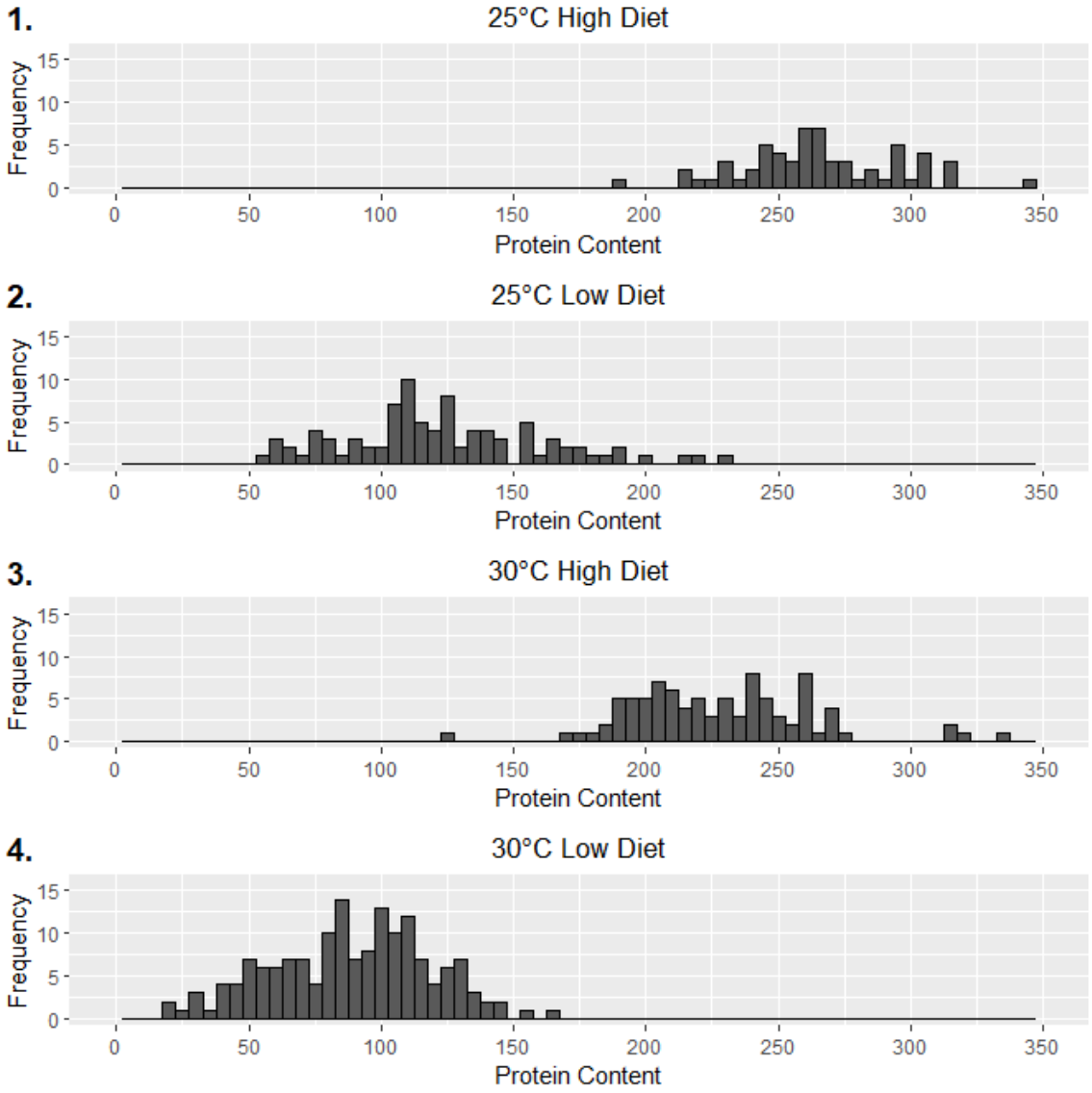

Figure 14. Total protein content frequency distributions for male mosquitoes from 4 development time experiments. Protein content units are micrograms.

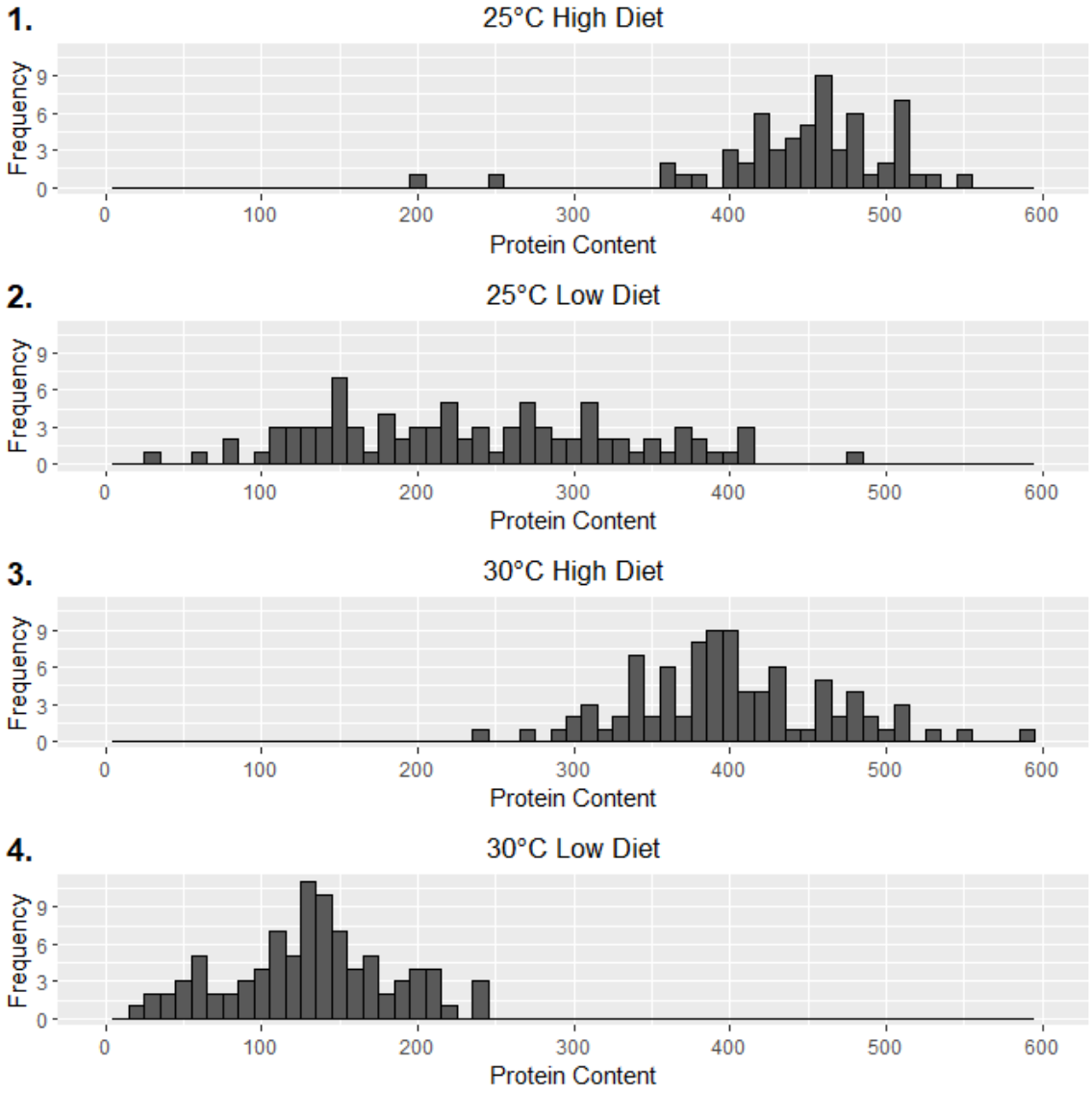

Figure 15. Total protein content frequency distributions for female mosquitoes from 4 development time experiments. Protein content units are micrograms.

Table 9. Summary of protein content data for male mosquitoes from 4 development time experiments. Protein content units are micrograms.

Experiment	N	Mean	S.E.	Minimum	Maximum	C.V.
25°C High Diet	61	265	3.73	192	344	11%
25°C Low Diet	90	124	4.01	57.3	232	30.7%
30°C High Diet	90	228	3.63	127	336	15.1%
30°C Low Diet	159	88.6	2.35	18	163	33.4%

Table 10. Summary of protein content data for female mosquitoes from 4 development time experiments. Protein content units are micrograms.

Experiment	N	Mean	S.E.	Minimum	Maximum	C.V.
25°C High Diet	60	447	7.65	197	545.6	13.2%
25°C Low Diet	90	235	10.1	26.7	476	40.9%
30°C High Diet	90	401	6.75	244	594	16%
30°C Low Diet	90	132	5.41	23.4	244	38.9%

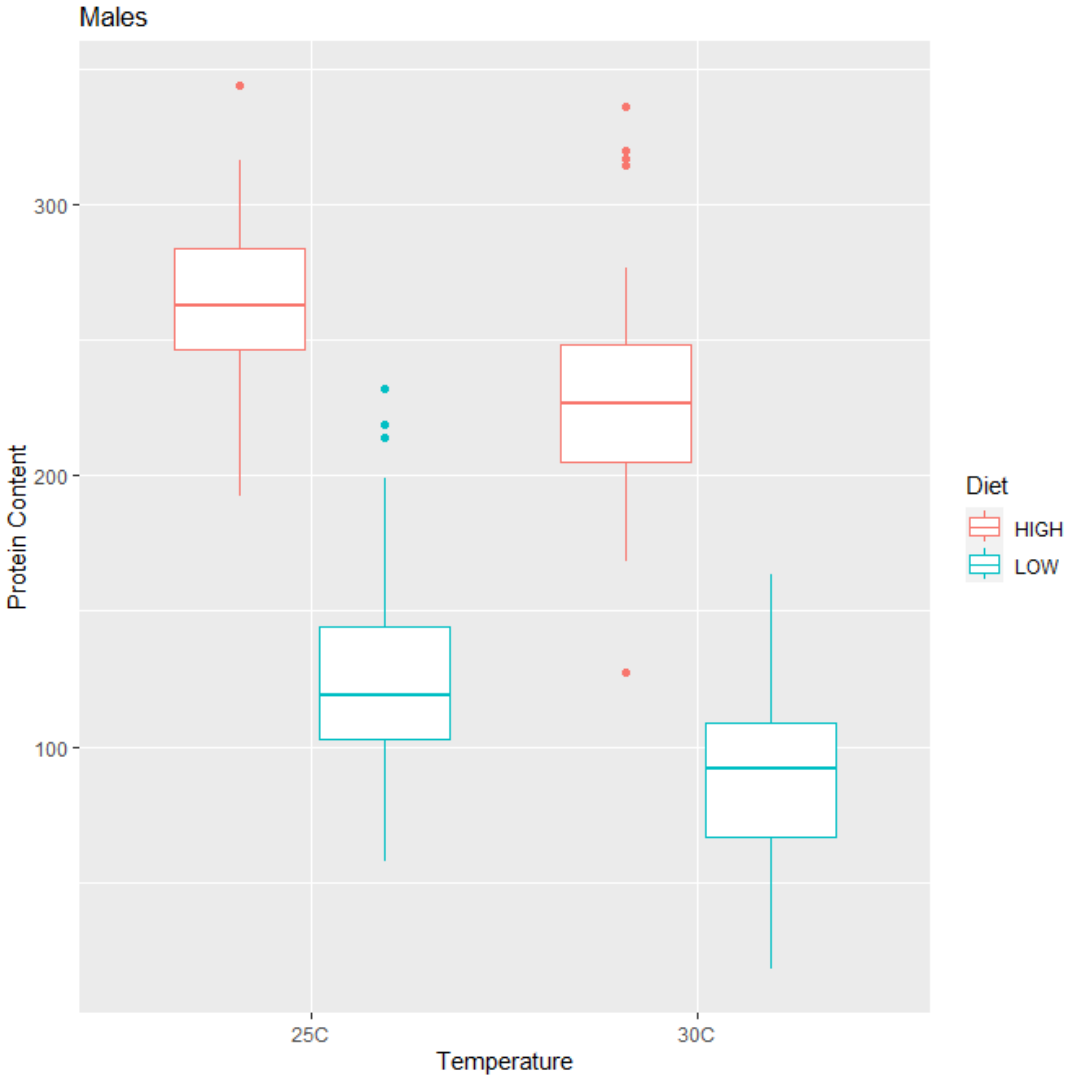

Figure 16. Distribution of male mosquito protein content in each experiment. Each box represents the Interquartile range with the horizontal line representing the median and each dot represents an outlier. Protein content units are micrograms.

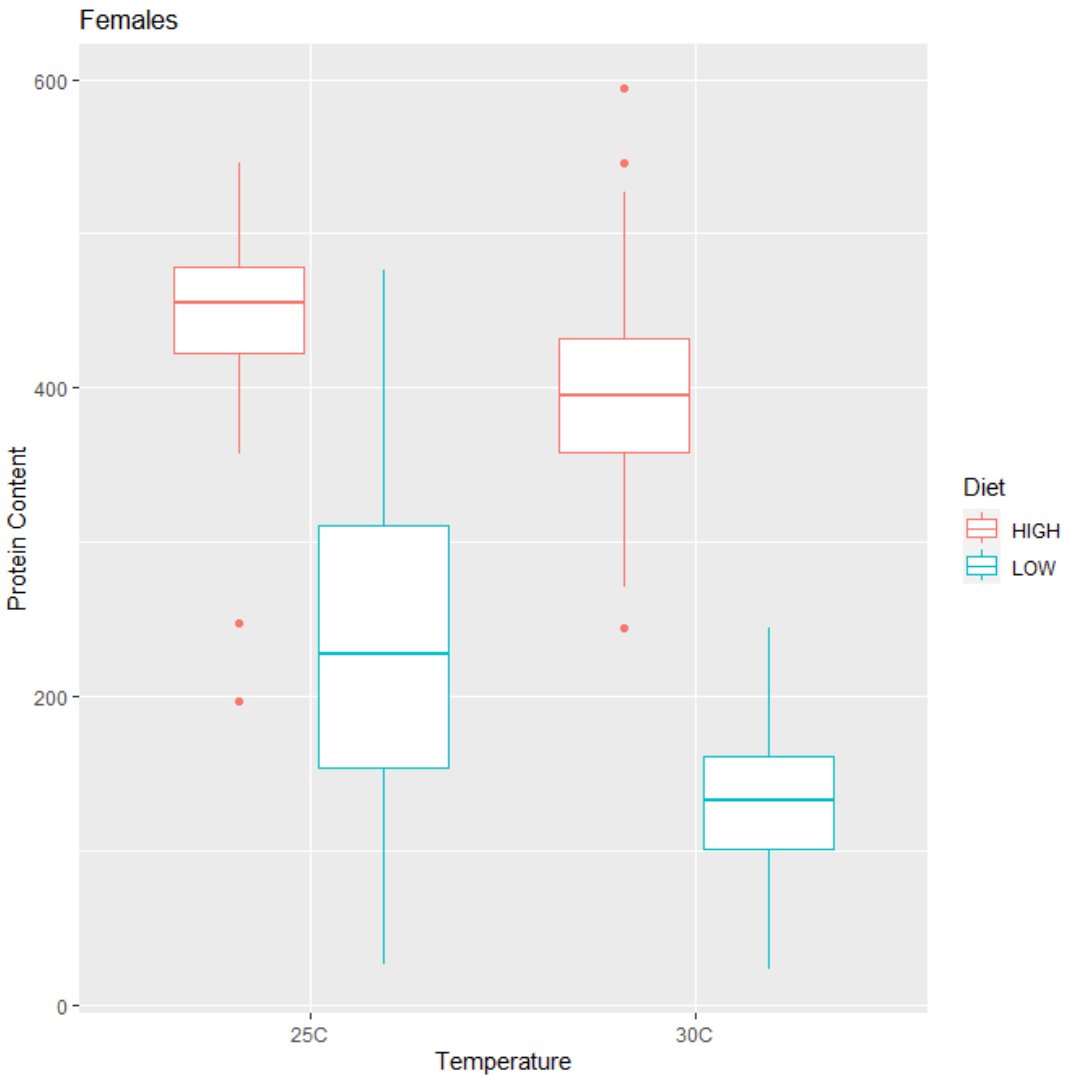

Figure 17. Distribution of female mosquito protein content in each experiment. Each box represents the Interquartile range with the horizontal line representing the median and each dot represents an outlier. Protein content units are micrograms.

There was evidence of mild non-normality but no evidence for unequal variances among the distributions of protein content in males. It has been shown that under mild non-normality and large sample sizes the ANOVA is still robust enough to use (Zar, 1996). A two-way ANOVA was performed to analyze the effect of diet and temperature on male protein content. A two-way ANOVA revealed that there was not a significant interaction between the effects of diet and temperature (interaction: $F_{1,396} = 0.081$, $P = 0.776$, Figure 15). There was a significant effect of diet ($F_{1,396} = 1772$, $P < 0.001$, Figure 18) and temperature ($F_{1,396} = 111.9$, $P < 0.001$, Figure 18) on male protein content.). Pairwise comparisons of esterase activity between each experiment revealed a significant difference between all combinations (Table 11). Both higher temperature and low diet had a negative effect on the average protein content.

There was evidence of non-normality and unequal variance among the distributions of protein content in females. Non-parametric Welch's ANOVA was used to determine if diet or temperature had a significant effect on protein content. Individual Welch's ANOVAs were performed for each of the four experiments. Temperature had a significant effect on protein content under both the High diet regime ($F_{1,133} = 20.8$, $P < 0.001$, Figure 19a) and the Low diet regime ($F_{1,136} = 81.2$, $P < 0.001$, Figure 19a). Diet had a significant effect on protein content at 25°C ($F_{1,147} = 280$, $P < 0.001$, Figure 19b) and at 30°C ($F_{1,170} = 968$, $P < 0.001$, Figure 19b).

A two-way ANOVA was performed to analyze the effect of diet and temperature on protein content. A two-way ANOVA revealed that there was a significant interaction between the effects of diet and temperature (interaction: $F_{1,326} = 12.96$, $P < 0.001$, Figure 17). Pairwise comparisons of esterase activity between each experiment revealed a significant difference between all combinations (Table 12). The results for females paralleled the results for males. Both higher temperature and low food diet had a negative effect on the average protein content.

Table 11. Results of the Tukey's test, comparing male protein content of each diet and temperature combination.

Pairwise Comparison	p-adjusted
25°C High Diet - 30°C High Diet	< 0.001
25°C High Diet - 25°C Low Diet	< 0.001
25°C High Diet - 30°C Low Diet	< 0.001
30°C High Diet – 25°C Low Diet	< 0.001
30°C High Diet - 30°C Low Diet	< 0.001
25°C Low Diet - 30°C Low Diet	< 0.001

Table 12. Results of the Tukey's test, comparing female protein content of each diet and temperature combination.

Pairwise Comparison	p-adjusted
25°C High Diet - 30°C High Diet	< 0.001
25°C High Diet - 25°C Low Diet	< 0.001
25°C High Diet - 30°C Low Diet	< 0.001
30°C High Diet – 25°C Low Diet	< 0.001
30°C High Diet - 30°C Low Diet	< 0.001
25°C Low Diet - 30°C Low Diet	< 0.001

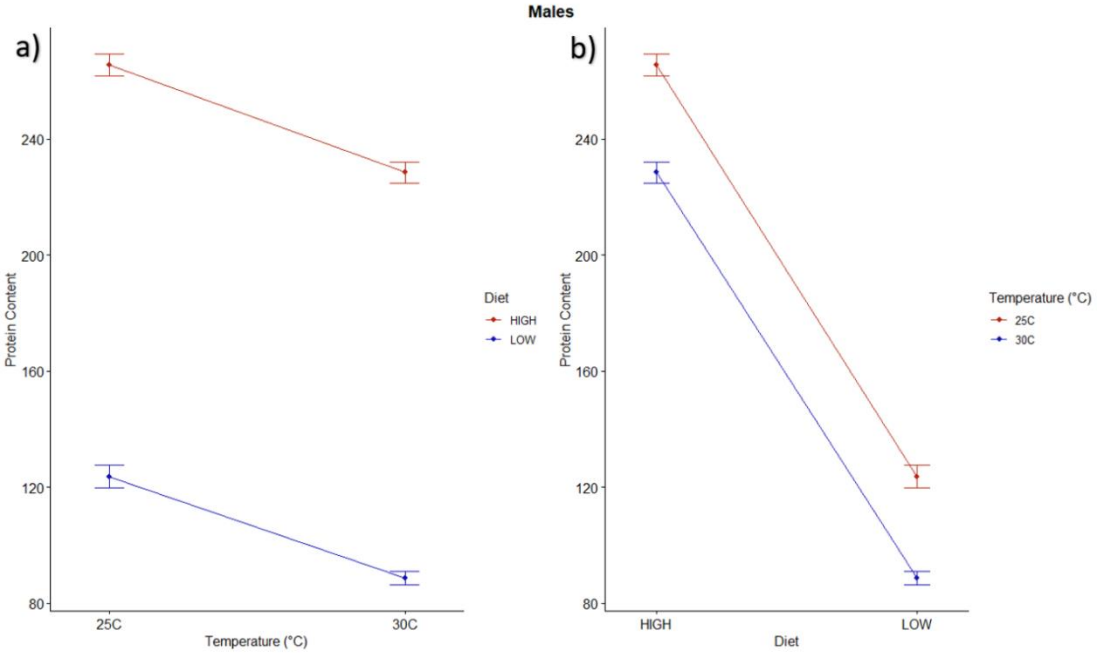

Figure 18: Interaction plot comparing mean protein content in male mosquitoes for each experiment. Error bars represent mean +/- S.E.

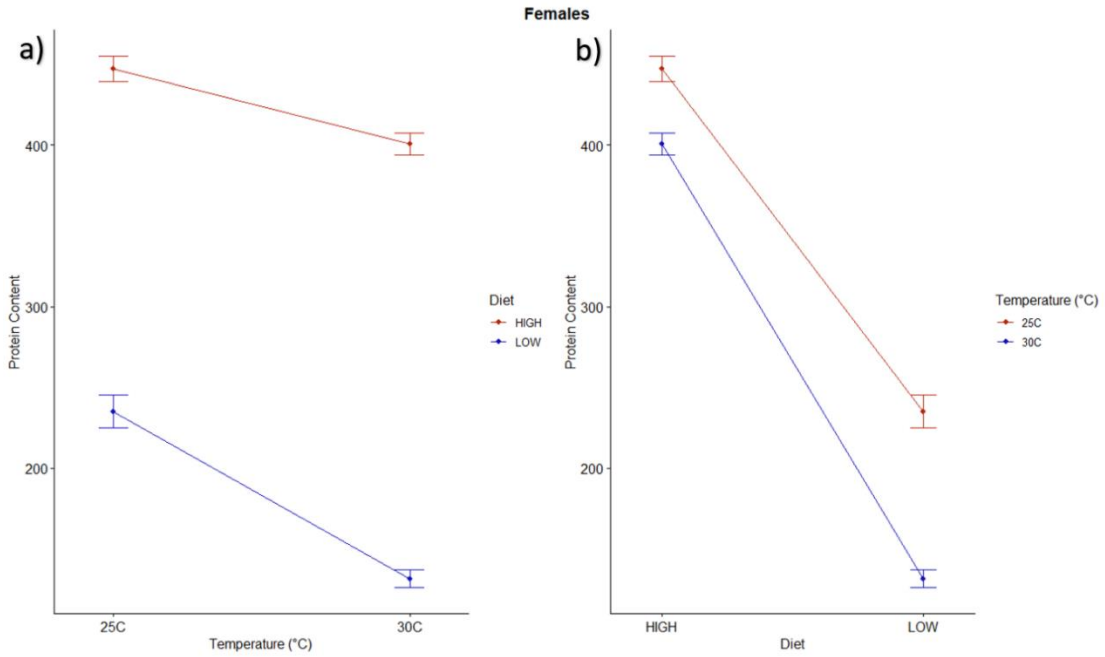

Figure 19: Interaction plot comparing mean protein content in female mosquitoes for each experiment. Error bars represent mean +/- S.E.

Relationships Between Esterase Activity and Development Time

Linear regression was used to test if there was a significant relationship between esterase activity and development time in males. There was a significant negative relationship between esterase activity and development time in the 25°C High diet experiment ($F_{1,59} = 7.86$, $p = 0.007$, $R^2 = 0.118$, Figure 20.1) indicating that males with higher esterase activity tended to develop faster than males with lower esterase activity. None of the other regressions of development time on esterase activity were significant: 25°C Low diet ($F_{1,88} = 0.214$, $p = 0.645$,

R^2 = 0.002, Figure 20.2); 30°C High diet ($F_{1,88}$ = 0.006, p = 0.94, R^2 < 0.001, Figure 20.3); 30°C Low diet ($F_{1,157}$ = 0.359, p = 0.550, R^2 = 0.002, Figure 20.4).

As was observed for males there was also a significant negative relationship between esterase activity and development time in the 25°C High diet experiment in females ($F_{1,58}$ = 9.13, p = 0.005, R^2 = 0.136, Figure 21.1). The results for females in the other experiments also followed the same pattern observed for males. None of the other regressions of development time on esterase activity were significant: 25°C Low diet ($F_{1,88}$ = 0.472, p = 0.494, R^2 = 0.005, Figure 21.2); 30°C High diet ($F_{1,88}$ = 2.30, p = 0.133, R^2 = 0.025 Figure 21.3); 30°C Low diet ($F_{1,88}$ = 1.97, p = 0.164, R^2 = 0.022, Figure 21.4).

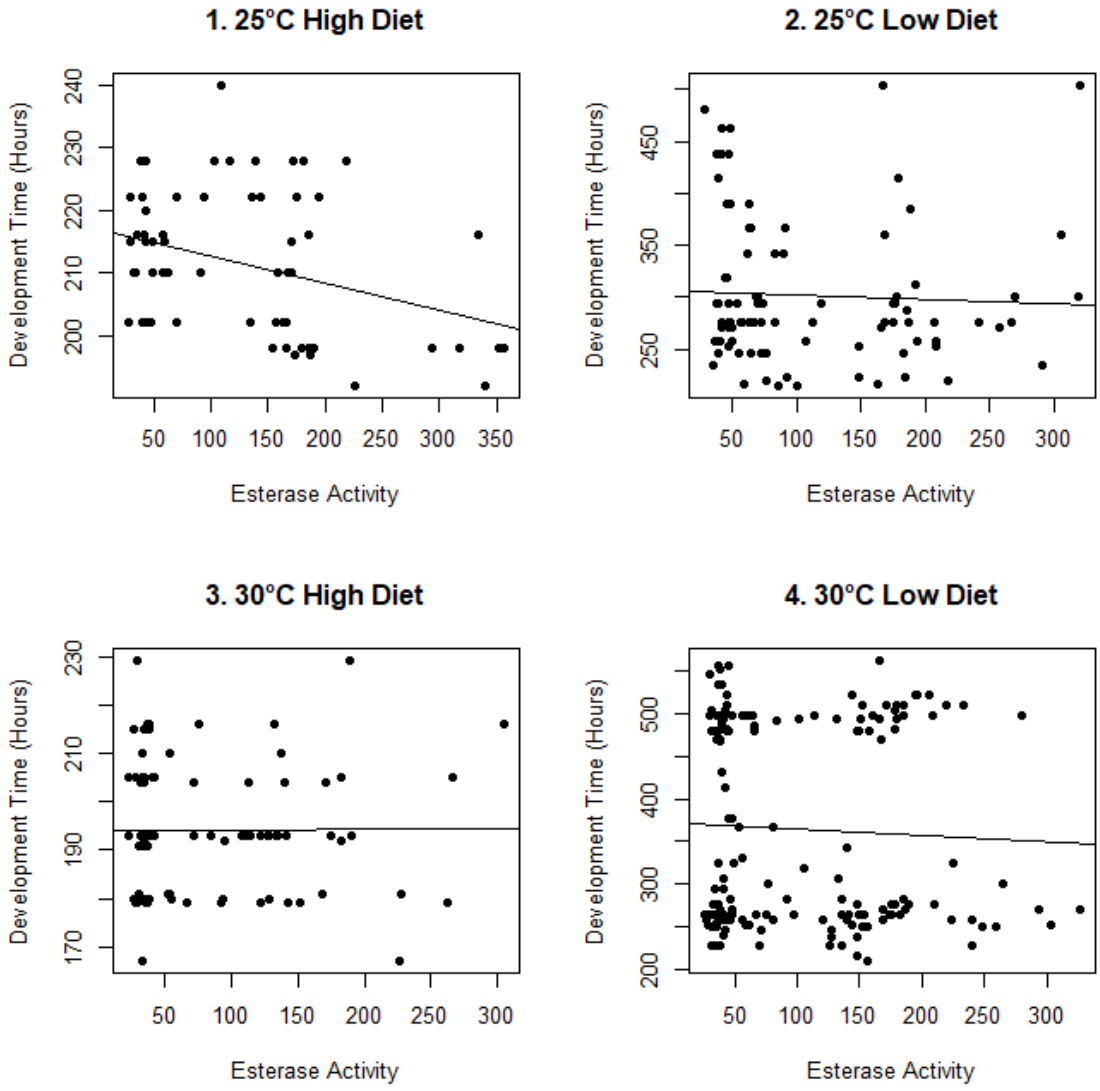

Figure 20. The relationship between esterase activity and development time for male mosquitoes.

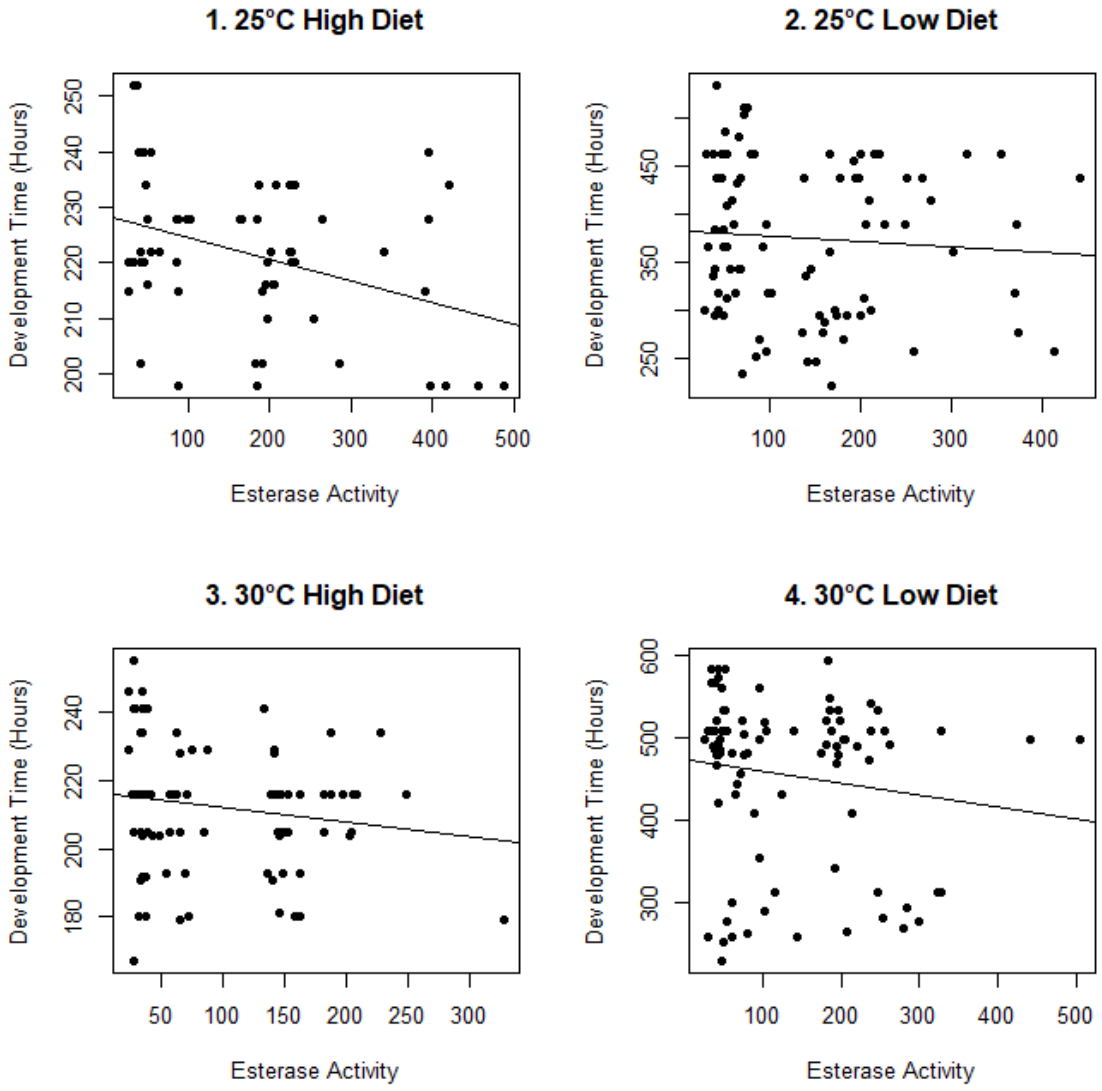

Figure 21. The relationship between esterase activity and development time for female mosquitoes.

Relationships Between Esterase Activity and Protein Content

Linear regression was used to test if there was a significant relationship between esterase activity and protein content for individuals reared under the four experimental conditions. For males no significant relationship between esterase activity and protein content was observed in any of the experiments (Table 13).

The results of the regression analyses for females in the four experiments were notably different than the results for males (Table 14). The slopes of the regression lines were negative in all four of the experiments (Figure 23). There was a significant negative relationship between esterase activity and protein content in the 30°C Low diet experiment. While the result of the regression analyses in the other three experiments was not significant, the p-values were < 0.1 in two of the experiments and < 0.2 in the third experiment (Table 14).

Table 13. Summary of regression analysis results between esterase activity and protein content in males.

Experiment	F-value	P-value	R^2
25°C High Diet	$F_{1,59} = 0.077$	0.782	0.001
25°C Low Diet	$F_{1,88} = 1.59$	0.211	0.018
30°C High Diet	$F_{1,88} = 2.30$	0.133	0.025
30°C Low Diet	$F_{1,157} = 0.699$	0.404	0.004

Table 14. Summary of regression analysis results between esterase activity and protein content in females.

Experiment	F-value	P-value	R^2
25°C High Diet	$F_{1,58} = 3.25$	0.077	0.053
25°C Low Diet	$F_{1,88} = 1.74$	0.19	0.019
30°C High Diet	$F_{1,88} = 2.96$	0.089	0.033
30°C Low Diet	$F_{1,88} = 11.41$	0.001	0.115

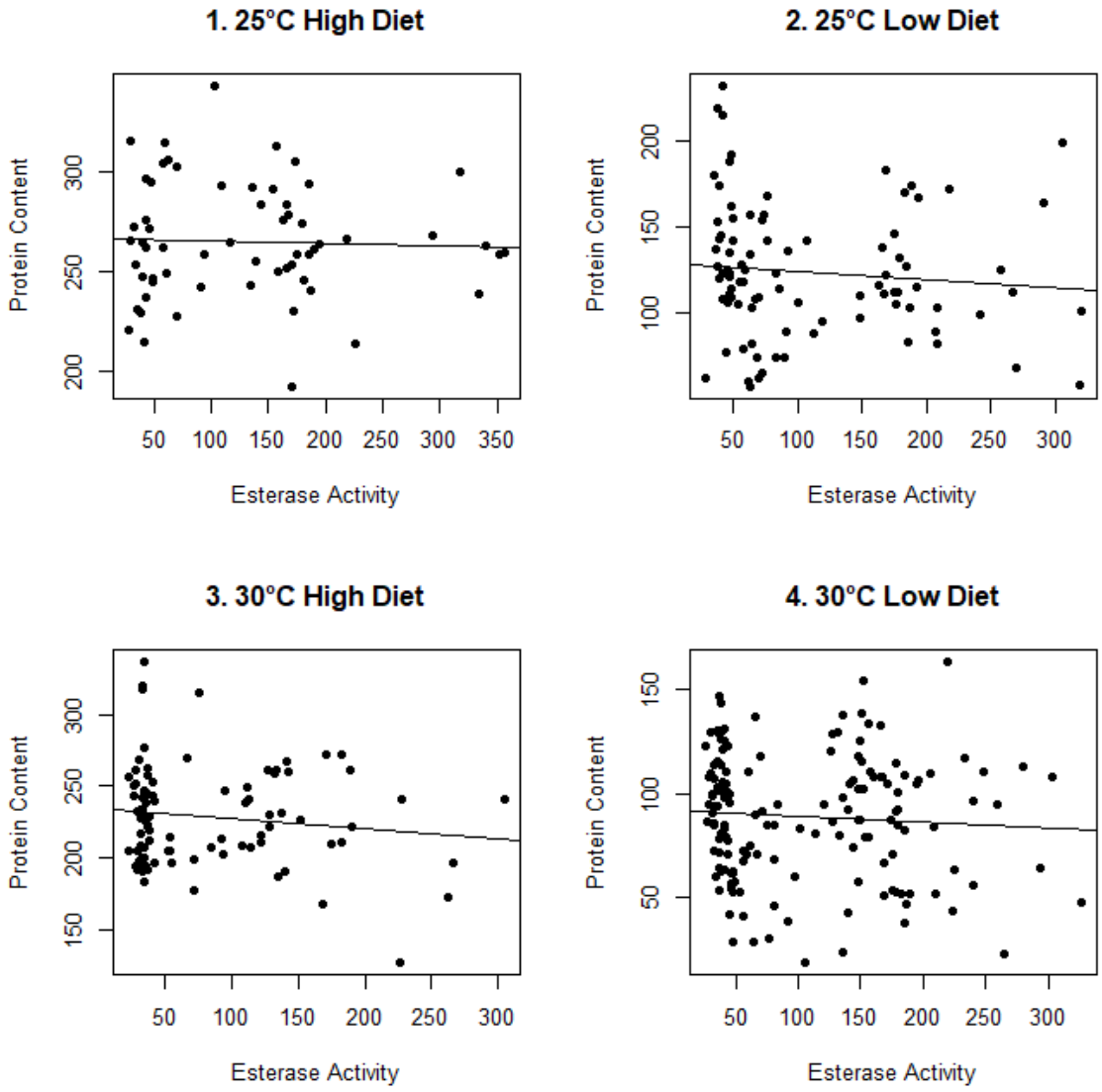

Figure 22. The relationship between esterase activity and protein content for male mosquitoes.

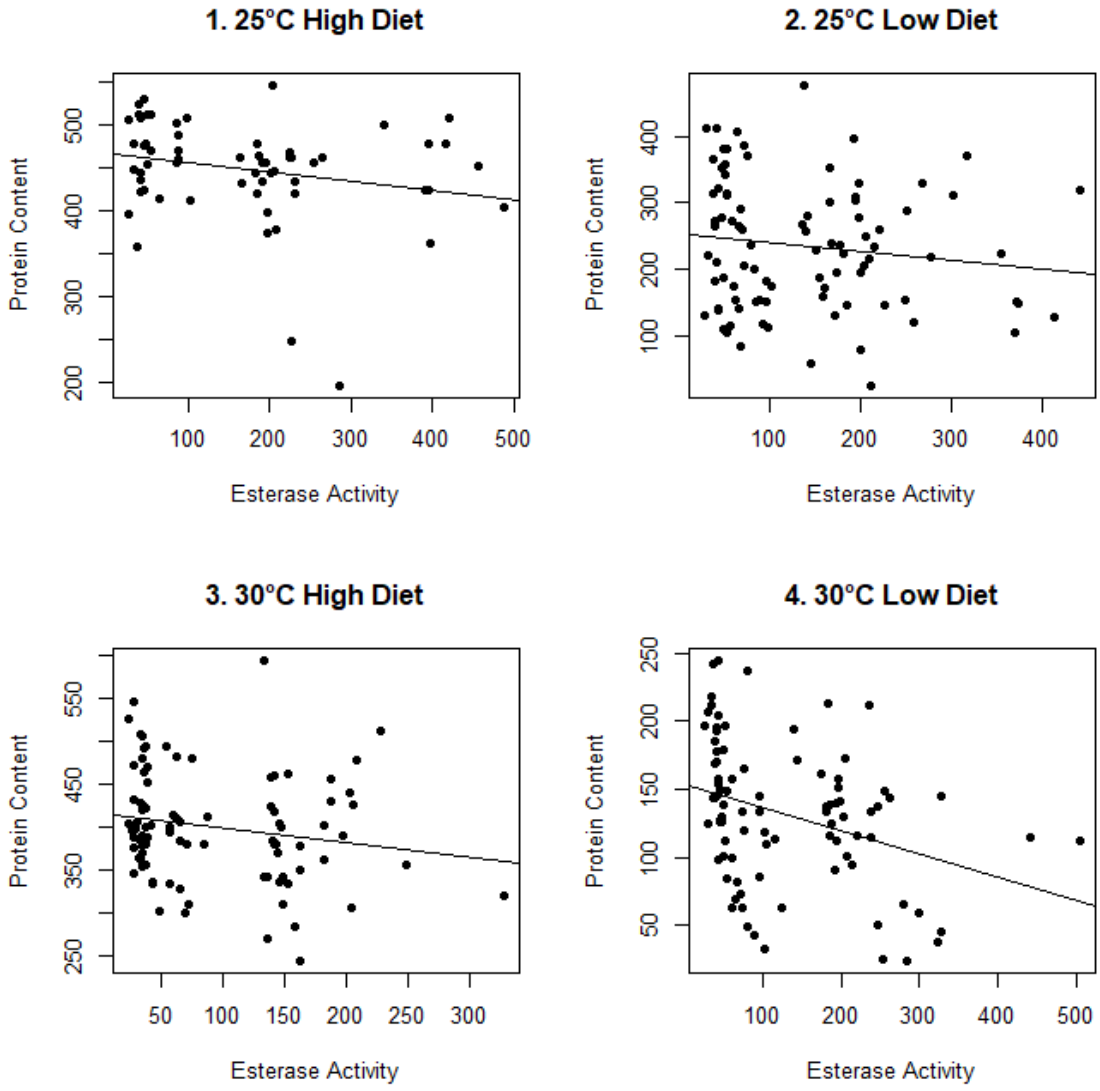

Figure 23. The relationship between esterase activity and protein content for female mosquitoes.

Relationships Between Development Time and Protein Content

Linear regression was used to test if there was a significant relationship between development time and protein content in males. Significant positive relationships between development time and protein content were observed in the 30°C High diet experiment ($F_{1,88} = 18.8$, $p < 0.001$, $R^2 = 0.176$, Figure 24.3) and in the 30°C Low diet experiment ($F_{1,157} = 34.56$, $p < 0.001$, $R^2 = 0.18$, Figure 24.4). The relationships between development time and protein content were not significant in the 25°C High diet experiment ($F_{1,59} = 0.071$, $p = 0.791$, $R^2 = 0.001$, Figure 24.1) or in the 25°C Low diet experiment ($F_{1,88} = 2.10$, $p = 0.151$, $R^2 = 0.023$, Figure 24.2).

In females statistically significant positive relationships between development time and protein content were observed in all four experiments. Table 15 summaries the results of the regression analysis.

Table 15. Summary of regression analysis results between development time and protein content in females.

Experiment	F-value	P-value	R^2
25°C High Diet	$F_{1,58} = 5.39$	0.024	0.085
25°C Low Diet	$F_{1,88} = 37.9$	< 0.001	0.301
30°C High Diet	$F_{1,88} = 65.8$	< 0.001	0.428
30°C Low Diet	$F_{1,88} = 59.9$	< 0.001	0.405

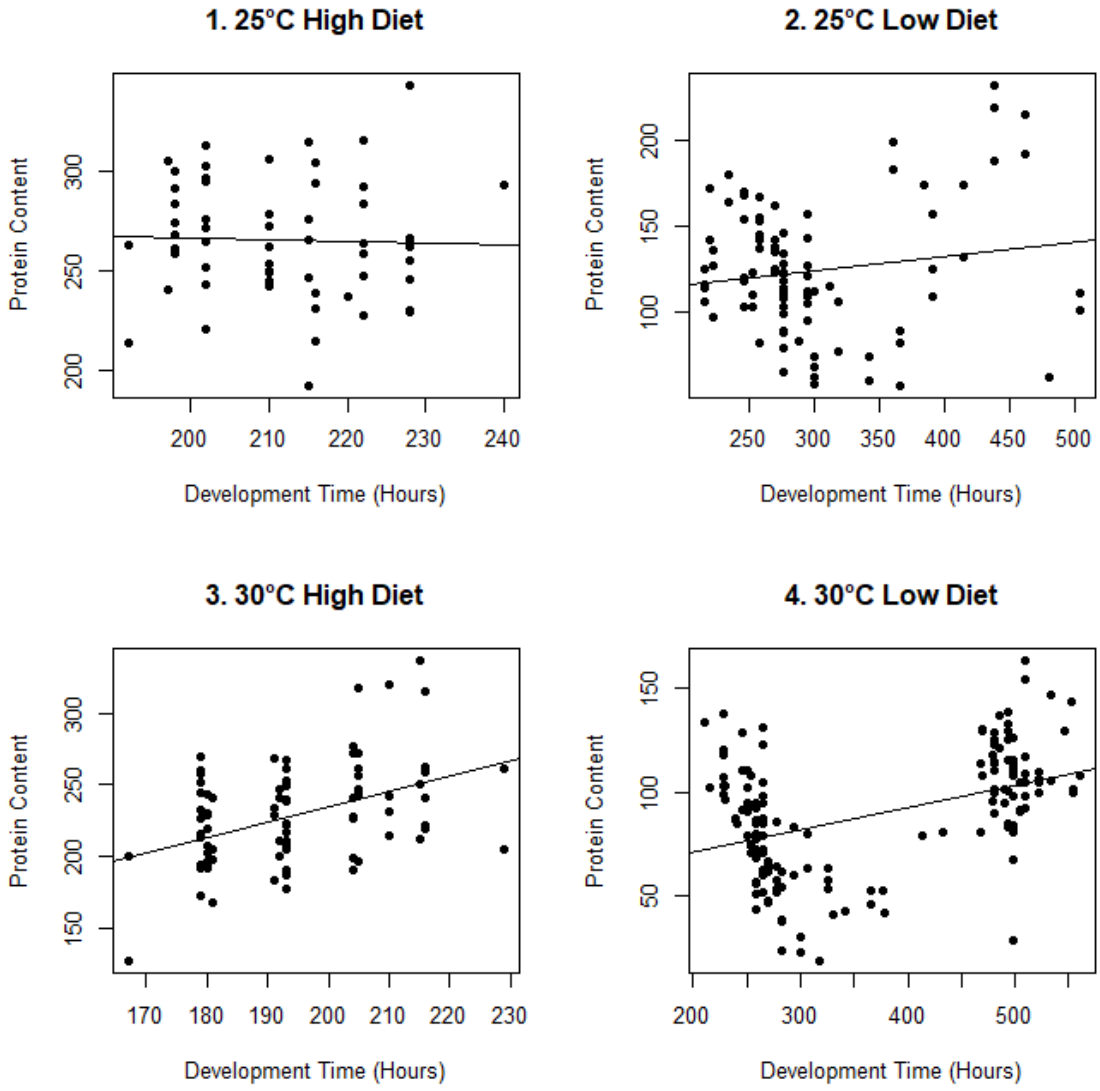

Figure 24. The relationship between development time and protein content for male mosquitoes.

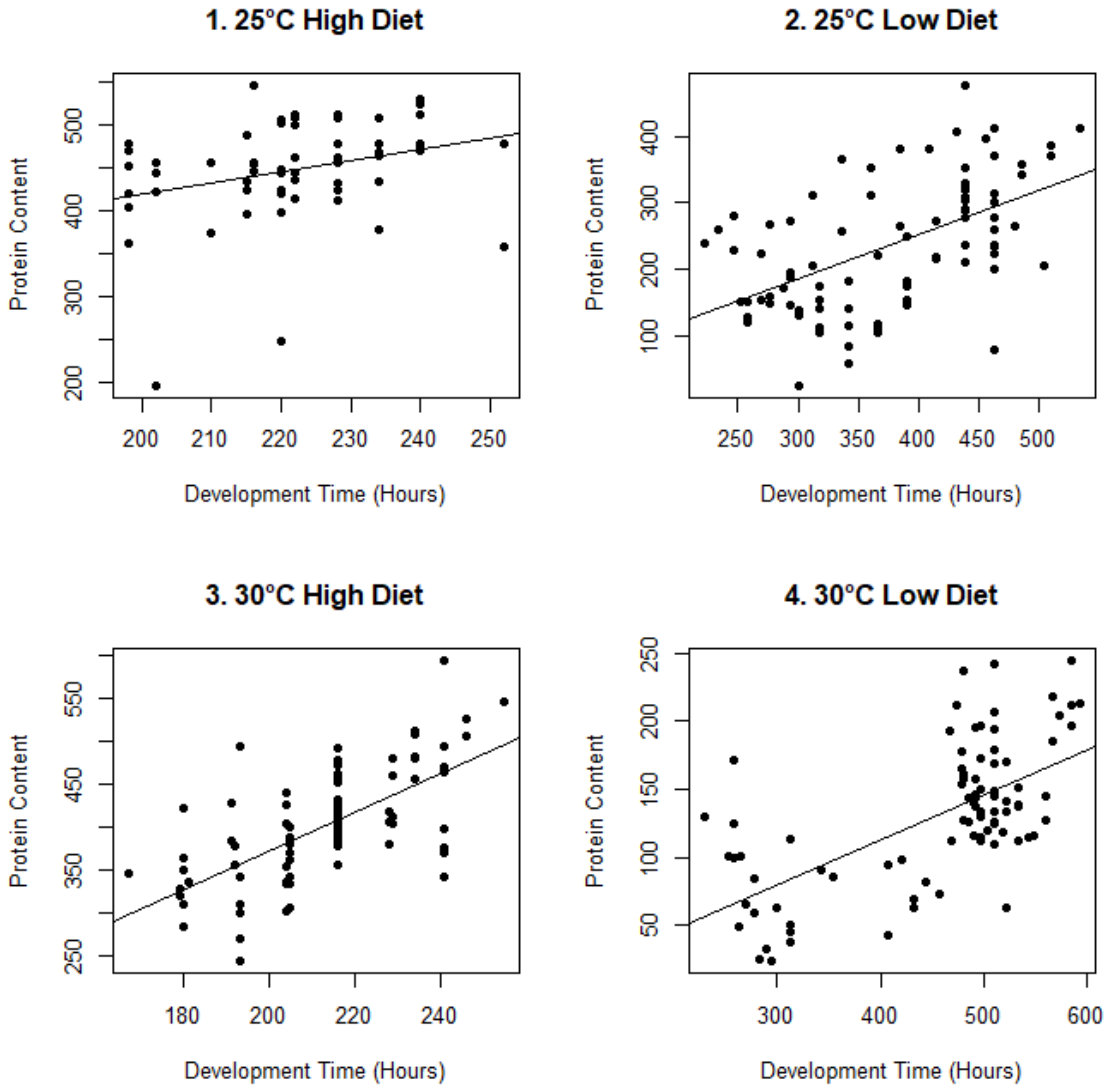

Figure 25. The relationship between development time and protein content for female mosquitoes.

Partial Correlation Analyses

Because of the significant relationships between protein content and development time noted above we wanted to determine if the relationship between protein content and development time might have influenced the relationships of each of these variables to esterase activity in the four experiments (the primary question of the study). Partial correlation analyses were run to determine the relationship between each pair of variables while controlling for the third and compared to the results of each linear regression analysis. In both males and females from all four experiments, the results of the partial correlation analyses matched those for the linear regression analyses, with one exception. For females in the 25°C High diet experiment, the relationship between protein content and development time when controlling for esterase activity was not significant ($r = 0.228$, $n = 60$, $p = 0.082$). This p-value is still below 0.1 and relatively close to the linear regression p-value of 0.024.

Electromorph Frequencies

Electrophoresis was done on males and females from both High diet experiments to identify the electromorph phenotype of each adult that completed development. The A2B2 electromorph had the highest frequency and the B1 electromorph had the lowest. 87% of all individuals tested expressed the A2B2 electromorph, 9% A2B2/B1, and 4% B1 (Figure 26). Considering the low frequency of individuals with the A2B2/B1 and B1 electromorphs in these experiments the results were not analyzed separately for each electromorph

type, and electromorph frequencies were not determined in the other two experiments.

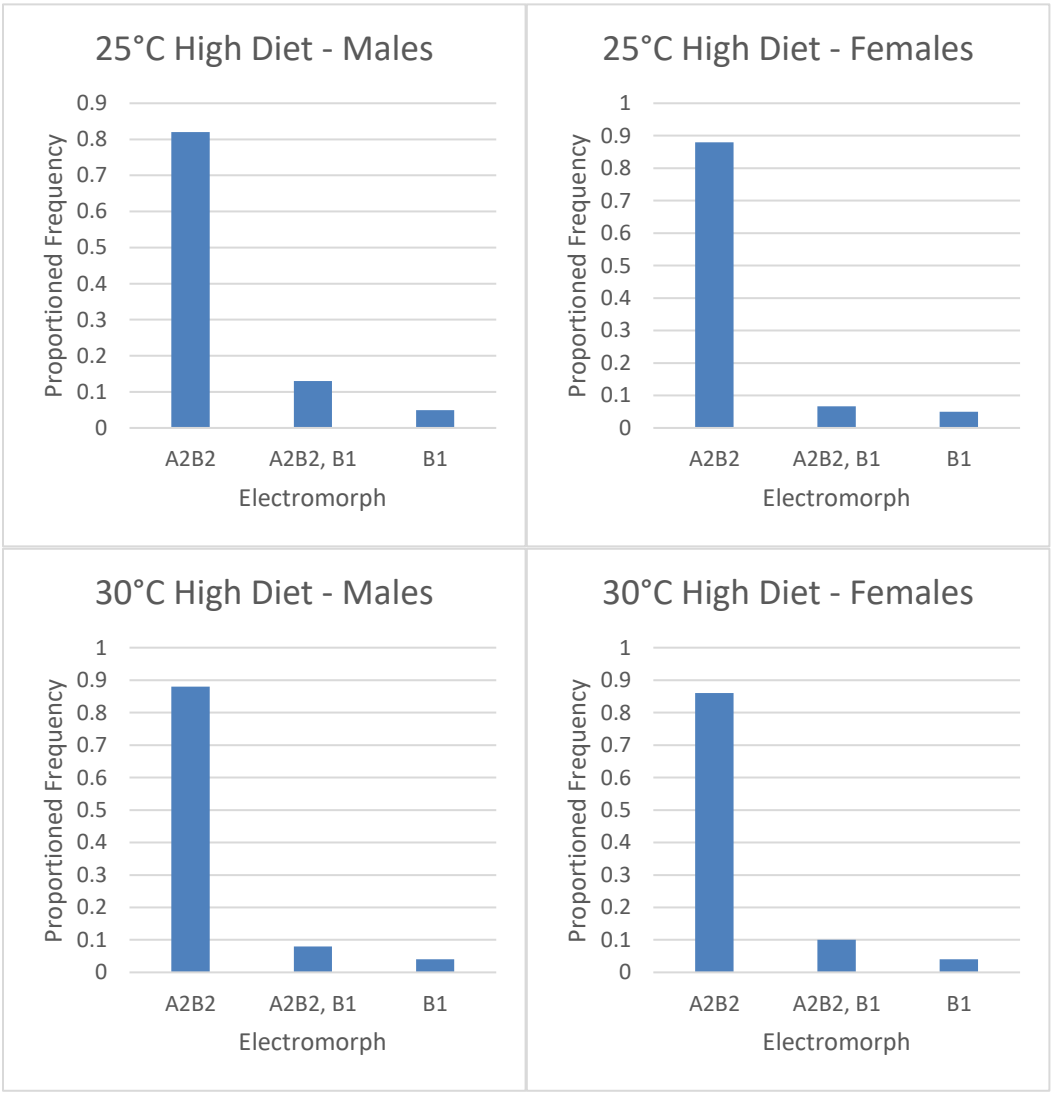

Figure 26. Proportioned frequency distribution of electromorphs.

CHAPTER FOUR

DISCUSSION

Ffrench-Constant and Bass (2017) outlined the components necessary for effective studies of potential fitness costs associated with resistance to insecticides (see Introduction). These authors admit that no study to date includes all these components, however the present study includes most of them. The Canyon Crest strain was established from a field collection of a substantial number of individuals so that the different genotypes from the natural population are represented. Preliminary work established that the strain exhibited a continuous range of esterase activities among individuals, and a substantial proportion of individuals in the strain exhibited esterase activities similar to the mean esterase activity of a standard laboratory susceptible strain. Some individuals in the CC strain had esterase activities 8-10X higher than the mean for susceptible strain individuals. It was therefore possible to examine the relationship between a continuous range esterase activity phenotypes and quantitative phenotypes associated with fitness among individuals with the same natural genetic background. Finally, in the present study individuals with different esterase activity phenotypes associated with different levels of resistance to insecticides competed directly against one another. The one component not fulfilled in the present study was that individuals did not compete in their natural field environment. This is certainly the most difficult component to incorporate into studies of the effects of different phenotypes on fitness, and is a long-

standing problem in the study of adaptive traits. Nevertheless, the combinations of temperatures and diets used in the present study did accomplish the goal of creating environmental conditions that seemed to increase the stress on developing larvae. Direct effects of sub-optimal temperature and diet, and the interaction of these factors with competition among individuals, affected the mean development time and protein content and resulted in increased variation in protein content (a measure of body size) at the adult stage and development time among individuals.

Reduction in mean adult protein content, an increase in mean development time, and an increase in the coefficient of variation of these measures were used as indicators of increased stress on developing larvae. The 25°C High Diet experiment represents the least stressful conditions while the 30°C Low Diet experiment represents the most stressful conditions. The mean development time in the 30°C Low Diet experiment was 73% longer in males and 105% longer in females than the 25°C High Diet experiment (Tables 3 & 4). The coefficient of variation of development time in the 30°C Low Diet experiment was 26.7% larger in males and 15.7% larger in females than the 25°C High Diet experiment (Tables 3 & 4). Both temperature and diet had a significant effect on mean development time in both sexes. The mean protein content in the 30°C Low Diet experiment was 67% less in males and 70% less in females than the 25°C High Diet experiment (Tables 9 & 10). The coefficient of variation of protein content in the 30°C Low Diet experiment was 22.4% larger in males and 25.7%

larger in females than in the 25°C High Diet experiment (Tables 9 & 10). Temperature and diet had a significant effect on development time in both sexes. Individuals in the 30°C Low Diet experiment had the lowest mean protein content and took longest to develop. Overall, the different experiments appear to be successful in imposing a physical stress on the developing larvae. The hope was that these stressors would increase competition among individuals with different esterase activity phenotypes. If there is an association between the potential fitness costs of different esterase activity phenotypes and phenotypes for traits likely associated with fitness, then it should be easier to detect under enhanced competition.

The primary focus of the study was to determine if there were trade-offs between esterase activity level and development time, and esterase activity and protein content (body size). The assumption is that for a multivoltine insect species, more rapid development time and larger body size would be associated with higher fitness. For females there were significant positive relationships between development time and protein content in all four experiments. Thus, there appears to be a trade-off between the phenotypes presumed to be related to higher fitness. More rapid development results in smaller individuals under most conditions.

The only experiment where there was a significant relationship between esterase activity and development time was the 25°C High diet experiment in both sexes. This experiment represents the least stressful conditions.

Mosquitoes reared in the 25°C High diet experiment also had the highest average esterase levels among all 4 experiments (Tables 5 & 6). Interestingly, in these conditions, mosquitoes with relatively higher esterase activity developed faster. In all other experiments this relationship was not seen. This could mean that under the normal rearing conditions, mosquitoes with higher esterase levels had an advantage but under more stressful conditions this advantage disappeared. We have no idea whether this apparent advantage would occur under field conditions, however the possibility that individuals with higher esterase activity could have a fitness advantage under certain environmental conditions in the absence of insecticide treatment further complicates models of the dynamics of esterase-based resistance.

The only experiment where there was a significant negative relationship between esterase activity and protein content was the 30°C Low diet experiment in females. Mean protein content among females in this experiment was only about a third that of females in the high diet experiments. In this case female mosquitoes with relatively higher esterase activity tend to be smaller, suggesting a fitness trade-off. Though not statistically significant, negative relationships between esterase activity and protein content were observed for females in the other three experiments as well. In both High diet experiments p-values were less than 0.1, and in the 25°C Low diet experiment the p-value was less than 0.2. Females naturally need more resources to develop since female adults are larger than males and need to spend energy on egg production. Additionally, females

take longer to develop than males. If there is an energy cost to increased esterase production, it would likely first appear in females. This is supported by the results of the pilot development time study described earlier. The only adults to emerge in the 25% diet experiment were males. The larvae were no longer fed after 7 days which resulted in high mortality. The female larvae did not receive enough food to fully develop. If the same is happening in the current study, the added competition from males could have affected the female larvae with high esterase levels more.

In conclusion, the only apparent trade-off to high esterase levels is seen in females. Females with higher esterase activity tend to have a smaller body size. This is likely due to competition and is mainly apparent in the most stressful conditions. This could be due to the need to allocate more nutritional resources to producing esterases, or to some other indirect effect of higher esterase activity on development. This can be quite a significant fitness cost since female body size can have a significant impact on egg production. Several studies have reported survival rate, blood-feeding success, fecundity, and bloodmeal size all increase with larger body size of females from several different mosquito species (Ameneshewa & Service, 1996).

It is important to note that this trade off was observed over relatively narrow ranges of esterase activities. Esterase activity ranges were similar in all four experiments, but the ranges were somewhat higher for females, which exhibited a mean range of 17.7-fold, than for males, which exhibited a mean

range of 12.6-fold (Tables 5 & 6). Individuals from a highly resistant laboratory strain subjected to selection with insecticide each generation over a number of years exhibited a mean esterase activity 120-fold higher than the mean esterase activity of a standard susceptible strain (Ferrari & Georghiou 1990). Esterase activities of this magnitude have not been observed in field populations of Culex, and the mean and range of esterase activity in the CC strain is comparable to values observed in Riverside-San Bernardino populations in the early 1990's and early 2000's (Milligan 1995, Spier unfinished Master's thesis CSUSB).

The evolutionary history of the B1 and A2-B2 esterase electromorphs in California may also help explain their current effects on fitness. In Southern California, the active B1 electromorph was first detected in the mid-1970's and must have been present early in the decade (Georghiou & Pasteur 1978). The active A2-B2 electromorph was observed at low frequency in the Riverside area in 1985, when the B1 electromorph was the predominant active esterase in the area (Raymond et al. 1987). Since the appearance of the A2-B2 electromorph Est B1, has declined in frequency while another esterase, A2-B2, has increased. Samples from a Riverside field population in 1987 found 56.6% individuals assayed had esterase B1, 1.0% had A2-B2, and 1.8% had A2-B2,B1 (Raymond et. al., 1987). In 1993, field samples that expressed at least one of these three allozymes from Riverside showed 44.7% had B1, 26.1% had A2-B2, and 29.2% had A2-B2,B1 (Milligan, 1995). Lab reared Canyon Crest individuals collected in 2015 showed 24% had B1, 66% had A2-B2, and 10% had A2-B2,B1. The

Canyon Crest individuals from this experiment showed 4% had B1, 87% had A2-B2, and 9% A2-B2,B1, demonstrating the frequency of B1 also declined under lab rearing. A similar pattern of allelic replacement has been well documented in resistant *Culex pipiens* populations in southern France, where the frequency of the once common esterase A1 electromorph has declined while the A4-B4 electromorph has increased (Guillemaud et. al., 1998). In Southern California the allelic replacement of B1 by A2-B2 has occurred during a period when direct use of OP insecticides for larval mosquito control has been greatly reduced in favor of bacterial insecticides such a *Bacillus thuringiensis israelensis*. While it is possible that OP insecticides being used for other purposes may be inadvertently introduced into mosquito breeding sites, the selection pressure on esterase genes has probably been reduced considerably. This, coupled with the observed decline of B1 in lab strains reared without insecticidal selection, suggests that individuals carrying the B1 esterase have lower fitness than individuals carrying A2-B2. In our experiments very few individuals had the B1 esterase, making any fitness effects due predominantly to esterase activity variation in A2-B2.

Another complicating factor is the possible evolution of fitness modifiers that reduce the adverse fitness trade-offs that might be associated with resistance to insecticides. Studies have demonstrated that modifier genes can reduce adverse fitness affects over time (Ffrench-Constant and Bass, 2017). The peach potato aphid (*Myzus persicae*) is able to switch on and off the expression of their amplified E4 esterase genes that confer resistance. When insecticides

are not present, they stop overproducing esterase E4 but can turn it back on when exposed to insecticides. Another well documented case is in the Australian sheep blowfly, *Lucilia cuprina*. Mckenzie (1994) showed overwintering diazinon-resistant blowflies have greater mortality than susceptible blowflies. However, when resistant blowflies have a fitness modifier their mortality is equivalent to susceptible blowflies. The possible existence of fitness modifiers associated with esterases in *Culex* have not been investigated, but the conditions favorable to the evolution of fitness modification have existed for at least the past three decades during which OP insecticides have not been widely used to control larval stage populations.

A number of approaches have been used to investigate the potential trade-offs between elevated esterase activity and phenotypic traits with potential effects on fitness. A unique feature of the present study is that it represents the first attempt to relate quantitative variation in esterase activity among individuals with variation in phenotypic traits that have the potential to affect fitness. Most previous studies have treated all individuals with a particular esterase allozyme pattern as equivalent and have not measured esterase activity of individuals. In some cases esterase activities of individuals were measured when characterizing a strain, but potential fitness trait comparisons were done between strains that differed in mean esterase activity, not among individuals in the same strain that differed in esterase activity.

Rivero et. al. (2011) compared the levels of stored energy resources (lipids and sugars) in a susceptible strain with levels in two strains exhibiting elevated esterase activity for different esterase allozymes in female *Culex pipiens*. Both strains exhibited about 7-8-fold higher mean esterase activity then the susceptible strain and a range of esterase activities among individuals. While the strain with the A4-B4 esterase allozyme exhibited about 30% lower total energy reserves than the susceptible strain, the strain with the B1 esterase allozyme did not differ significantly from the susceptible strain in the level of energy reserves. While esterase activity was measured on individual mosquitoes from each strain, the levels of lipids and sugars was measured on pools of individuals, so potential relationships between esterase activity and levels of energy resources was not investigated. While the authors did not do a statistical analysis of larval development time and mortality among the strains used, they noted that the percentage of larvae that completed development was lower in the strain with the B1 esterase (54% compared with 75-80% for the other strains) and that the proportion of males was higher (70% compared with 45-50% for the other strains).

Gazave et. al. (2001) compared the frequency of females with or without active esterase allozymes in *Culex pipiens* overwintering in a French cave during two time periods. The first period was comprised of the first 50 sampling days and the second period was comprised of the remaining 144 days. The esterase allozymes present in the cave population were A1, A2-B2, and A4-B4. The

individuals from the first period represented the first arrivals at the beginning of the overwintering period. The authors speculated that individuals arriving during the second period represented mosquitoes migrating from other overwintering sites in search of a more suitable site. There was a significant decrease in the frequency of mosquitoes exhibiting elevated esterase activity during the second period. The authors speculated that the lower frequency of individuals with active esterase allozymes arriving later was due to a survival cost for mosquitoes with elevated esterase activity. They believed that resistant individuals were more likely to die in an original overwintering site with poor conditions or they were not able to survive the migration to the new cave, but other explanations are possible.

In some cases, resistance to insecticides has been shown to have no fitness cost and even provide an advantage when insecticides are not present. McCart et. al. (2005) compared egg, larval and pupal viability, and the number of eggs laid of a DDT resistant strain and a susceptible strain of *Drosophila melanogaster* at two different temperatures. To make the genetic backgrounds of the strains similar the resistant strain was backcrossed with the susceptible strain for five generations before measurements were made. For all measures homozygous resistant flies were significantly superior to susceptible flies. Additionally, heterozygous flies where resistance was inherited from the female had higher viability and development rate in larvae and pupae than heterozygotes where resistance was inherited from the male. However, the

development rate advantage was not apparent at the higher temperature. This study suggests that insecticide resistance can be associated with higher fitness even when the insecticide is not present, and that maternal effects may affect the relationship between resistance and fitness. Smith et. al. (2011) investigated this further by comparing pre- and post-copulatory component differences in resistant and susceptible males from two different strains with different genetic backgrounds. To produce pairs of resistant and susceptible strains with different genetic backgrounds the laboratory susceptible Canton-S strain and a wild caught susceptible strain were both backcrossed with a wild caught resistant strain. Reciprocal crosses were then done between the resistant and susceptible strains with the same genetic background. While there were some significant differences between males from the resistant and susceptible strains in competitive mating ability and body size, it was dependent on the strain's genetic background. In one genetic background, resistant males were significantly smaller than susceptible males and only achieved 22% of matings when competing with susceptible males. In the other genetic background resistant males were larger but exhibited no significant difference from susceptible males in mate competition experiments. This illustrates the importance of using individuals with the same genetic background when comparing components of fitness between resistant and susceptible individuals. Fitness costs found in one genetic background might not be found in a different genetic background. It should also be noted that while a number of studies have used repeated

backcrossing to a susceptible strain to place the gene or genes for resistance on the susceptible strain genetic background, this process would eliminate possible fitness modifier genes that existed in the genetic background of the resistant strain.

To discuss the implications of the present study it is helpful to review some aspects of the role of active esterases in resistance to OP insecticides in *Culex quinquefasciatus* that were presented in the Introduction. Since the molecular mechanism of elevated esterase enzyme activity is gene amplification, individuals with the same esterase electromorph have the potential to vary in esterase activity because of differences in the number of esterase gene copies they carry (Ferrari 2015). For tandemly repeated genes that segregate as a block in genetic crosses, variation due to copy-number differences is analogous to variation caused by allelic differences at a locus, with each copy-number class defining a different “allele” (Ferrari & Georghiou 1991). Each copy-number allele can produce a different amount of esterase enzyme, resulting in a different esterase activity phenotype in an enzyme activity assay. Since esterases can detoxify certain OP insecticides by sequestration (Hemingway and Karunaratne 1998), the level of resistance is dependent on the amount of esterase enzyme present. As seen in the present study, and in previous studies, field populations often show a continuous distribution of esterase activities that vary over a fairly wide range (Miligan 1995, Pietrantonio et al. 2000, Ferrari 2015). Ferrari (2015) estimated that there were at least 11 different esterase activity variants (=

esterase activity alleles) represented in a sample of 26 chromosomes carrying *Estβ1* gene amplifications isolated from a field population of *Culex pipiens* (see details in Introduction).

As discussed above, most studies of the potential fitness cost of active esterase allozymes treat all individuals with the same electromorph as a homogeneous class with respect to esterase activity and fitness. Even in studies where esterase activities of samples of individuals are measured, mean values for some components of fitness are usually compared between resistant and susceptible strains that differ in mean esterase activity. My study took a different approach. The main question addressed was whether there is a relationship between the degree of fitness cost and the levels of esterase activity exhibited by individuals within a population. In my experiments I observed a significant negative relationship between esterase activity and protein content for females reared under the most stressful experimental conditions (30°C Low diet). While this relationship was not significant under less stressful conditions, in two of the experiments p-values were less than 0.1, and less than 0.2 in the third experiment. These results support the hypothesis that fitness costs increase with increasing esterase activity in females. As noted above, this relationship was observed over a much narrower range of esterase activities than would likely exist is a population that was under strong insecticidal selection.

As discussed in the Introduction, population genetic models have been widely used to understand the factors affecting the evolution of insecticide

resistance in populations of arthropod pests of agriculture and vectors of disease. Such models have investigated a variety of Resistance Management Strategies, patterns of insecticide use that might potentially slow or prevent the increase in frequency of genes for resistance in populations. As with all models in science, the predictions of such models of the evolution of resistance depend on the assumptions about the parameters used. In population genetic models of the evolution of resistance important parameters include the genetic basis of resistance (i.e. the degree of dominance of alleles conferring resistance or susceptibility) and the relative fitness values of different genotypes and their resistance phenotypes. In the vast majority of such models, resistance is assumed to have a monogenic basis with two alleles, one that results in a susceptible homozygote and one that results in a resistant homozygote (REX Consortium 2010). This results in at most three genotypes, resistance phenotypes, and relative fitness values (depending on the dominance of the resistance allele). Further, the relative fitness values could differ in environments with or without insecticide application. The important assumption of many models, and which prompted present study, is that the resistance genotype/phenotype carries a fitness cost in generations without insecticide treatment. If there are many resistance alleles conferring different levels of resistance and with different fitness costs models of resistance evolution would need to be more complex.

Models of the evolution of resistance due to active esterases should incorporate the widespread variation in esterase activities present within esterase electromorphs in natural populations. Models should also incorporate the relationships between fitness and the level of esterase activity in the presence of insecticide, and between fitness costs and esterase activity during periods when insecticides are not being used. More research on the relationships between esterase activity variation and fitness, in both the presence and absence of insecticidal selection, could lead to improved models of the dynamics of amplified esterase genes in populations (see Taylor 1983, Tabashnik 1990, Ferrari 1996, REX Consortium 2010).

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