EFFECT OF LARVAL ENVIRONMENT ON SOME LIFE HISTORY PARAMETERS IN *ANOPHELES GAMBIAE S.S.* (DIPTERA:CULICIDAE)

by

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ABSTRACT

The effects of larval density, nutrition and cannibalism risk on some life history parameters of *Anopheles gambiae* larvae were evaluated in the laboratory. Adult body size was inversely correlated with larval density whereas larval mortality and mean age at pupation varied across experiments. When density increased, the secondary sex ratio shifted toward female bias. Effects of different types of nutrition on larval life were compared by providing larvae with algae *Chaetophora* sp., fish food or both. The fish food generated the highest mortality, longest developmental time and produced smaller adults. Mortality and developmental time was higher with algae diet. With regard to somatic body reserves, algae-fed larvae had more sugar and lipid and full diet mosquitoes had more glycogen and protein reserves. In a separate set of experiments with cannibalism pressure the mortality rate and developmental time decreased but larval activity and body size increased compared to risk-free larvae.

Keywords: *Anopheles gambiae;* cannibalism; *Chaetophora* sp.; larval density; adult body size; somatic body reserve; life history

DEDICATION

To my parents

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CHAPTER 1: INTRODUCTION

Mosquito larval ecology and its impact on larval life are considered as a key component for malaria control. The common question is: what do larvae eat and how does nutrition impact adult life? In addition, does food directly impact adult life or does it act in concert with other factors to determine vector competence (see Glossary)?

Mosquito larvae are found in different types of habitats, artificial (e.g. containers, hoof prints, drains) and natural (e.g. ponds, tree-holes, small pools). Each species is particular in choosing its habitat and habitat preference varies from species to species. In addition, some species may choose different habitats under different environmental conditions. For example, species that normally breed in large water bodies may sometimes breed in small container under adverse conditions. Habitats may also be a limiting factor for vector species if there are cannibalistic conspecifics present.

Mosquito larval habitats nurture different kinds of microorganisms, particulate organic matter or detritus, biofilms etc., which are good food sources for mosquito larvae (Gimnig *et al*. 2001). Nutrition from such habitats may also affect subsequent adult fitness (McCombs 1980). Some mosquito species feed principally on the thick biofilm that covers all submerged surfaces, including

those of plants (periphyton), stones (epilithon) and sediments (epipelon) (Clements 1992). These biofilms consist of a consortium of bacteria, fungi and algae embedded in a polysaccharide matrix (Lock 1990, Gimnig *et al*. 2002). Many workers have found that algae are one of the most important food sources for mosquito larvae (e.g. Rejmankova *et al.* 1996, Rohani *et al*. 2004, Tuno *et al.* 2005, Wallace & Merritt 1999). Lamborn (1922-3) suggests that mosquito larval habitats depend up on algal distribution, which means water bodies that have algae in them are most likely the favourable breeding grounds for mosquitoes. But there are some non-digestive algal species which can act as controlling agents for mosquito larvae. Non-digestive algae can slow down the digestion and eventually kill the larva by slowing growth rate. The dilemma of accepting or rejecting algae, food or non-food, depends on the algal species as well as mosquito species.

In their natural habitats, larvae usually compete with conspecifics, larvae from other mosquito species and other aquatic organisms (Knight *et al*. 2004, Schneider *et al.* 2000). Their survival also depends on the presence of predators such as larvae of many aquatic organisms including, aquatic insects, older instars of conspecifics or heterospecific etc. In such environments competition, cannibalism and predation are all very common phenomena (Siddiqui *et al.* 1976). Predation and cannibalism pressure is enhanced by ecological factors related to the low availability or scarcity of alternative food when density of a population is high in a specific habitat at a given time. Cannibalism is a special

form of predation that occurs within a species. Cannibalism is reported to occur frequently among malaria vectors (Koenraadt and Takken 2003). Inter or intra specific competition and cannibalism will influence the resulting density of the adult mosquito population (Koenraadt *et al*. 2004). Even when cannibalism does not occur, the presence of fourth instar apparently causes significant reduction in developmental rate of first instar larvae (Koenraadt and Takken 2003).

The purpose of this study is to elucidate the effect of different larval environments where both biotic and abiotic factors may affect larval life and subsequent adult life. Factors such as, nutritional conditions may affect the adult fitness; for instance, different types of food are stored as different types of body reserves which would affect survival, gamete maturation, reproduction etc. Competition with conspecifics at high density and low-density environments with limited food supply may affect the resulting adult densities, which eventually determine the spread of mosquito-vectored diseases. Again, factors such as, predation or cannibalism risk may lead to early metamorphosis and, hence, impact adult fitness.

1.1 STUDY ORGANISM: *Anopheles gambiae sensu stricto*

Anopheles gambiae sensu stricto was used as the study organism and has been cultured in Dept. of Biological Sciences, Simon Fraser University, BC for approximately 10 years after originally being collected in Tanzania. *Anopheles*

gambiae s. s. is considered to be the most effective human malaria vector of sub-Saharan Africa (WHO 1990).. Each year approximately 350-500 million cases of malaria occurs worldwide, killing 1-3 million people (WHO 2005). The World Health Organization, WHO, reported one million deaths of young African children in the year 2008. According to WHO a child dies of malaria in every 45 seconds in Africa, which sums up to 20% of all childhood deaths.

Anopheles gambiae is a member of a species complex that consists of at least seven morphologically indistinguishable species of genus *Anopheles*. The species complex is also known as *Anopheles gambiae sensu lato*. Among all seven members of this species complex, *Anopheles gambiae s.s.* and *Anopheles arabiensis* are the most efficient vectors of *Plasmodium falciparum*; considered as the most virulent parasite of human malaria (Levine *et al.* 2004).

An. gambiae's capacity of rapidly colonizing small pools of rain water, capability of harboring the parasite *Plasmodium falciparum* in its body under a wide range of environmental conditions, its acute anthropophilic nature and finally, extensive endophilly and endophagy (feeding and resting indoors, respectively) [about 95% indoor resting catch in Kenya (Minakawa et al. 2002)] habits make it the primary vector of malaria. *Anopheles gambiae s.s*. is an anautogenous (require blood meals to produce eggs) species. Protein from blood meals is essential for egg production.

Anopheles gambiae s.s. is a holometabolous insect that passes through two completely different habitat stages during its life cycle, aquatic and terrestrial. In the aquatic stage, larvae hatch from singly laid eggs. The larval period varies from 6 to more than 25 days depending on environmental conditions including temperature, rainfall, habitat types, availability of food, presence of predator in habitats, etc. In an ideal environment where food is available, larvae grow very fast accumulating a lot of food. Larvae pass through four stages, from first to fourth instar, the fourth instar moults to pupa. The pupal stage normally lasts 1 or 2 days. The newly hatched adults either seek a sugar meal or mate. Females take blood meals (from humans) for each batch of eggs produced. There are 50-200 eggs per batch. The number of eggs produced decreases with age. Following oviposition, eggs take two to three days to hatch. Adult life span ranges from seven days to a month or more depending on the environmental conditions and the availability of sugar and blood meals (Roitberg B. personal communication, Stone et al. 2009). Adult males feed extensively on plant sugar whereas females feed on both plant sugar and blood meal depending on availability and need (Foster 1995).

In nature, larvae perform best in clear water with lots of vegetation. These habitats are generally man made, or natural, such as; hoof prints pools, temporary or stagnant water pools, containers, stream edges, marshes etc. Conditions in larval habitats have significant effects on adult body size and body reserve which ultimately determines reproduction and vector competence. For

instance, females derived from poorly-nourished larvae require more sugar meal and might have to engorge on blood two or even three times for ovarian maturation (Macdonald, 1956); also egg production could be affected throughout life if they receive poor nutrition at larval stage (Mathis, 1935).

Laboratory studies to date on *Anopheles gambiae* have mainly focused on adult biology, genomics and disease transmission, whereas, a relatively small number of experimental studies have been conducted on larval ecology. Therefore, in this study, efforts were taken to determine the factors in the larval life that regulate adult survivorship, body size and body reserve.

1.2 OVERVIEW OF THE THESIS

Vector competence of a mosquito depends on both of its aquatic larval and terrestrial adult life stages. Parameters such as vector capacity, life span, survivorship, etc. of an adult mosquito depend on the physical and biotic characteristics of larval habitats, which include size and shape of the habitats, water depth, etc. as physical characteristics and food, crowding, presence of predators, competition etc. as biotic properties. Both the biotic and physical characteristics are crucial for success as a vector. In this study, I evaluated three different aspects of larval life, food, competition and predation of *An. gambiae s.s.* that are important regulators of vector capacity of the adults.

In Chapter 2, I examined whether the growth and survivorship of *Anopheles gambiae* larvae was density dependent. A series of experiments was conducted in a laboratory setting with different larval densities. Crowding effects on larval life were measured by applying three different factors: food amounts, larval density and container size.

In chapter 3, I manipulated various diets to larvae of the same age under laboratory conditions. For mosquitoes, energy requirements for maintenance, growth, swimming, development etc. in larval stage depends crucially upon the availability of the nutrients in the habitat. In nature, larval habitats of *Anopheles gambiae s.s*. frequently harbour algae (Minakawa *et al.*2004). Some of those algal habitats, are favored by *An. gambiae* larvae. The impact of the algal diet on larva towards successful adulthood was assessed. Protein, carbohydrates and lipid reserves were measured to compare the difference among the somatic reserves of adults from the different diets.

In chapter 4, I evaluated the effect of cannibalism threat on *Anopheles gambiae* larvae. Larvae of *Anopheles gambiae* can become cannibalistic under conditions such as low food density, high larval density and loss of habitat through evaporation. On the other hand, simple predatory behavior even in an ideal environment can lead to cannibalism. In a high-risk environment, larvae readily mimic dead insect carcasses, or dive to deeper water and remain there for long

time (Tuno *et al*. 2004) to avoid predators. Here, I used a fourth instar larva constrained by a mesh tube as a predator threat to the younger instars assuming that the younger instars would receive some kind of cue of the possible danger. The density of the younger instars was low in terms of the container size. The prediction was, in such larval environment where larvae are under threat of cannibalism, interaction between the individuals, food consumption and larval activity would be different from that which would occur in a low-risk environment. The effect of this cannibalism threat on the life history parameters of growing larva was evaluated.

In all the experiments, life history parameters such as length of larval period or mean age at pupation, mortality, and body size (by measuring wing length) at eclosion were documented and compared.

The last chapter summarizes the results of the three experiments and concludes the effect of larval habitats on vectorial capacity of *Anopheles gambiae s.s.* and finally suggests some possibilities for future studies.

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CHAPTER 2: EFFECTS OF LARVAL DENSITY AND DIFFERENT AMOUNTS OF FOOD ON LARVAL LIFE HISTORY TRAITS IN *ANOPHELES GAMBIAE S.S.*

ABSTRACT: Mosquito larval habitat determines fitness, survivorship, fecundity and vector capacity of emerging adults. Effects of density, nutrition and cannibalism in larval habitats on some life history parameters were measured in laboratory experiments. Larval density was positively correlated with larval mortality but that varied across experiments with regard to mean age at pupation. In addition, increased density skewed sex ratios that favored females.

Keyword Index: larval density, *Anopheles gambiae s.s*., competition, conspecific, sex- ratio.

[1]

[1] A similar version of this chapter has been prepared for submission to the Journal of Vector Ecology

2.1 INTRODUCTION

Food and space in a habitat regulate competition among conspecifics and are considered as the controlling factors of adult survivorship, fecundity and vector capacity. Developmental time and mortality increase and adult body size decreases if food is scarce (Hawley 1985a, Bradshaw & Holzapfel 1992, Renshaw *et al*. 1993, Mahmood *et al.* 1997, Nekrasova 2004). As a consequence, adult survivorship, male competence, fecundity, flight capacity, mating capacity, vector capacity etc. are also affected by nutritional constraints (Wada 1965, Steinwascher 1982, Reisen *et al.* 1984, Fisher *et al*. 1990, Davies 1991, Rowe *et al*. 1994).

Density is another regulator of adult body size and survivorship. Larval development rate, adult survivorship and adult body size all decrease as larval density increases (Schneider *et al*. 2000, Gimnig *et al*. 2002, Wiwatanaratanabatr & Kittayapong 2009). Density dependent development in an artificial temporary habitat that may dry out quickly, such as a hoof print nursery, will thus increase competition among conspecifics, which eventually affects adult fecundity and vectorial capacity.

There are few studies that evaluate the effects of larval density on adult body size wherein a fixed amount of food per capita is employed. This is important because one can not say if larval density per se is the factor that impacts adult size or if it simply alters decreases the amount of food per individual. Put another way, information on the relationship between density dependent or density-independent food availability and their interactions is lacking. There is one field study in Kenya, where the effect of an additional input of food, or of first instar larvae, to the pupal production was measured (Subra & Mouchet 1984). It was found that, adding food promoted a significant increase in the number of pupae but adding larvae did not.

In optimal foraging theory, individuals become more opportunistic when intra and interspecific competition is high and preferred food resources are scarce. ODT (optimal diet theory) predicts well for foragers that feed on immobile prey rather than mobile prey (Sih & Christensen 2000) and ODT works well in the laboratory but may be too simple to make accurate predictions given the complexities inherent in the field (Zach & Smith 1981). In the field where there are alternate choices diet breadth may expand. When diet breadth is wide inter and intraspecific competition may reduce and as such niche overlap declines. But for cannibalistic or opportunistic cannibals, a conspecific is often the first choice for feeding if food is scarce and density is high: this tactic provides nutrition and reduces competition.

For years ecologists have tried to determine the impact of intraspecific competition during the larval stage on the adult fitness and performance at higher densities (Paaijmans et al. 2009). From the perspective of medical entomology, the outcome of intraspecific competition for a vector species is critical to disease prevalence because density-dependent intraspecific competition may alter vector capacity of the adults. Hence, laboratory and field studies should focus on the importance of crowding in the larval stage to find out the effects on many different life history parameters of larvae and adults.

In this study, I evaluated the interactions among same-age larvae in a given environment with specific amounts of food. A series of experiments were conducted to determine the optimum amount of food for mosquito larvae to reach to the adulthood. The effect of larval density on some of the life table attributes, such as, larval mortality, development and adult body size (wing length) were measured. Here, food was given on a per capita basis and also instar basis. Assuming 1st and 2nd instar larvae consume less food (1st instar = 2-2.5 mm, 2nd instar = 3.5-5 mm, 3rd instar = 7-9.5mm, 4th instar = 10-12 mm); the total amount of food per capita per biomass in each tub was then smaller compared to the 3rd and 4th instars, where, the amount was increased as larvae grew 80-90% of their body size and where most biosynthesis take place in this stage (Briegel 2002).

The near optimum amount of food for a larva to reach adulthood was determined. This amount was used in other experiments with different larval densities and

container sizes to elucidate the interactions, competition and cannibalism among the same age larvae of *Anopheles gambiae s. s.*

2.2 MATERIALS AND METHODS

2.2.1 Experimental Design

Three approaches were taken to evaluate information regarding the effect of larval density and different volumes of food on larval life of *Anopheles gambiae s.s.* The experiments were conducted as follows:

1) An iterative approach to determine the optimum amount of food for a solitary larva
2) Different numbers of larvae/container with a fixed amount of food
3) Different numbers of larvae with a fixed amount of food in larger size containers

In my first experiment, I tried to determine optimum amount of food for the larvae to reach adulthood. In the second experiment, that amount was given to different densities of larvae. In the final experiment, food amount and larval densities were fixed but container size and water volume was changed. Following are the descriptions for all three experiments:

1) Determining optimal food level for a solitary larva

Here I used a single larva per container. The container size was 3.75cm × 3.75cm × 6 cm and each larva had full access to 14.06 cm² space (the surface area) in its tub. Food was provided in two different amounts: low food (LF) and high food (HF). Each amount had three different sub-amounts, for example, low food treatment had LF1, LF2, LF3.

The amount of food was doubled in every set from the previous set. The amount also increased across instars.

I ran a preliminary set of experiments with a very broad range of food provision (min-0.003mg to max 5 mg per larva) that led to the following experimental provision values:

Instars	LF1	LF2	LF3	HF1	HF2	HF3
1st	.003 mg	.006 mg	.012 mg	.1 mg	.2 mg	.4 mg
2nd	.006 mg	.012 mg	.024 mg	.2 mg	.4 mg	.8 mg
3rd	.012 mg	.024 mg	.048 mg	.4 mg	.8 mg	1.6 mg
4th	.024 mg	.048 mg	.096 mg	.8 mg	1.6 mg	3.2 mg

Nutrafin ® Basix Staple Tropical Fish Food *ad libitum* was given to the larvae. The food was measured with a Cahn Electrobalance in microgram units. Food

was provided on a per-instar basis assuming 2 days for each instar. For each amount there were 5 replicates, such as, LF 1 had 5, LF 2 had 5 replicates for a total of 30 replicates. There was an internal control with no food for all the treatment levels to confirm that the food that was provided was the only source of nutrients. The amounts that worked best were chosen for treatments 2 and 3.

2) Different numbers of larvae/container with fixed amount of food

In this experiment, the amount of food and container size was fixed. Two different larval densities were chosen namely low density or LD (10 larvae), and high density or HD (200 larvae).

I choose the size of the containers in such a way so that in low-density treatments each larva had access to approximately 14.06cm² surface space as in experiment 1. Therefore, the container size I picked was 12 cm × 12 cm × 12 cm. Hence, in low density tubs one larva had 14.4 cm² surface area, which is close to 14.06 cm² whereas in high density tub the surface space was 0.72 cm² for each larva. Each density had 3 replicates and an internal control per replicate.

3) Different numbers of larvae with fixed amount of food in larger size containers

Here, I repeated the experiment above but with much larger containers to see if density treatment effects were container-size specific. This experiment provided more space via more water volume and greater surface area for the larvae. In this experiment the fixed variables were volume of food (from experiment 1) and number of larvae (from experiment 2). The container size was 25.4cm × 38.1cm × 6.35 cm. Therefore, in low density treatments, each larva was provided 96.77 cm^2 surface areas whereas in high density a larva was provided 4.83cm^2 surface space in the tub. Density effect of this larger surface area was then compared with the result of experiment 2.

2.2.2 Rearing Condition

All the experiments were conducted at Simon Fraser University, Burnaby, B.C., Canada, in a walk-in Conviron™ growth chamber with the chamber temperature 30˚c ± 2˚c and humidity 75-80% RH. Photoperiod was 14:10 (L: D).

The water level in each rearing vessel was held constant at 5cm deep per container. The water level was checked everyday and evaporated water was replaced by adding distilled water whenever needed. The containers were placed in a random manner by position to minimize possible side by side effect.

The total-larval period was set to 10 days; larvae that failed to pupate in that time period were discarded. The cages were 30cm × 30cm × 30 cm Plexiglas™ cages

with mesh on 5 sides and cotton sleeves to access the cage on one side. Emerged adults were collected whenever observed and placed in small labeled cuvettes. All of the cuvettes were placed in a refrigerator at 4˚C after adult collection for two hours to kill the adults for further study.

2.2.3 Parameters:

The following parameters were measured for all three experiments:

i) Larval Mortality:

Larval mortality from each treatment was measured by recording all dead larvae each day per container. The dead larvae were removed whenever they were noticed. Missing larvae from each treatment were also counted as dead.

ii) Mean age at pupation

Mean age at pupation was expressed by *t*, where,

t = No. of days to pupation × No. of larvae pupated / total larvae pupated (Lyimo *et al*. 1992). Collected pupae from each treatment were kept in a small cage marked with the treatment names until emergence. Pupae that failed to hatch were discarded.

iv) Wing length

The pupae were checked twice per day for eclosion. Upon emergence, adults from each treatment were separated by sex and placed in the previously labeled microcentrifuge tubes. All tubes were placed in the refrigerator at 4˚C.

Adult body size was determined by measuring the wing length (both wings) of the adult mosquitoes. The wing length measure was the distance from axial incision to the apical margin, excluding fringe of scales (Rohani *et al*. 2004). The mean of the both wings was calculated. Wing length is considered as an indicator of body size because it is directly proportional to dry body weight (McCombs 1980). Differences in wing length by sex were recorded.

Micorscope measurements:

Mag	Ocular Units	mm	mm/ocular units
6x	13.6	24	
16x	**12.5**	**8**	**0.64**
40x	12.1	3	

2.2.4 Statistical Analyses

All the data showed a normal distribution, therefore, parametric tests (Student's t-test, Analysis of Variance, and Tukey's honestly significant difference test) were used to test the significance of the treatments.

Statistical analyses were conducted using JMP 8.0 (SAS Institute Inc. 2005). Graphs were generated using GraphPad Prism 5.00 (GraphPad Software Inc. 2005).

2.3 RESULTS

1) Fixed larval density with different amounts of food

Results showed no significant differences in case of the parameters observed here among the three different amounts of food from low food (LF) (p value =0.2385, F ratio = 0.1847) and high food or HF (p value = 0.3982, F ratio =0.7649) treatments. Therefore, results of LF1, LF2 and LF3 were pooled to calculate the average and termed as LF. Similarly the average for HF was used.

In case of the internal control, mortality was 100% at 1st instar. For low food treatment the mortality was 100% in all three amounts of LF (LF1, LF2 and LF3) before pupation. For high food treatments the mean mortality was 20%. Mean age at pupation (*t*) in case of HF treatment was 8.22.

In HF treatments the male to female sex ratio was 49:51. Females were larger than males (Fig. 2.1) ($p = <.0001^*$; F ratio = 22.693). In case of females: mean ± SE = 4.800 ± 0.046 and for males mean ± SE = 4.461 ± 0.054 when $\alpha = 0.05$.

Larvae from low food treatments died in their 1st and 2nd instars in case of single larva treatment but under higher larval densities the lowest amount performed the best. In experiments 2 and 3, larval densities were higher than experiment 1, e.g. low density = 10 larvae, high density = 200 larvae. When food was provided on a per capita basis the larger amounts of food from the HF treatments caused larval death by polluting the water when larvae reached to the later instars. Therefore, the lowest food amount among the three LF amount was chosen for experiments 2 and 3.

2) Different numbers of larvae/container with fixed amount of food

Mean mortality in low density LD (LD1, LD2 and LD3) was 60.55% and high density HD (HD1, HD2 and HD3) was 96.44% (Table 2.1 and 2.2). Mean age at pupation in the case of LD was 8.38, and HD was 7.16 (Table 2.1, 2.2).

Male and female sex ratio was 22:78 and males were protandous, emerging with a trend toward smaller body size. Adults from LD treatment were larger than those from HD treatment (Fig. 2.2) (Table 2.1). Lower density produced bigger adults with longer developmental time.

3) Different numbers of larvae with fixed amount of food in larger size containers

Mean larval mortality from LD treatment groups was 6.66% and for HD treatment groups was 42.33% (Fig. 2.3) (Table 2.1 and 2.2). Mean age at pupation in LD was 6.27 and HD was 9.8 d (Fig. 2.4) (Table 2.1 and 2.2). Adults from LD treatments were significantly (Table 2.1 and 2.2) larger than those of HD treatments (Fig. 2.5).

2.4 DISCUSSION

For species with complex life histories (e.g. larval stage in water and adult stage in terrestrial habitats) such as mosquitoes, larval environment plays a vital role in determining adult fitness. Changes in larval density and food availability can affect larval development and therefore, affect adult body size. In this study,

crowding produced significantly smaller adults in both sexes with elevated mortality.

Larval mortality

In the density dependant experiments (Experiments 2 and 3) the mortality was higher in the high-density treatment. In these experiments food and density were the two limiting factors. Density was the major cause of mortality where all individuals had access to the entire amount of resources. But in experiment 1 (one larva per container) nutritional stress was the reason of high mortality. Here, larva had access to the food but the amount was not sufficient for them to reach to pupation. This result contradicts insights from the mathematical model of Dye (1984b) where he concluded that all mortality is density dependent. However, Legros *et al*. (2009) stated that, density dependent mortality occurs when all individuals have access to the food in the environment but the amount is such that each individual obtains, or is likely to obtain, a suboptimal share; a form of scramble competition (Smith and Smith 2006).

Mean age at pupation

Life-history theory suggests that, the timing of metamorphosis is of major importance (Rowe and Ludwig 1991, Abrams *et al*. 1996) because individuals

experience a strong reduction in fitness if they fail to reach a specific stage before a set time (Rudolf & Rodel 2007).

In experiment 2, the mean age at pupation was lower in HD treatments, but the result was opposite in experiment 3. A possible explanation for this could be the different surface areas for a larva provided in HD treatments. In experiment 2, a larva from HD treatment had access to 0.72cm² surface area but in experiment 3, a larva from HD treatment had access to 4.83cm² surface area. Therefore, HD treatments from experiment 2 had higher larval density per cm² space than that of experiment 3. Some larvae from HD treatments in experiment 3 remained in the 3rd or 4th instar till the 10th day. These larvae never pupated even if they were kept in the container for longer periods of time. They remained in the 3rd or 4th instar for over a month and eventually died without pupating. Arrivillaga & Barrera (2004) found that 3rd instar of *Aedes aegypti* can survive in a food stressed condition for a significant number of days. Larvae that pupated early might choose to delay under such nutritional stress because they could consume more from the total volume of food given for all the larvae. The larvae that never pupated were receiving less food than required per day and were forced to draw on energy reserves but never could accumulate sufficient energy to develop to older instars or pupae stages. Day and Rowe (2002) published a model, which is an extension of Wilbur-Collins model for amphibian development, where they included a threshold size for eclosion below which maturity is not possible.

Cannibalism was observed in the larvae with delayed or no pupation. Time of metamorphosis thus expanded under several limiting factors. This kind of plasticity of growth and age at metamorphosis can lead to differences among individuals of the same cohort in survival, vector capacity and fecundity.

Wing length

Body size is a direct result of density and nutritional conditions in the larval habitat (Hawley 1985, Lyimo *et al.* 1992, Lounibos *et al.* 1993). Lower density produced larger body sized mosquitoes in both experiments 2 and 3. Although previous studies showed that density-dependent development produces smaller-bodied adults (Gimnig *et al*. 2002, Lyimo *et al*. 1992, Koella and Lyimo 1996).

In experiment 2, the male and female ratio at emergence was 22:78 (mainly from LD treatments), which varies greatly from the typical 50:50 ratios. The reason for such a high female ratio is unknown. One possible reason could be cannibalism; male larvae were apparently cannibalized more than female larvae. Adult body size showed that females were larger than the males. In such cases, males were also smaller than females during the larval or pupal stage. Smaller larvae are always more susceptible prey than larger larvae. Hence, most of the male larvae were cannibalized by the larger larvae.

Sex-ratio distortion may also be caused by meiotic drive. Studies showed that, in culicines, such as, *Culex quinquefasciatus* and *Aedes aegypti*, a Y(M)-linked gene caused a change in sex-ratio in favor of males (Wood & Newton 1991, Sweeny & Barr 1977, Hickey & Craig 1966, Craig *et al*. 1961) producing lower percentage of females from 45% to as low as 0% females or no females at all. Since my results occurred with a change in environment, this mechanism is probably not the key driver here.

On the other hand in some insect families, female-biased sex-ratio at high larval densities has been observed. Cipollini (1991) stated that male-biased mortality at higher larval densities produced female-biased sex-ratio in case of a weevil, *Acanthoscelides obtectus*. In this study, the magnitude of density-dependent larval development and mortality thus caused the distorted sex-ratio.

There is a chance that the female-biased sex ratio could be some experimental error or environmental effect such as chamber effect, container effect etc. for this specific study. However, the result was consistent throughout all the experiments.

In conclusion, the experiments suggested that the amount of food consumption was crucial for a larva to successfully complete larval life in a set time period. However, larval mortality and adult body size (measured in wing length) was strongly dependent on larval density.

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Table 2-1 Effect of two larval densities, low density (LD) and high density (HD) on larval mortality (% of dead larvae), mean age at pupation (no. of days to pupate) and wing length (mm) of *Anopheles gambiae* s.s. * indicates a statistically significant effect (ANOVA α = 0.05)

Source	DF	Sum of squares	F ratio	p value
Larval Mortality (2)	1	18718.593	4.732	0.0449*
Larval Mortality (3)	1	39265.333	134.046	<.0001*
Mean age at pupation (2)	1	2328.113	9.078	0.0049*
Mean age at pupation (3)	1	166.294	41.614	<.0001*
Wing Length (2)	1	3.511	0.655	0.4209
Wing Length (3)	1	9.397	26.393	<.0001*

(2) = Experiment 2: Different numbers of larvae/container with fixed amount of food per capita

(3) = Experiment 3: Different numbers of larvae with fixed amount of food per capita in larger size containers

Table 2- 2 Effect of two larval densities, low density (LD) and high density (HD) on larval mortality (% of dead larvae), mean age at pupation (no. of days to pupate) and wing length (mm) of *Anopheles gambiae* s.s. Means with different letters are significantly different (Tukey's HSD α = 0.05)

Source	Treatment	n	Mean ± SE
Larval Mortality	2 (LD)	30	96.258 ± 1.579 (A)
	2 (HD)	600	65.444 ± 14.076 (B)
	3 (LD)	30	6.666 ± 2.795 (A)
	3 (HD)	600	52.444 ± 2.795 (B)
Mean age at pupation	2 (LD)	30	9.678 ± 2.279 (A)
	2 (HD)	600	2.295 ± 0.899 (B)
	3 (LD)	30	6.245 ± 0.384 (A)
	3 (HD)	600	9.886 ± 0.412 (B)
Wing Length	2 (LD)	50	3.456 ± 0.032
	2 (HD)	19	3.406 ± 0.052
	3 (LD)	27	3.758 ± 0.085 (A)
	3 (HD)	28	3.281 ± 0.039 (B)

2 = Experiment 2: Different numbers of larvae/container with fixed amount of food per capita

3 = Experiment 3: Different numbers of larvae with fixed amount of food per capita in larger size containers

FIGURE LEGENDS

Figure 2-1 Comparison of mean wing length (mean ± SD) of male and female of *Anopheles gambiae s.s.* from Experiment 1, where specific amount of food was given to one larva / container

Figure 2-2 Differences of mean wing length (mean ± SD) of adults of *Anopheles gambiae s.s.* from treatments LD (Low Density) and HD (High Density) of Experiment 2 (different numbers of larvae/container with specific amount of food)

Figure 2-3 Comparison of mortality (mean ± SD) of *Anopheles gambiae s.s* from LD (Low Density) and HD (High Density) treatments from Experiment 3, where number of larvae and amount of food was fixed using larger containers (25.4cm × 38.1cm × 6.35 cm)

Figure 2-4 Mean age (mean ± SD) at pupation for *Anopheles gambiae s.s* from LD (Low Density) and HD (High Density) treatments in Experiment 3, where number of larvae and amount of food was fixed using larger containers (25.4cm × 38.1cm × 6.35 cm)

Figure 2-5 Wing length (mean ± SD) of *Anopheles gambiae s.s.* from LD (Low Density) and HD (High Density) treatments of Experiment 3, where number of larvae and amount of food was fixed using larger containers (25.4cm × 38.1cm × 6.35 cm)

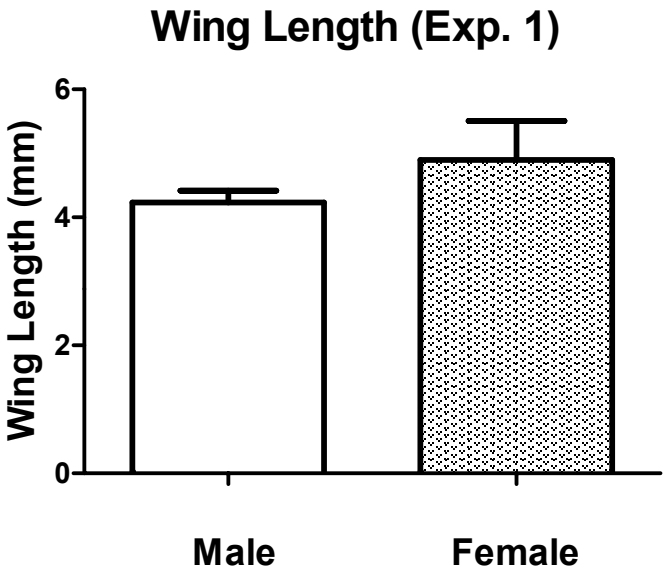

Figure 2-1

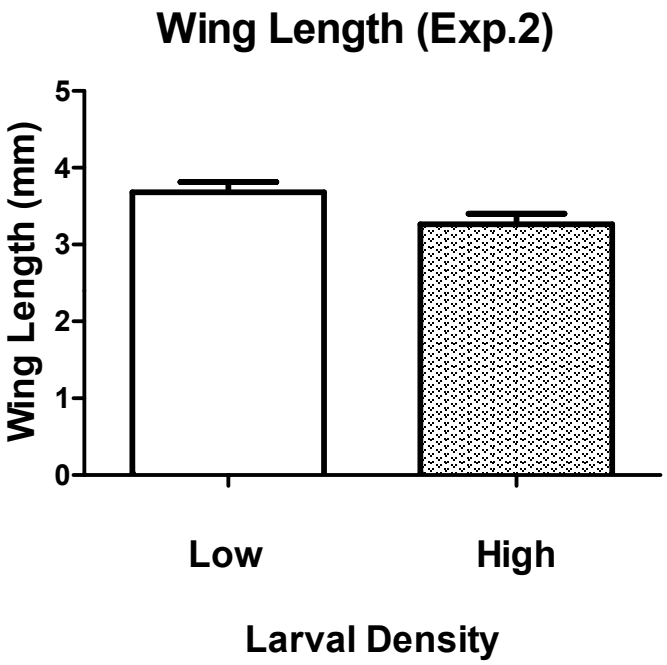
Wing Length (Exp.2)
Wing Length (mm)
5
4
3
2
1
0
Low
High
Larval Density

Figure 2-2

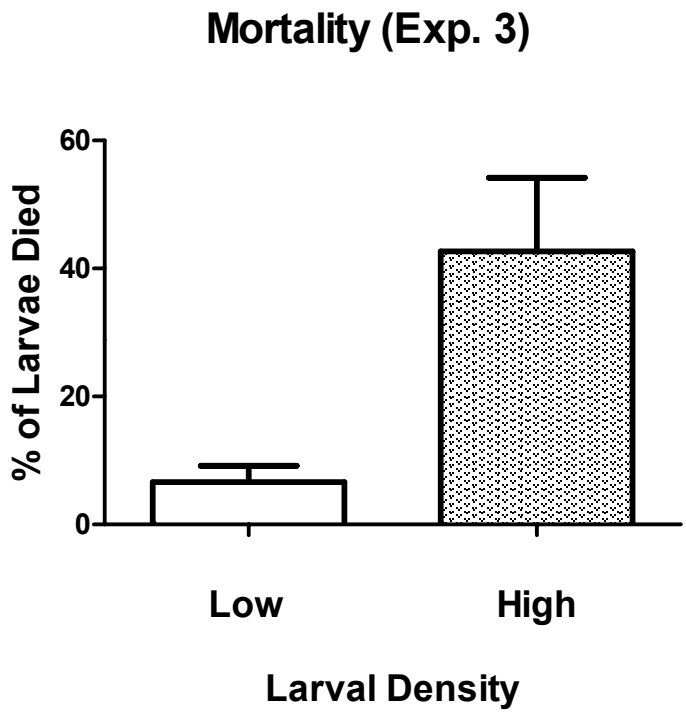
Mortality (Exp. 3)
% of Larvae Died
60
40
20
0
Low
High
Larval Density

Figure 2-3

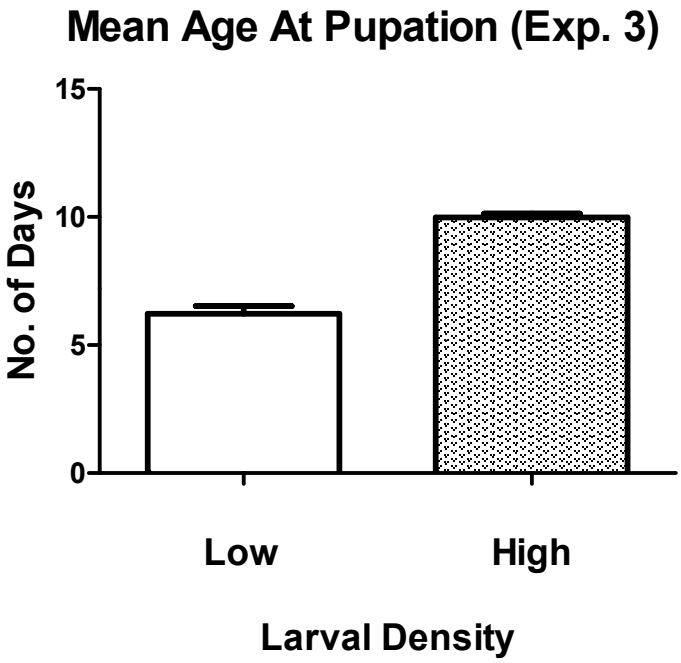
Mean Age At Pupation (Exp. 3)
No. of Days
15
10
5
0
Low
High
Larval Density

Figure 2-4

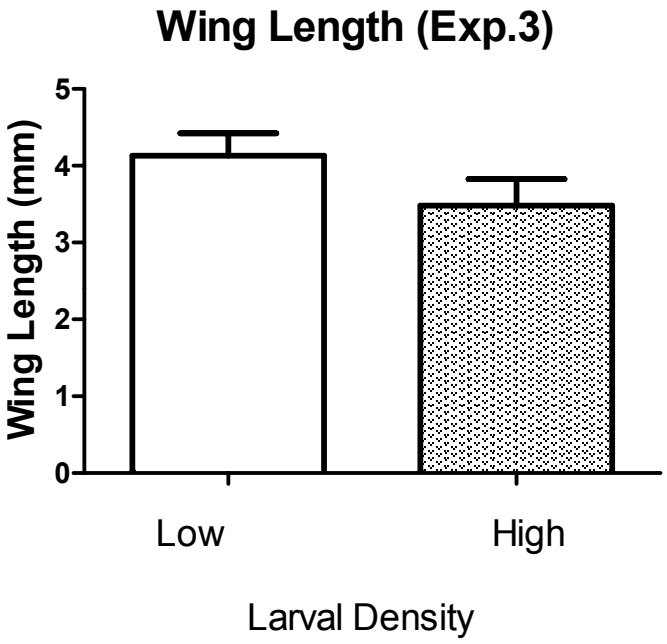
Wing Length (Exp.3)
Wing Length (mm)
5
4
3
2
1
0
Low
High
Larval Density

Figure 2-5

CHAPTER 3: EFFECTS OF ALGAE DIET ON SOME LIFE HISTORY PARAMETERS OF *ANOPHELES GAMBIAE S.S.* (DIPTERA:CULICIDAE)

Abstract

The co-existence of algae and mosquito larvae in a habitat depends on the type of algal food source, toxic or nutritious, as well as the mosquito species. The effect of algal diet on various life history parameters of both larva and adult of *Anopheles gambiae s.s.* was examined by providing algae *Chaetophora* sp. as food for larvae of *An. gambiae s.s*. Nutrafin fish food (F) was used as a control, and a combination of algae and fish food (AF) was also provided to compare with the algae (A) diet. Larvae from the fish food (F) group had the highest mortality and longest developmental time and produced smaller adults. Mortality rate and mean age at pupation was higher in algae (A) treated larvae than those treated with combination food (AF). In the case of somatic body reserves at emergence, algae-treated adults had significantly more sugar, AF treated had greater glycogen and protein and F treated adults had more lipid reserves.

Key Words: *Anopheles gambiae*, *Chaetophora* sp., teneral body reserve, mean age at pupation, adult body size[2]

3.1 Introduction

Adult fitness and vector capacity of mosquitoes are related to larval environment. Nutrition in the larval environment determines adult survivorship and vector competence. Growth rate, longevity and other factors that affect vectorial capacity may also depend upon larval feeding rates. Previous research has shown that increased feeding rates in larval stages produces larger adults. Numerous researche has demonstrated that larger mosquitoes have greater fecundity (Briegel 1990, Steinwascher 1982, Washburn *et al.* 1989, Lyimo & Takken 1993), greater survival (Nasci 1987, Packer & Corbet 1989a), more blood feeding success (Nasci 1990) and larger egg clutches (Renshaw 1994) and small conspecifics. Blackmore & Lord (2000) and Roitberg & Gordon (2005) found that the relationship between body size and fecundity was significant and positive in case of *Aedes albopictus* and *Anopheles gambiae*, respectively.

It is well documented that mosquito larvae feed upon the surface micro layer of water bodies that consists of extensive amounts of bacteria, algae, heterotrophic protists, micro invertebrates etc. Cyanobacteria (once called blue-green algae

[2] A similar version of this chapter has been prepared for submission to the Journal of Medical Entomology

but now considered bacteria) are widely distributed in mosquito habitats and have been found in mosquito guts (Thiery *et al*. 1991, Khawaled *et al*. 1989). For example, six species of blue-green algae were isolated from *Anopheles albimanus* midgut in Mexico (Martinez *et al.* 2002).

Algae can be a nutritious diet item or toxic to a mosquito larva. Some algae are only partially digested in the mosquito gut; as a result, larvae feeding on those non-nutritious or less digestible algae have high mortality rates and low growth rates in their later instars. Some algae are tolerant to larvicides; hence, mosquito larvae ingesting those algae as principal diet components suffer less from this larvicidal effect. The association of algae and mosquito larvae in a habitat, is thus depends on the digestibility of the algal species. If an algal species is digestible and nutritious then the coexistence of mosquito larvae and algae is favored. Recent studies have focused mostly on the larvicidal effect of algae in mosquito control biology (Berry *et al*. 2008, Marten 2007, Abdelhameed *et al.* 1993 etc.) and less on the nutritious algae.

Mosquito larvae may filter algae from the water column, scrape them from the surface of containers or aquatic plants, or scoop them from the bottom of aquatic habitats (Marten 2007). Mosquito biologists first discovered algae during gut content analysis of mosquitoes (Boyd and Foot 1928, Senior-White 1928). Among all the mosquito species, anophelines are most commonly associated with algal prevalence (Tuno et al. 2005). Gut contents of *An. quadrimaculatus*

showed a high concentration of algae even when other microorganisms were also abundant in the pond water. Many other recent studies showed that anopheline larvae in general are strongly associated with algae in natural habitats (Rejmankova *et al*. 1996, Minakawa *et al.* 1999, Wallace and Merritt 1999, Gimnig *et al.* 2001, Tuno *et al*. 2005, Bond *et al*. 2005).

Algae that are common in mosquito habitats in nature have species from mostly Chlorophyta, a division of green algae, comprising more than 8000 species in the Chlorophyceae class. However, not many algal species have been identified from larval habitats to date. Gut content analysis from third and fourth instar larvae from natural habitats in Kisumu, Kenya using a bar-coding approach revealed that *Anopheles gambiae* larvae fed on specific groups of algae (Chlorophyta, Chlorophyceae Class) despite the wide range of microorganisms available in natural habitats (Garros *et al.* 2008). In another study, the highest concentration of third and fourth instars and pupae of *An. gambiae* was sampled in conjunction with the unicellular epizoic green algae, *Rhopalosolen* species (Chlorophyta; Chlorophyceae) (Tuno *et al*. 2005).

Chaetophora sp. used in this study is common in small artificial water tanks and is a member of Chlorophyceae Class as discussed below. It grows very readily in fish aquariums and larval rearing trays. I used this local *Chaetophora* sp. because experiments on *Anopheles gambiae* are frequently carried out in laboratory settings where *Chaetophora* sp. are present. My results could differ if I

had used algal species commonly found in the mosquito habitats in East Africa, though I have no specific reasons to conclude so. Moreover, in nature there are very many algal species from Chlorophyceae class associated with anopheline larvae.

The classification of *Chaetophora* is as follows:

Domain: Eukaryota

Kingdom: Plantae

Division: Chlorophyta

Class: Chlorophyceae

Order: Chaetophorales

Family: Chaetophoraceae

Genus: *Chaetophora* sp.

Energy reserves derived from larval food may contribute to adult nutrition and therefore, adult survivorship and fecundity (Zhou *et al*. 2004). Algal biomass consists mainly of common bio-molecules such as carbohydrates, proteins, lipids etc. as well as all elements essential for herbivore nutrition (Sterner & Hessen 1994). In a previous study, Gimnig *et al*. (2002) showed that *An. gambiae* larvae can greatly reduce algal biomass and alter algal community composition at the surface micro layer of newly formed artificial habitats, suggesting algae as a nutritious food source. In case of *Anopheles gambiae*, Briegel (1990) found that

teneral reserves were isometric with body size and fecundity is significantly greater in larger females (Briegel *et al.* 2002).

In this study, the relationship of different types of larval nutrition and adult body reserve was examined by providing algae as larval food to *Anopheles gambiae s.s.* larvae. The potential effect of algae diet was compared to a fish-food diet via impacts on larval mortality, mean age at pupation, wing length, and adult body reserves.

3.2 Materials and Methods

3.2.1 Rearing Conditions

The experiment was conducted at Simon Fraser University, Burnaby, B.C., Canada in a walk-in Conviron™ growth chamber where the chamber temperature was 30˚C ± 2˚C and humidity from 75-80% RH. Photo period was held constant at 14:10 (L: D).

Groups of twenty 1st instar larvae were placed into 12.7cm×12.7cm×15.2cm square rearing white plastic tubs with distilled water 5cm deep. The different treatment containers were positioned in a random manner. The water levels were checked everyday and adjusted by adding distilled water whenever needed.

Different plastic pipettes were used for algae and non-algae treatments to avoid contamination. Pupae from each treatment were transferred to cages labeled with date and treatment names. The cages were 30cm×30cm×30cm Plexiglass™ boxes with mesh on 5 sides and cotton sleeves to access the cages on one side. Emerged adults were collected once each day and placed in small cuvettes. All the properly labeled cuvettes were placed in a refrigerator at 4˚C after adult collection for 2-3 hours to kill the adults for further study.

3.2.2 Experimental Design

To determine the efficacy of algae as larval food, larvae were fed one of the three following diets:

i) Algae,

ii) Fish food (control treatment)

iii) A combination of algae and fish food.

There were four replicates for each diet (4×3=12) with internal control groups with fish food for each one and the experiment was replicated four times.

3.2.3 Measurement of Food

Nutrafin ® Basix Staple Tropical Fish Food *ad libitum* was given to the larvae in the appropriate treatments. The food was previously measured with the Cahn Electrobalance © in microgram units. The food allotments were as follows:

1st instar = 0.003mg/larva (2 days)

2nd instar = 0.006mg/larva (2 days)

3rd instar = 0.012mg/larva (2 days)

4th instar = 0.024mg/larva (2 days and onward)

Algae *Chaetophora* sp. was cultured in plastic trays (12cm×12cm×6cm) for several weeks in the Conviron chamber. Algae was collected from the rearing trays and transferred to Petri dishes in a drying oven at 50˚C for several days. Dried algae was crushed into powder using a mortar and pestle and then weighed on a Cahn microbalance. The mass of dried algae provided to the larvae was same as the fish food allocation above. Weighed food was placed in small, labeled cuvettes for use in the experiment.

Food was given on larva/capita/biomass basis. Each larva received the same amount of food for two consecutive days (Considering the larval period as 8 – 10 days).

3.2.4 Parameters

The following parameters were recorded:

i) Larval mortality,
ii) Mean age at pupation,
iii) Wing length and
iv) Teneral reserves

i) Larval Mortality:

Larval mortality from each treatment was measured by recording the dead larvae every day per container. The dead larvae were removed whenever noticed. Missing larvae from each treatment were also counted as dead. Missing larvae could be due to cannibalism.

ii) Mean Age at Pupation

Time required for pupation of larvae from each treatment was calculated.

Mean age at pupation is expressed by *t*, where,

t = No. of days to pupation × No. of larvae pupated / total larvae pupated (Lyimo *et al*. 1992).

Collected pupae from each treatment were kept in a small cage marked with the treatment names until emergence. Pupae that failed to hatch were discarded.

iii) Wing Length

Wing length is considered as an indicator of body size because it is directly proportional to dry body weight (McCombs 1980). Wing length was defined as the distance from axial incision to the apical margin, excluding fringe of scales (Rohani *et al*. 2004). Wing lengths were measured by using the compound microscope (16X magnifications on WILD Heerbrugg microscope # 69066) equipped with an ocular micrometer. After measuring, the dried mosquitoes were placed back in the centrifuge with the wings (if possible) for body reserves analyses.

v) Teneral Reserves

Dead adults (after measuring the wing length) were removed from the freezer and thawed for further analysis. Proprotion of teneral reserves (stored carbohydrates, protein and lipid) was measured by conducting the following biochemical analyses (see Appendix) and absorptions were read on a Beckman Du 640 spectrophotometer:

- Bradford Assay for reserved protein measurement (absorbance at a wavelength of 595nm),

- Anthrone Assay for quantification of glycogen and sugar (at a wavelength of 625 nm), and

- Vanillin Assay for measuring the amount of lipids (at a wavelength of 525 nm)

The procedures described by Van Handel (1985a, 1985b) and Van Handel and Day (1988) are well established.

3.2.5 Statistical Analyses

A one-way ANOVA was used to test the effects of algal diet, fish food diet and combination food diet on mortality, mean age at pupation and body size. The sum of squares, F ratios and p values were reported. Tukey's Honestly Significant Difference (HSD) test and Student's t tests were used for comparing means, with a 0.05 significance level.

A MANOVA was used to evaluate the interactions in the body reserves from three different food treatments because the response variables (absorbance rate) were not independent. Data for proportional sugar, glycogen, lipid and protein

concentration were also not normally distributed; therefore, they were transformed by arcsine transformation before conducting the MANOVA.

Statistical analyses were conducted using JMP 8.0 (SAS Institute Inc. 2005). Graphs were generated using GraphPad Prism 5.00 (GraphPad Software Inc. 2005).

3.3 Results

i) Larval Mortality

The highest mortality was in the fish food treatments (F), followed by A (algae) and AF (algae and fish food) (Fig 3.1). The differences were significant in case of AF and F, and also AF and A. But the mortality from A and F were not statistically significant (Table 3.1, 3.2). In the 2^{nd} instar, mortality was high in F, but in 3^{rd} and 4^{th} instar mortality was higher in A than the other treatments (Fig. 3.2). Proportion of cannibalism was significantly higher in (F) (Fig 3.3).

ii) Mean Age at Pupation

Post-hoc analysis showed that the lowest mean age to pupation was in case of AF, followed by A and F (Fig. 3.4). ANOVA showed that the responses were not

statistically significant in case of A and F but in case of A and AF it was significantly different (Table 3.1, 3.2).

iv) Wing Length

Algae-fed adults were larger than those from the other two diets. Smallest adults were generated by fish-food-fed larvae (Fig. 3.5). The differences between A and F were statistically significant (Table 3.1, 3.2). However, with regard to the combination food (AF) and algae (A), the difference was not statistically significant although adults from treatments A were larger than the adults from treatments AF on average. In all three treatments wing length was affected by sex; e.g. females were larger than the males but the difference was not significant (Fig 3.6).

v) Teneral Reserves

Adults from fish food (F) treatments had more lipid and protein reserves and less sugar and glycogen reserves (Fig 3.7, 3.8, 3.9 and 3.10) at emergence. The interaction between the food types and the distribution of calories between the four classes of reserves was significant in case of AF, F and A (Table 3.3). Adults from A had more sugar and lipid reserves at emergence than the adults from AF (Fig 3.7 & 3.9). The difference was statistically significant (F = 12.369, p value =

<.0001* DF = 3, n = 18). With regard to glycogen and protein reserves, adults from AF had more reserves than adults from A (Fig 3.9 & 3.10).

3.4 Discussion

This study demonstrated that for *Anopheles gambiae s.s.,* larval mortality, mean age at pupation and wing length was significantly affected by protein and non-protein diets where food allotment was limited and was provided larva/capita basis. *Chaetophora*-fed larvae had higher mortality but emerged as larger bodied adults than fish food-fed larvae. Increased mortality means either the algae *Chaetophora* sp. was indigestible, therefore, toxic to the larvae or had lower food-value. On the other hand, adults emerged larger from the algae treatment, which confirms *Chaetophora* as a digestible food. Moreover, those first instar larvae reached 3rd instar in three or four days, which was one or two days faster than larvae from the other food treatments. But then they often remained in the later instars (3rd or 4th) for a long time. This higher mortality of larva or arrested larval growth in later instars could be explained as either due to insufficient amounts of algal food for the total number of larvae present in the culture tray or poor quality food, possibly lacking in some nutrient critical for metamorphosis. Thus a life history trade-off can be hypothesized between high growth with good

food (the *Daphnia* strategy) and slower growth on poor food (the *Diaptomus* strategy) (Sterner & Hessen 1994). Another explanation would be the requirement of different nutrients in different instars or ages, such as, more carbohydrates in the first and second instars and more protein in the later instars. My assumption is that the ratio of fish food (protein diet) and algae (carbohydrates) F:A consumed was identical across instars, i.e. they did not adjust intake ratios.

Overall, my results suggest that AF diet provided balanced nutrition that led to lower mortality and mean age at pupation. On the other hand F and A diets provided insufficient amount of nutrition, which caused higher mortality. However, algae fed larvae produced larger adults. Below I discuss the specific attributes that include mortality, energy reserves and wing length from each diet.

3.4.1 Larval Mortality

The mortality was higher in treatments A and F. In AF treatments, larva received both algae and fish food, i.e. both carbohydrates and protein: this mixed diet led to sufficient stored energy to facilitate pupation and eclosion and to reduce mortality presumably through somatic maintenance. On the other hand, the algae-fed and fish-food-fed larvae sequestered mostly carbohydrates or protein, respectively. Moulting from one instar to the next, pupation and eclosion requires

considerable energy and this comes from the body reserves via food intake. Single diet treatments might not provide sufficient energy or critical nutrients larvae need to attain adulthood or fecundity. In this experiment, most of the larvae from treatments A died after reaching to 3rd instar, the highest mortality was in 4th instar (Fig 3.2). This result contradicts that of Tuno *et al*. (2005), where he found that *Rhopalosolen* sp. (Chlorophyta) fed larvae of *An. gambiae* had lower mortality and more rapid developmental. However, the amount of *Rhopalosolen* sp. provided as food to the larvae was not fixed or known. In my study, the food amount was controlled. Larvae from treatments A had rapid developmental until 3rd instar. Perhaps the young instars fed or foraged more on algae than the older instars. A similar result was observed in *Culex pipiens*, which graze on alga *Selenastrum* sp. significantly more in younger stages (Gophen & Gophen, 1986). In case of AF treatments, larval death increased with the size too, possibly because of insufficient amount of food as algae treatment since later instars consumed a large amount of food before pupation.

There were missing larvae in all treatments, more in F followed by AF (Fig 3.3). The most likely reason for this is cannibalism. *Anopheles gambiae s.s.* larvae and the entire *Anopheles gambiae s.l.* complex is known to be cannibalistic (Koeraadt & Takken 2003). The onset of cannibalism might occur because of the low food availability (Balfour 1921). Koenraadt and Takken (2003) reported that in a food stressed condition, first instar larvae are consumed by fourth-instar larvae of the *Anopheles gambiae* complex.

In this study, cannibalism was observed after the larvae reached to 3rd instar. In some of the fish food treatment trays larvae were missing (1 per tub) every single day until the last one pupated. There was a possibility that the last larva from those treatments was consuming the others. Although this assumption could be wrong, there could be several larvae preying on each other under food-stressed condition.

Putative cannibalism was not significant in treatment A, even though the mortality was high in 3rd and 4th instars. However, larvae from this treatment rarely cannibalize each other. By contrast, in treatments F and AF the amount of protein intake was greater from the onset than from the A diet. As larvae matured and grew, the amount of protein in the diet may not have been sufficient to sustain their high rate of growth and thus they supplemented their diet via cannibalism. AF had more protein reserves than F (Fig 3.9). Furthermore, if there is greater variance in body size then there are more opportunities for cannibalism. Though I did not collect data on variance in body size my observations suggest a more uniform distribution of body size in the A treatment.

3.4.2 Wing Length

Adults from treatments A were larger than from the other two treatments. There could be two explanations for this. Since the number of days to pupation for larvae from treatments A was higher, their body size was larger too, which means that body size was positively correlated with the mean age at pupation. The other explanation is that algae might serve as higher quality food sources for anopheline larvae, which produced significantly larger adults than the other treatments. Body size is a direct result of density and nutritional conditions in the larval habitat (Hawley 1985, Lyimo *et al.* 1992, Lounibos *et al.* 1993). Wallace and Merritt (1999) showed that adult female *An. quadrimaculatus* reared in algal clump treatment had longer wing lengths than females in the non-algal treatments. Tuno *et al*. (2005) also found that water bodies with the green algae *Rhopalosolen* sp. yielded larger mosquitoes than other habitats without the algal flora. Females were significantly larger than their counterpart males. Body size might alter the vectorial capacity too, because larger adults have longer life span and greater fecundity (Takken *et al.* 1998, Blackmore & Lord 2000, Roitberg & Gordon 2005). Therefore, it can be concluded that larger body size is advantageous to the vector species and increases vectorial capacity.

3.4.3 Teneral Reserves

Algae-fed larvae stored significantly more sugar as body reserves than the others. Larvae from treatments AF had the least sugar reserves but higher

glycogen levels. High sugar level might be the reason for producing larger adults from treatments A. Carbohydrate reserves from the larval life are critical sources of energy during the first gonotrophic cycle of adult mosquitoes (Zhou *et al*. 2004). The same study showed that in the case of *Aedes aegypti*, during the first gonotrophic cycle, about 60% of the glycogen and sugar stores were metabolized, with about 9% being transferred to the eggs, about 30% lipid was metabolized, with about 65% being transferred to the eggs, about 33% of labeled protein and 72% of labeled amino acid stores were metabolized, with about 9% being transferred to the eggs and the rest were oxidized. Several studies showed that larger females had greater fecundity and higher survival rate along with more body reserves (Takken *et al.* 1998, Blackmore & Lord 2000, Roitberg & Gordon 2005).

Sugar feeding is an important behaviour for adult male and female mosquitoes (Manda *et al.* 2007) to gain energy for flight, survival and fecundity. Nutrients acquired through sugar feeding can contribute to both somatic and reproductive metabolic reserves (Walker 2008). Briegel (1990) stated that provision of sugar in the female diet increases the lipid content of somatic reserve and this increased somatic lipid reserves are then allocated towards reproduction to produce yolk lipids. In this study, algae fed larvae had more sugar and lipid content to support this statement. In adult mosquitoes, sugar feeding increases the fecundity more than blood feeding alone (Gary 2005). There is evidence that moderate amounts

of sugar increase the lifespan of *Anopheles gambiae* females too (Straif and Beier 1996, Okech et al. 2003).

The majority of calories in both sexes of *An. gambiae* were derived from lipids (71% in females and 72% in males), while glycogen accounted for the smallest proportion of total caloric content (8% in females and 7% in males). In Walker's study on this same colony, the larvae were restricted to fish food diet only; therefore, the proportion of calories at emergence was more in favor of lipid and protein (Walker 2008). Contrast this with the algae diet in this experiment; carbohydrate provided sufficient nutrients to get the larvae to the 3rd instar earlier than the other two treatments. After the 3rd instar larvae grew bigger the necessity of protein might become more significant, which eventually slowed down the pupation. However, the algae diet produced larger adults at the end and might be sufficient for completing at least one gonotrophic cycle since its lipid level was high.

Glycogen absorption on the other hand was the lowest in treatment A. Adults from AF treatments had the highest absorption rate of glycogen. Glycogen reserves varied over a large range depending on motor and short-time feeding activities, compared to the protein and lipid reserves which had a fixed linear relationship with body size (Timmermann & Briegel 1998).

Lipids are essential for egg maturation. They also provide the offspring enhanced resistance to starvation. The storage of lipid was highest in mosquitoes from treatments F followed by treatments A. But adults from treatments A grew larger than the adults from F. Briegel *et al.* (2002) found that larger females of *Aedes aegypti* transferred most of their pre-blood meal lipid (9mcal/oocyte) into the ovaries whereas smaller females transferred a considerably lower amount (7 mcal/oocyte) of lipid and total fecundity reached 450 eggs in large females and 280 eggs in small females. Here, I observed that algae-fed larvae produced adults with larger body size, which might give them greater fecundity. Further study is required to determine whether this observation can be generalized.

Protein is an essential nutrient for an organism. In this study, the combination diet provided the more protein than F and A treatments. Larval protein increased linearly with body size, although biosynthesis revealed that 70-88% of the final protein values and 80-92% of final lipid values attained in 4^{th} instar (Timmermann & Briegel 1998). Larvae from the algae diet were deprived of protein during later instars, therefore they had delayed pupation with increased mortality and less protein reserve.

In conclusion, it appears that algae *Chaetophora* sp. had positive effect on the larval life and subsequent adult life of A*nopheles gambiae s.s.* by producing larger adults which might have increased fecundity and greater survival. Overall,

the combination food diet (AF) performed the best in terms of all other life history parameters examined here.

3.5 Literature Cited

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Table 3-1 Effect of three different diets: algae (A), fish food (F) and combination of algae and fish food (AF) on larval mortality (% of dead larvae), mean age at pupation (no. of days to pupate), and wing length (mm) of *Anopheles gambiae* s.s. * indicates a statistically significant effect (ANOVA α = 0.05)

Source	DF	Sum of squares	F ratio	p value
Larval mortality	2	1.090	21.712	<.0001*
Mean age at pupation	2	100.732	74.441	<.0001*
Wing length	2	3.955	6.885	0.0022*

Table 3-2 Effect of three different diets: algae (A), fish food (F) and combination of algae and fish food (AF) on larval mortality (% of dead larvae), mean age at pupation (no. of days to pupate), and wing length (mm) of *Anopheles gambiae* s.s. Means with different letters are significantly different (Tukey's HSD α = 0.05)

Parameter	Treatment	n	Mean ± SE
Larval mortality	Algae	80	0.887±0.018 (A)
	Algae+ Fish Food	80	0.693±0.044 (AB)
	Fish Food	80	0.937±0.005 (A)
Mean age at pupation	Algae	80	13.367±0.167 (A)
	Algae+ Fish Food	80	10.262±0.194 (C)
	Fish Food	80	16.960±0.178 (B)

Wing length	Algae	18	6.375±0.072 (A)
	Algae+ Fish Food	44	6.222±0.038 (A)
	Fish Food	12	5.825±0.170 (B)

Table 3-3 Repeated measures analysis (MANOVA) of the absorbance (nm) of body reserves (sugar, glycogen, protein and lipid) after emergence of *Anopheles gambiae s.s.* adults from three different diets: algae (A), fish food (F) and combination of algae and fish food (AF). * indicates a statistically significant effect (α = 0.05)

Source	df	F	p value
Sugar	6	6.967	0.0002*
Glycogen	6	4.538	0.0025*
Protein	6	9.791	<.0001*
Lipid	3	21.948	<.0001*

Figure Legends

Figure 3-1 Larval mortality (mean ± SD) of *Anopheles gambiae s.s.* larvae from three different food treatments; algae (A), algae & fish food (AF) and fish food (F)

Figure 3-2 The proportion of dead larvae of *Anopheles gambiae s.s.* per instar from three different food treatments: algae (A), fish food (F) and algae & fish food (AF)

Figure 3-3 Proportion (mean ± SD) of cannibalism among the larvae of *Anopheles gambiae s.s.* in three different food treatments: algae (A), fish food (F) and algae & fish food (AF)

Figure 3-4 Mean (mean ± SD) age at pupation of *Anopheles gambiae s.s.* larvae from three different food treatments: algae (A), fish food (F) and combination of algae and fish food (AF).

Figure 3-5 Mean (mean ± SD) wing length of *Anopheles gambiae s.s.* adults emerged from three different food treatments: algae (A), fish food (F) and both algae and fish food (AF)

Figure 3-6 Comparison of wing length (mean ± SD) between males and females of *Anopheles gambiae s.s.* emerged from three different types of larval food treatments: algae (A), fish food (F) and algae & fish food (AF)

Figure 3-7 Proportion of teneral sugar in *Anopheles gambiae s.s.* adults after emergence. Larvae were reared with three different diets: algae (A), fish food (F) and algae & fish food (AF)

Figure 3-8 Proportion of teneral glycogen in *Anopheles gambiae s.s.* adults after emergence. Larvae were reared with three different diets: algae (A), fish food (F) and algae & fish food (AF)

Figure 3-9 Proportion of teneral lipid of *Anopheles gambiae s.s.* adults that emerged from three separate diets: algae (A), fish food (F) and algae & fish food (AF)

Figure 3-10 Proportion of teneral protein in *Anopheles gambiae s.s.* adults after emergence from three different food treatments: algae (A), fish food (F) and algae & fish food (AF)

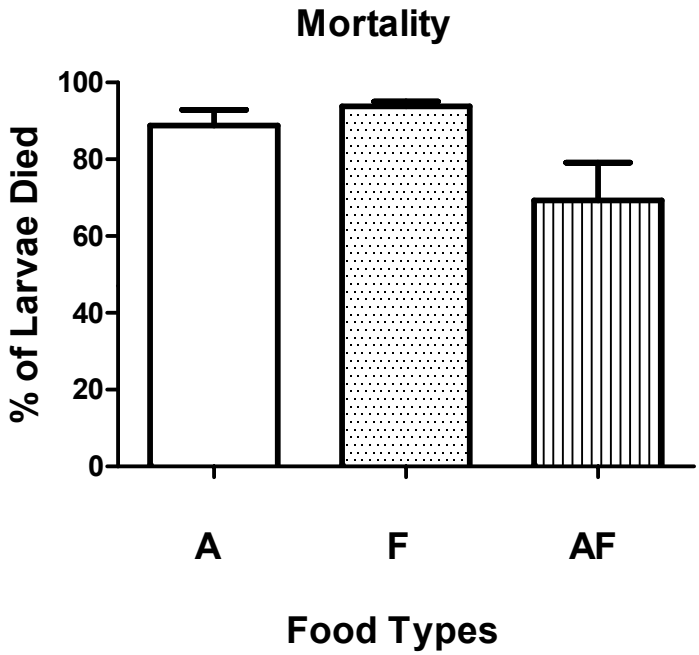

Figure 3-1

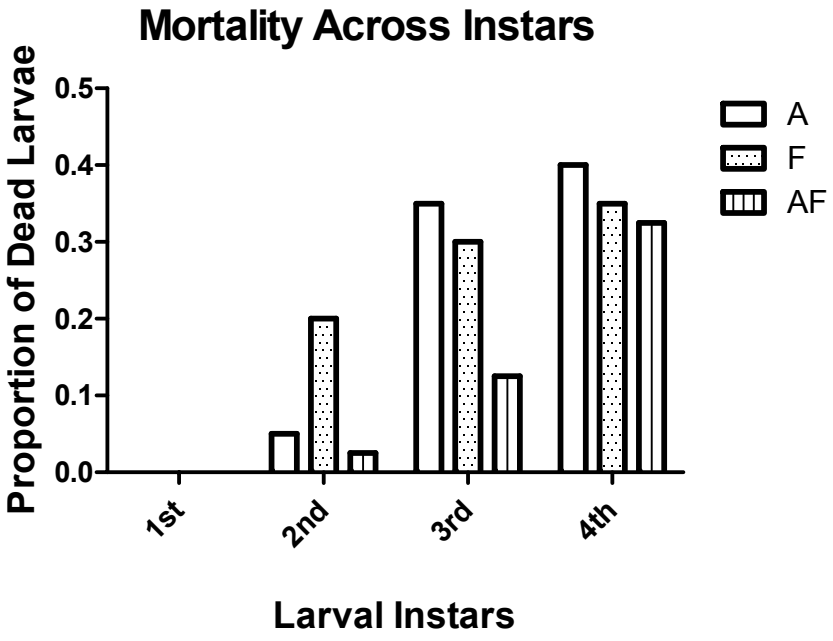
Mortality Across Instars
Proportion of Dead Larvae
0.5
0.4
0.3
0.2
0.1
0.0
1st
2nd
3rd
4th
Larval Instars
A
F
AF

Figure 3-2

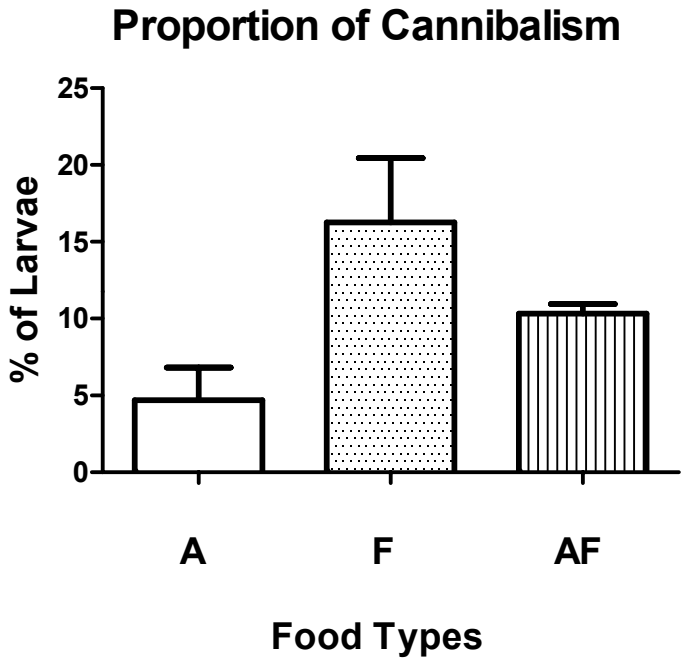
Proportion of Cannibalism
% of Larvae
25
20
15
10
5
0
A
F
AF
Food Types

Figure 3-3

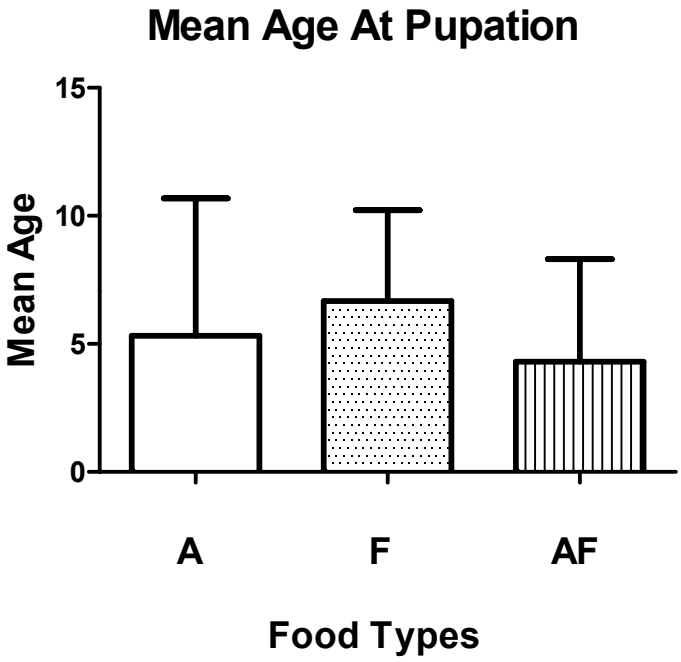

Figure 3-4

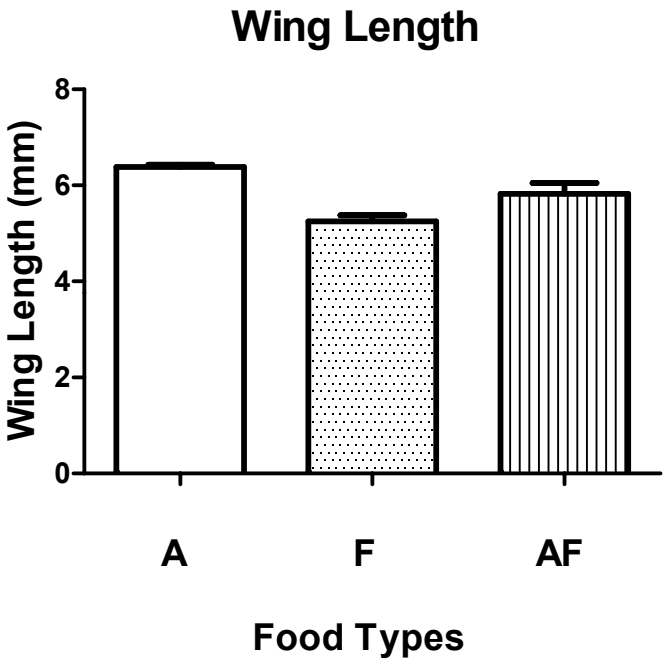

Figure 3-5

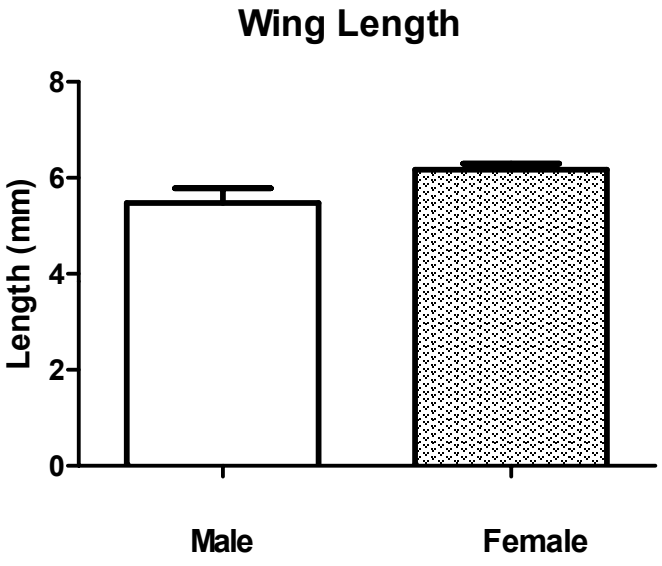

Figure 3-6

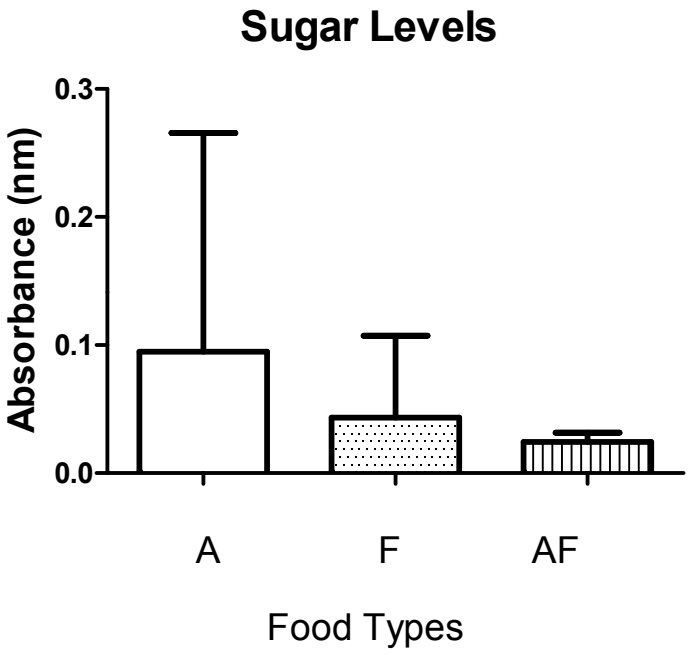

Figure 3-7

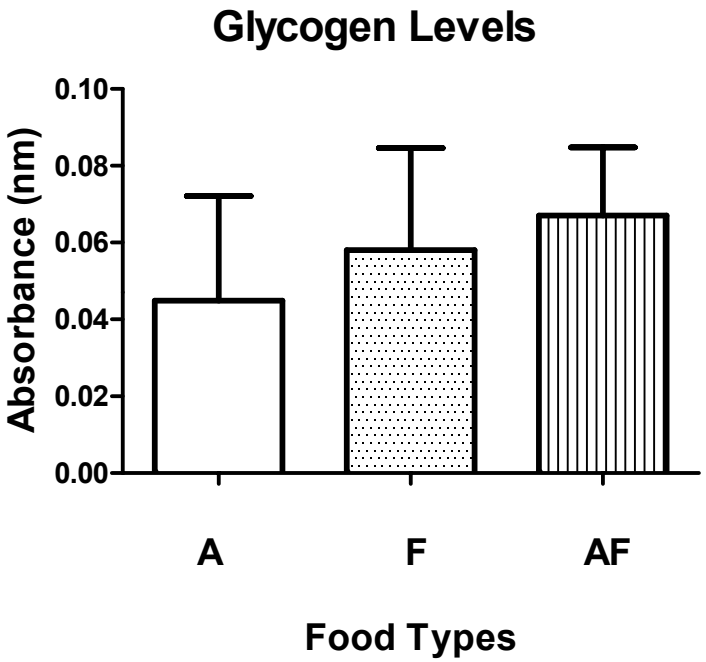
Glycogen Levels
Absorbance (nm)
0.10
0.08
0.06
0.04
0.02
0.00
A
F
AF
Food Types

Figure 3-8

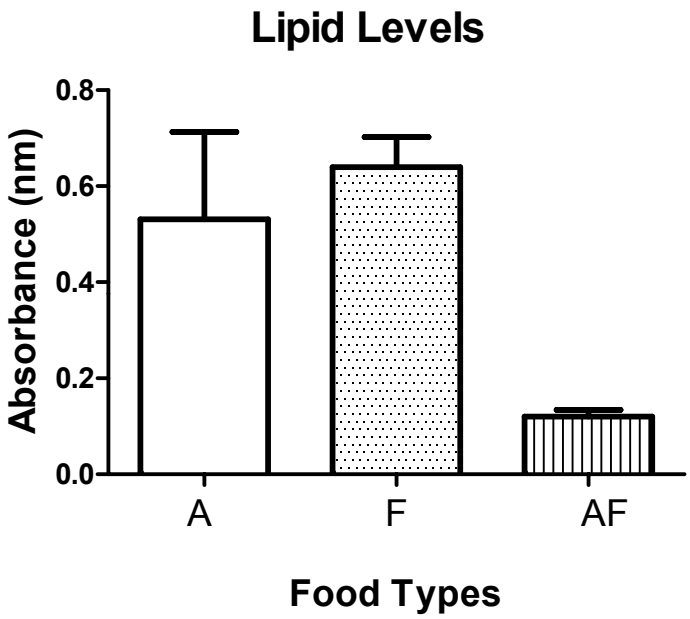

Figure 3-9

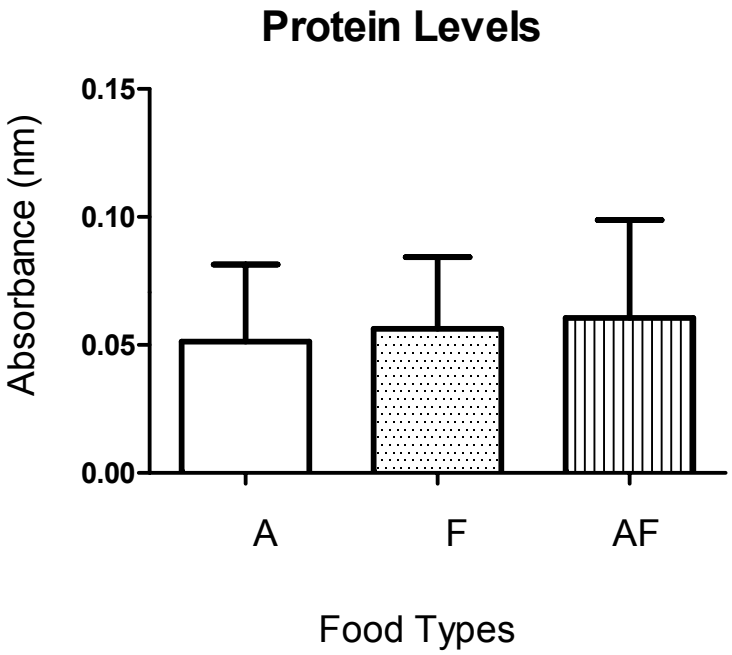
Protein Levels
Absorbance (nm)
0.15
0.10
0.05
0.00
A
F
AF
Food Types

Figure 3-10

CHAPTER 4: EFFECT OF THE PRESENCE OF OLDER INSTAR LARVAE ON YOUNGER INSTAR LIFE HISTORY TRAITS IN *ANOPHELES GAMBIAE* S. *S.* (DIPTERA:CULICIDAE)

4.1 ABSTRACT

Larval ecology has become an important factor in recent years in malaria control programs. In particular, density dependent factors can greatly impact adult mosquitoes from nursery sites. One such factor is cannibalism a common phenomenon in the larval habitat of *Anopheles gambiae s.l*. In this study, older instar larvae placed in mesh tubes were introduced to younger instars to create a predation risk cue. I examined the effect of this cannibalism cue on some life history attributes of younger instars. Larval activity of the younger instars in the presence of the older instar was also compared with the treatments older conspecifics was absent. Larval mortality and mean age at pupation was higher in the absence treatments. Larvae grew faster and larger and showed increased larval activity in the presence of cannibal's pressure compared to those of the

absence treatments. Further, adults emerged larger from the former versus the later treatments.

Keyword Index: *Anopheles gambiae s.s.,* cannibalism, predation cue, larval activity, adult body size.

3

[3] A similar version of this chapter has been prepared for submission to the Journal of Vector Ecology

4.2 INTRODUCTION

Cannibalism has long been reported among the malaria vectors in their larval stages. Balfour (1921) first reported that *An. gambiae s.l*. larvae become cannibalistic when deprived of food. Similar reports for many other malaria vectors from different parts of the world followed. Koenraadt and Takken (2003) reported that under food stressed conditions first instar larvae are consumed by fourth-instar larvae of the *Anopheles gambiae* complex. For organisms that undergo metamorphosis from a larval stage in one environment to an adult stage in a different environment, metamorphosis represents an opportunity to escape predation risk in the larval environment (Benard 2004). This kind of escape in the presence of cannibalistic older instars must have some impact on the adult body size and therefore, vector potential.

Individuals of the same age/size rarely cannibalize each other, whereas large size differences between larvae can result in an extremely high rate of cannibalism on the smaller individuals (Sherratt & Church 1994). This kind of size-dependent larval cannibalism has been observed in a number of mosquito species (Koenekoop & Livdahi 1986). In one study, Sherratt & Church (1994) found that eight–day-old fourth instar *Trichoprosopon digitatum* larvae were highly predatory on one-day-old instars of the same species, to the extent that more than two-thirds of the young instars were consumed within 24 hours. As a result, *T. digitatum* females tended to oviposit less in the habitats containing

eight-day-old larvae rather than the habitats containing one or two-day-old or no larvae.

Avoidance of cannibalism can impact reproductive success in some species. Female mosquitoes tend to avoid oviposition sites that contain potential predators, such as, non-mosquito aquatic organisms, heterospecifics or older conspecifics. Organisms that do not provide parental care following emergence of their offspring may attempt to place such offspring at safe oviposition sites, free of predators and competitors if there are choices. Thus the decision of female mosquitoes to oviposit in a safe place reduces the likelihood of cannibalism. Furthermore, the degree of cannibalism experienced in a population is generally not constant, but can vary with factors such as food level, larval density, relatedness between individuals and the size and age composition of the population (Fox 1975; Polis 1981). Thus, mosquitoes might improve their chances of ovipositing in a suitable site if they possess a more sophisticated discriminatory ability than simply detecting the presence or absence of conspecifics (Sherratt & Church 1994).

Should adults place their offspring in dangerous locations, mosquito larvae may assess level of danger and respond appropriately. Many aquatic insects including mosquito larvae use chemical alarm cues in the water to asses the risk level of predation. These cues include predator odors and alarm cues from damaged conspecifics (Peckarsky & Dodson 1980, Wisenden *et al.* 1997, Huryn & Chivers

1999, Kesavaraju & Juliano 2004, Ferrari *et al.* 2008). For instance, *Culex pipiens* mosquito larvae showed antipredator responses to the odor of predatory backswimmers *Notonecta undulata* and to alarm cues from damaged conspecifics (Sih 1986). Cues from damaged conspecifics represent a generalized response to predation cues (Chivers and Smith 1998, Wisenden and Chivers 2005).

Responses to predation (cannibalism) threat could take many forms but in each case could involve a tradeoff (e.g. reduced activity would lead to lower food intake). Tradeoffs between life history components are often mediated through behaviors (Krebs and Davies 1993). For instance, to balance increased risk of predation during periods of foraging activity with the benefit of greater food intake, many prey species decrease foraging activity in response to the presence of predators (McPeek 2004, Urban 2007). Energy and time required to avoid cannibalization may decrease the survival and increase the mortality and also have an overall impact on adult fitness.

In this study, I investigated the behavioural response and developmental time of younger instar larvae in the presence of an older conspecific as a potential predator. I also documented the generation time/developmental time of the 1st instars, larval mortality, and early or late metamorphosis in the presence of a late instar. Decreased developmental time (produces smaller and weaker adults) and increased mortality always have some effect on disease transmission. In

addition, I investigated the size of adults at emergence to see whether the larvae metamorphosed big or small in the presence of a 4th instar. Evidence showed that interactions between physiological condition (As measured by body size) and diet composition (Sugar vs. blood) affect trade-offs between somatic and reproductive functions (Briegel 1990).

In the presence of a late instar, young instar larvae of *An. gambiae* might adopt a low-risk behavior, such as less foraging, more resting etc. On the other hand, predation pressure may induce the trade-offs between reproductive success and survivorship. Larvae might metamorphose late, which means a delay in age-of-maturity at the cost of reproductive output or larvae could metamorphose early and small, and thus lower per cycle fecundity. Larvae either forage vigorously to metamorphose as a bigger adult or don't forage much to escape the predation and metamorphose as a small adult.

The possible hypothesized responses of *An. gambiae* larvae to the presence of cannibals are summarized in the table below (Table 4.1).

Table 4-1 Various adaptive hypotheses for responses of the young larvae to the presence of cannibalistic older conspecifics

Parameters	Mechanisms	Hypothesis	Null hypothesis
Larval mortality	i) Feeding	Mortality decreases when feeding increases	Feeding does not affect mortality
	ii) Growth rate	Larva grows fast to escape the cannibalism, which may decrease the mortality	Growing faster does not reduce mortality from cannibal
Age at pupation	i)Developmental time	By decreasing developmental time larva may avoid cannibalism	Short larval life does not reduce mortality from cannibals
Larval activity	i) Active search for food	Larva spends more time seeking food in order to grow fast	Increased foraging does not increase growth rate
	ii) Feeding	Feeding increases the chance of growing big	Does not affect larval life
	iii) Resting	Resting more saves the body reserves	Resting larva could be an easy prey for the older conspecifics
	v)Diving	Diving frequency should increase when predators are present. This reduces the chance of getting captured by the predator or older conspecific (Futami *et al.* 2008)	Larva acts irrespective of the presence of an older conspecifics
Pupal weight	i) Metamorphose earlier	Larva metamorphose earlier and smaller in the presence of older conspecific to escape cannibalism	Larva metamorphose irrespective of the presence of an older conspecifics
Adult body size	i) Emerging small	Extension of the pupal size response above	As above for pupae

4.3 MATERIALS AND METHODS

4.3.1 Laboratory rearing conditions

Larvae were reared in a walk-in Conviron™ growth chamber at Simon Fraser University, Burnaby, B.C., Canada, where the chamber temperature was 30˚C ± 2˚C and humidity was 75-80% RH throughout the experiment with the photo period of 14:10 (L:D).

First instar larvae were placed into 12.7cm×12.7cm×15.2cm square rearing white plastic tubs with distilled water 5cm deep. The water level was checked everyday and adjusted by adding distilled water whenever needed. A 5cm × 2.5cm round mesh tube was placed in the middle of each tub in such a way that no larva from in or outside the mesh tube could pass through it. Nutrafin ® Basix Staple Tropical Fish Food *ad libitum* was given to the larvae. The food was previously measured with the Cahn Electrobalance in microgram units.

4.3.2 Measurement of food

Food was provided on a larva/capita/biomass basis. Each larva received the same amount of food for two days in a row, (assuming the larval period of 8 days, each instar lasted two days). After the 8^{th} day, larva received the same amount as the fourth instars were provided. The food measurement was as follows:

1st instar = .003mg/larva (2 days)

2nd instar = .006mg/larva (2 days)

3rd instar = .012mg/larva (2 days)

4th instar = .024mg/larva (2 days and onward)

Measured food for each treatment was properly labeled and stored in small plastic cuvettes (1.5 ml). Half of the pupae from each treatment were placed in cages to emerge as adults, whereas, the other half were collected in cuvettes as pupae to measure the dry weight. The cages were 30cm × 30cm × 30cm Plexiglass™ cages with mesh on 5 sides and cotton sleeves to access the cages on one side. Emerged adults were collected whenever seen and placed in small cuvettes. All the labeled cuvettes were placed in a refrigerator at 4˚C for at least 2 hours to kill the adults for further study.

4.3.3 Experimental design

There were two treatment sets, one had a 4th instar larva inside the mesh tube, and the other was an empty mesh tube. The 1st instars (20 in each tub) were placed in the experimental tubs after hatching. The 4th instar larvae inside the mesh tube were taken from the regular *Anopheles gambiae s. s.* colony. After collection, the 4th instar larvae were placed in a glass bowl for half a day to starve and then placed inside the mesh tube. The rationale behind starving the 4th instar

was, starved larvae would look for food by wriggling more and might produce more chemicals by sensing that other larvae were nearby. Older conspecifics are known to release some chemical cues of predation under food stressed condition (Lima & Dill 1990; Chivers *et al*. 1996; Lima 1998; Wisenden 2000; and Kusch *et al*. 2004). The 4th instar was replaced if they pupated. Food was given everyday outside the mesh tube so that the 4th instar inside the mesh tube probably remained starved.

4.3.4 Parameters

The following parameters were evaluated during and after the experiment:

i) Larval mortality,

ii) Mean age at pupation,

iii) Larval activity,

iv) Dry weight of pupae, and

v) Wing length

i) Larval mortality:

Larval mortality from each treatment was measured by recording the number of dead larvae every day per container.

ii) Mean age at pupation

Time required for pupation of larvae from each treatment was calculated. Mean age at pupation is expressed by *t*, where,

t = No. of days to pupation × No. of larvae pupated / total larvae pupated (Lyimo *et al*. 1992).

iii) Larval activity

Larval activity was measured by visual observation twice per day but only after the larvae reached 3rd instar, since 1st and 2nd instars were very difficult to observe. The time period for each observation was 60 seconds/tub. There were two observation periods/day; one in the morning and one in the afternoon. Altogether there were 5 consecutive observations days starting after the larvae reached to the 3rd instar. Therefore, in each experiment, each experimental tub was observed 10 times. The experiment was repeated at least four times to yield an appropriate number of sets of results for statistical analysis. Responses hypothesized in Table 4.1 were measured as follows:

a) Active food seeking: Number of larvae actively seeking for food was counted.

b) Feeding: Number of larvae feeding was counted.

c) Wriggling: Number of larvae wriggling was counted in the presence and absence of the 4th.

d) Diving: Number of 3rd and 4th instar larvae diving was recorded.

e) Resting: Number of larvae stayed still either on the surface of the water or bottom of the water was recorded.

iv) Dry weight of pupae

After pupation, pupae from each treatment were collected in small, previously-weighed cuvettes. All cuvettes were labeled and placed in a rack or beaker and then put into the drying oven to desiccate over anhydrous calcium sulfate for dry weight determinations (0.001mg sensitivity on a Cahn Electrobalance) (Gimnig *et al*. 2002) at the temperature of 50˚c for at least 48 hours. All the dried pupae from the two different treatment groups were weighed separately.

v) Wing length

Wing length is considered a good indicator of body size because it is directly proportional to dry body weight (McCombs 1980). Wing length was defined as the distance from axial incision to the apical margin, excluding fringe of scales (Rohani *et al*. 2004). Wing lengths were measured by using the compound

microscope (16X magnifications on WILD Heerbrugg microscope # 69066) equipped with an ocular micrometer.

4.3.5 Statistical analyses

The mean mortality, developmental time/age at pupation, wing length, and dry weight of pupa were compared between treatments (i.e. mesh tube with 4^{th} instar vs. no 4^{th} instars) by t-test. Analysis of variance (ANOVA) was used to test the effect of the presence of an older instar on the life-history of the younger instar larvae. The sum of squares, F ratios and p-values were reported. Tukey's Honestly Significant Difference (HSD) test was conducted to determine if the effects of the two treatments on larval life were significantly different or not. The least square mean values were reported for HSD and Student's t-tests.

For larval activity, MANOVA was used to determine the interactions among the dependent variables (i.e. responses) because their expression is not independent. Since total number of larvae in different tubs varied over time, the arcsine square root transformation was used to the percentage values to normalize the test results before applying MANOVA analysis.

Statistical analyses were conducted using JMP 8.0 (SAS Institute Inc. 2008) and graphs were generated using GraphPad Prism 5.00 (Graph Pad Software Inc. 2005).

4.4 RESULTS

Larval Mortality

Mortality was higher in the absence treatments versus those with a 4th instar present; 91.88% vs. 42.08%, respectively (Fig 4.1) (Table 4.2). About 50% of larval death occurred in absence treatments before younger instars reached 3rd instar.

Mean age at pupation

Larvae with the 4th instar present in the mesh tube grew faster than did the absence. Mean age at pupation for present and absent treatments was 3.91 and 7.725 respectively (Fig 4.2) (Table 4.2).

Dry weight of pupa

Dry weight of pupae differed between the two treatment groups. The average dry weight was 0.263 mg for pupa from treatments with 4th instars versus 0.129 mg for the controls (Fig 4.3) (Table 4.2).

Wing length

Adults from the treatments with caged cannibals emerged larger than the adults from absence treatments. The average wing length of the adults from the treatments with the 4th was 3.63 mm whereas in the 4th absent treatment the average was 2.509 mm (Fig 4.4) (Table 4.2).

Larval activity

Larvae from the two treatments showed significant differences (Table 4.3, 4.4) in most of their activities, e.g. feeding, filtering/foraging time, resting, wriggling and diving. In both the treatment groups larvae rested at the edge of the experimental tubs most of the time.

4.5 DISCUSSION

This study supported the notion that older instar cannibalistic larvae have considerable effect on various life-history traits of a mosquito species. In this

study, the presence of an older instar *Anopheles gambiae s.s*. larvae significantly affected some of the life history parameters of the growing larvae.

Larval mortality was significantly higher (50%) in the treatments with no 4th instars. The possible explanation of this unexpectedly high mortality could be the benign environment of the habitats, not competitive or threatfull enough to have the pressure of metamorphosing earlier. There were some missing larvae too, which could be due to cannibalism within cohorts. In a previous study, Koenraadt *et al*. (2004) found that the 4th instars were responsible for cannibalizing dead, younger instars (when the 4th was exposed to the younger instars). In this study, younger *Anopheles gambiae s.s.* larvae were indirectly exposed to the 4th, hence not subjected to cannibalism but showed more activity e.g., fed more, foraged more etc., which led them to grow fast and leave the aquatic environment sooner.

Mean age at pupation decreased significantly in the presence of a 4th instar **(**Fig. 4.2) because larvae probably grew faster either under the predation or cannibalism pressure. However, Knight *et al*. (2004) found that in the presence of predator and competitor *Anopheles quadrimaculatus* larvae took at least 2 days longer to pupate. But several other studies stated that organisms optimize their size and age at metamorphosis in response to the predation risk during the larval period (Koenraadt and Takken 2003, Werner 1986, Rowe & Ludwig 1991). This study also supports the latter hypothesis of reducing the developmental time to

escape predation. Thus the results met the predictions (Table 4.1) that by decreasing developmental time larva may avoid cannibalism.

Pupal weight differed greatly between two treatment groups. As larvae fed more in the presence of 4th instar, they metamorphosed as larger pupae at the end. As a result adults emerged larger from the treatments with cannibalism risk. This result agrees with other previous studies, where they stated that, rapidly growing larvae had a competitive advantage over small, slow ones (Lyimo *et al*. 1992), and the treatments produced larger adults in the presence of older instars (Wallace and Merritt 1999). It is well known that larger adults may have extended life span and greater fecundity as well as vector capacity (Reisen *et al*.1984; Hawley, 1985a, Nasci, 1986; Packer & Corbet, 1989; Briegel, 1990, Roitberg & Gordon 2005).

Larval activity

There are published observations wherein larvae of some mosquito species reduce their activity and behavioral responses in the presence of predators. One of these studies showed that *Toxorhynchites rutilus* larvae preyed on another container dwelling mosquito larvae *Ochlerotatus triseriatus* (Kesavaraju 2007). *O. triseriatus* larvae reduce movement, foraging, and time below the surface, and increase the frequency of resting at the surface when *T. rutilus* larvae are present in the same habitat. Similarly, larvae of the tree hole mosquito *Aedes triseriatus*

reduced filtering, browsing, and time below the surface in response to water that had held a feeding larva of the predator *Toxorhynchites rutilus* (Juliano and Gravel 2002).

A low risk behavior allocates more energy for stationary feeding in exchange of the reduction of movements to prevent loss of energy. The prediction of this study, larva would adopt low-risk behavior in the presence of a predator or older instar was not met. Instead larvae fed more and in doing so increased the chance of growing large and escape sooner from the unwanted environment. Larvae also foraged and filtered more in the presence of 4^{th} instar, whereas previous studies (Kesavaraju and Juliano 2008, Juliano and Reminger 1992) found the opposite results.

Larvae increased their movement significantly in the presence of the 4^{th} (Fig. 4.5). Ferrari *et al*. (Unpublished data) stated that in response to the predation cues, larvae either dramatically increase their number of wriggle movements, representing fleeing behavior, or dramatically decrease their number of wriggle movements, representing freezing behavior. The result of this study supported the fleeing behavior more than the freezing behavior to avoid danger in the risky environment.

Unlike all other activities, there was no effect of predation risk on diving. Mosquito larvae and pupae dive when their habitat is mechanically disturbed

(Gilles and De Meillon 1968, Clements 1999). Mosquito larvae also dive in the presence of predator (Sih 1979, Juliano and Gravel 2002). But in this study larvae did not dive much in the presence of a predator. Diving ability differs among the immature life stages of *An. gambiae* (Tuno *et al*. 2004, Futami *et al*. 2008). In disturbed water, 2nd instars dove more than any other instars. In this study only the 3rd and 4th instars were observed for the diving behavior and the response was not as predicted (Table 4.1).

Prolonged exposure to the predators across generations should select for adaptive behavioral responses. These responses should be threat sensitive to optimize energy budgets and survival. According to the threat sensitivity hypothesis, prey will alter their predation avoidance responses according to the magnitude of the threat, so that as risk of predation increases, time spent on predation avoidance behavior would increase too (Helfman 1989). Juliano and Gravel (2002) also stated that, consistent presence or absence of predators can select rapidly for divergence in prey behavior, including facultative behavioral responses to predators. Cannibalism in *An. gambiae* is an established phenomenon through generations, which might explain the quick altered behavioral responses in the presence of older instars. It is likely that cannibalism has been a common occurrence in the colony used in this experiment.

What kind of cues did larvae receive?

Aquatic organisms may respond to the water-carried chemical cues produced by the predators present in the same habitat by changing their behavior. Previous studies showed that aquatic prey can detect the presence of a predator via water-borne chemical cues (Lima & Dill 1990, Chivers *et al*. 1996, Kats and Dill 1998, Lima 1998, Wisenden 2000, and Kusch *et al*. 2004).

This study did not focus on the kind of cue larvae received that ultimately let them to change their behavior. However, there were some indications as to the nature of the cue that larvae received. Chemical cues were the most likely given that olfaction is the primary sensory modality in invertebrates. Visual cues were possible, however in this experiment the older conspecific was hidden inside the mesh tube. Although the pore size was not microscopic, therefore younger instars might be able to see the larger conspecifics and assume that the competitor or predator was near by. Another possibility was vibration; the younger instars might detect the vibrations in the water created by the older instars in such small containers. Older instar moved/wriggled frequently as the space created by the mesh tube was small/narrow, not enough to move around freely compared to the larval tray it was removed from where the sense of water depth and periphery was bigger than the tiny mesh tube. Older instars also moved frequently in search of food due to lack of food provision inside the mesh tube. All these movements caused vibration on a large scale. It is not clear however whether vibrations profiles from larger larvae can be discriminated from those of smaller, less dangerous individuals. Therefore, it is very hard to

determine which particular cue played the most significant role here. Future studies might reveal the answer.

In conclusion, this study demonstrated that risk of cannibalism can have significant impacts on the development of growing larva in a mesocosm. Instead of metamorphosing earlier and smaller larvae chose to grew faster and larger in the presence of an older conspecific to escape cannibalism. As a result emerging adults were bigger which might lead to greater fecundity and vector capacity. Of course, in the real world some of those individuals will be cannibalized leading to lower recruitment but of larger individuals.

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Table 4-2 Effects of the presence of an older instar (4th instar) larva on the mortality (% of dead larvae), mean age at pupation (no. of days to pupate) and wing length (mm) of the *Anopheles gambiae s.s.* larvae. * indicates result is statistically significant (ANOVA, α = 0.05).

Parameters	Sum of squares	F ratio	p value
Mortality	17018.153	49.072	<.0001*
Mean age at pupation	113.863	8.115	<.0001*
Dry weight of pupa	0.362	18.815	<.0001*
Wing length	13.871	106.560	<.0001*

Table 4-3 Effect of the presence of the older instar larvae on the larval activities of *An. gambiae s.s*. * indicates result is statistically significant (MANOVA, α = 0.05).

Larval activity	Sum of squares	F ratio	p value
Resting	6076.891	43.832	<0.0001*
Feeding	1095.497	17.679	0.0002*
Filtering/Foraging	1494.732	36.964	<0.0001*
Wriggling	984.007	4.311	0.0471*
Diving	139.656	0.613	0.440

Table 4-4 Means of different larval activities of the larvae of two different treatment groups, one with 4th instar as a predator inside the mesh tube versus the treatment with no 4th instar inside the mesh tube. Means with different letters are significantly different (Tukey's HSD, $\alpha = 0.05$).

Parameters	Treatment group	Mean ± SE
Resting	With the 4th instar	46.054 ± 2.567 (B)
	Without the 4th instar	68.384 ± 2.130 (A)
Feeding	With the 4th instar	16.359 ± 1.632 (A)
	Without the 4th instar	9.110 ± 0.821 (B)
Filtering/Foraging	With the 4th instar	20.795 ± 1.485 (A)
	Without the 4th instar	10.241 ± 0.965 (B)
Wriggling	With the 4th instar	15.090 ± 1.146 (A)
	Without the 4th instar	11.385 ± 1.697 (B)
Diving	With the 4th instar	1.698 ± 0.690 (A)
	Without the 4th instar	0.877 ± 0.405 (A)

FIGURE LEGENDS

Figure 4-1 Larval mortality of *An. gambiae s.s.* larvae between two treatments, one with an older instar larva as a possible predator to the younger instars in a mesh tube and the other treatment without an older instar

Figure 4-2 Effects of the presence of an older instar larva on the mean age at pupation of *An. gambiae s.s.* larvae

Figure 4-3 Differences in the dry weight of pupae of *Anopheles gambiae s.s.*, from two different treatments, one with an older instar larvae and the other had no older instar larvae inside the mesh tube

Figure 4-4 Effects of the presence of an older instar larva in the larval environment on the emerging adult's wing length (mm) of *An. gambiae s.s.* larvae

Figure 4-5 Differences in larval activity (Resting, feeding, foraging, wriggling and diving) in between two treatments, in one treatment there was a 4^{th} instar larvae inside the mesh tube as a possible threat to the younger instars, the other treatment had none

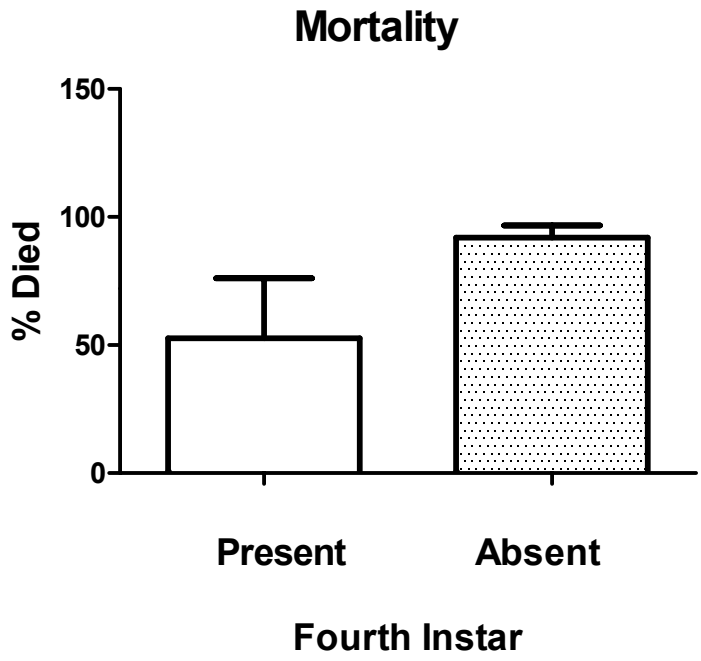

Figure 4-1

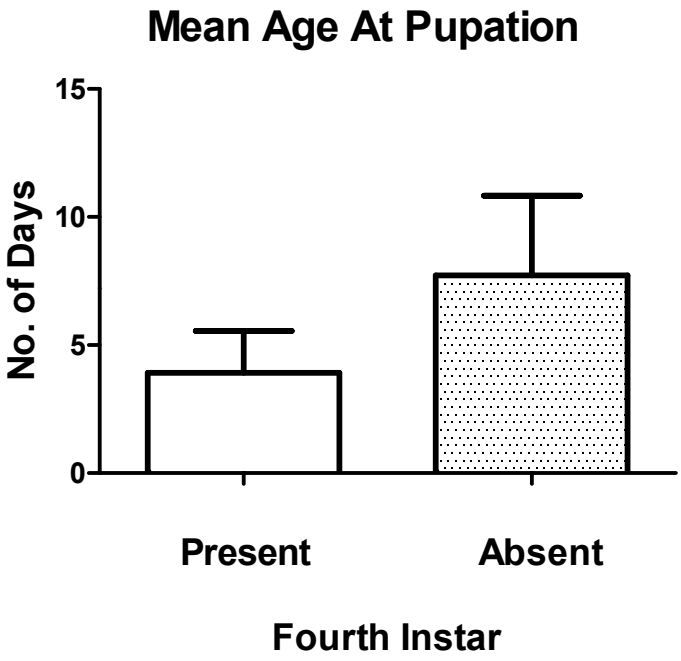
Mean Age At Pupation
No. of Days
15
10
5
0
Present
Absent
Fourth Instar

Figure 4-2

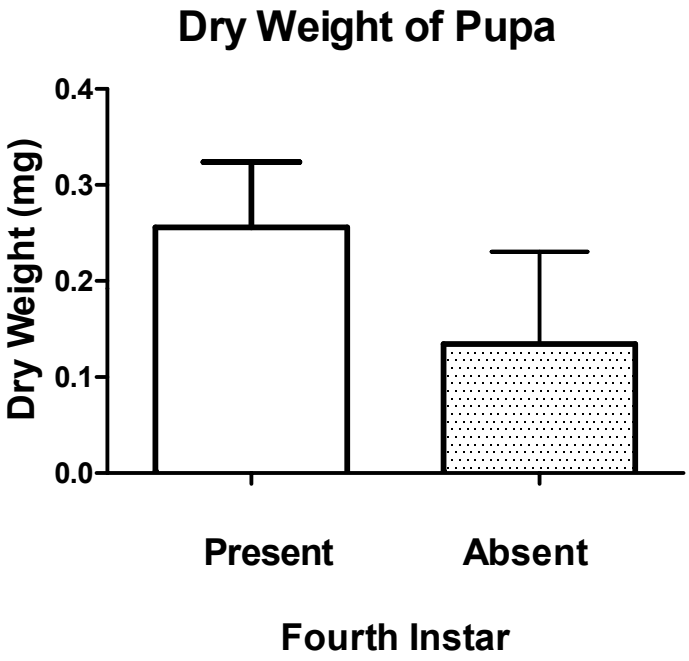
Dry Weight of Pupa
Dry Weight (mg)
0.4
0.3
0.2
0.1
0.0
Present
Absent
Fourth Instar

Figure 4-3

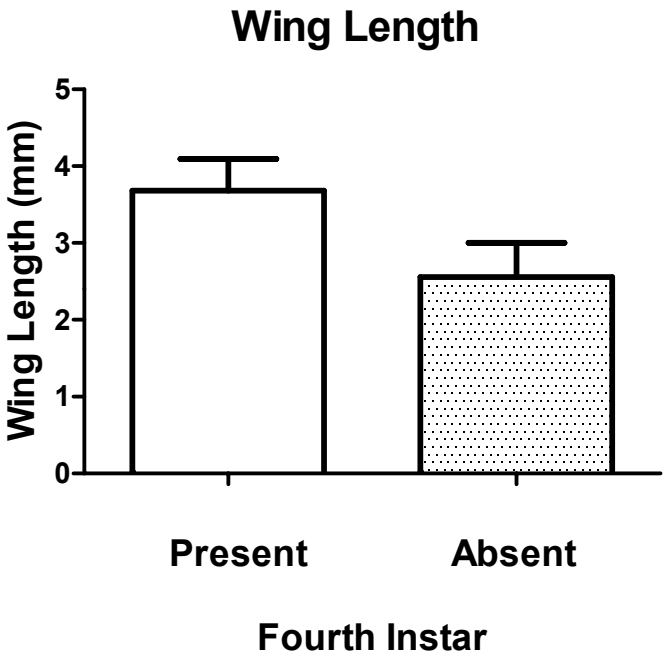
Wing Length
Wing Length (mm)
5
4
3
2
1
0
Present
Absent
Fourth Instar

Figure 4-4

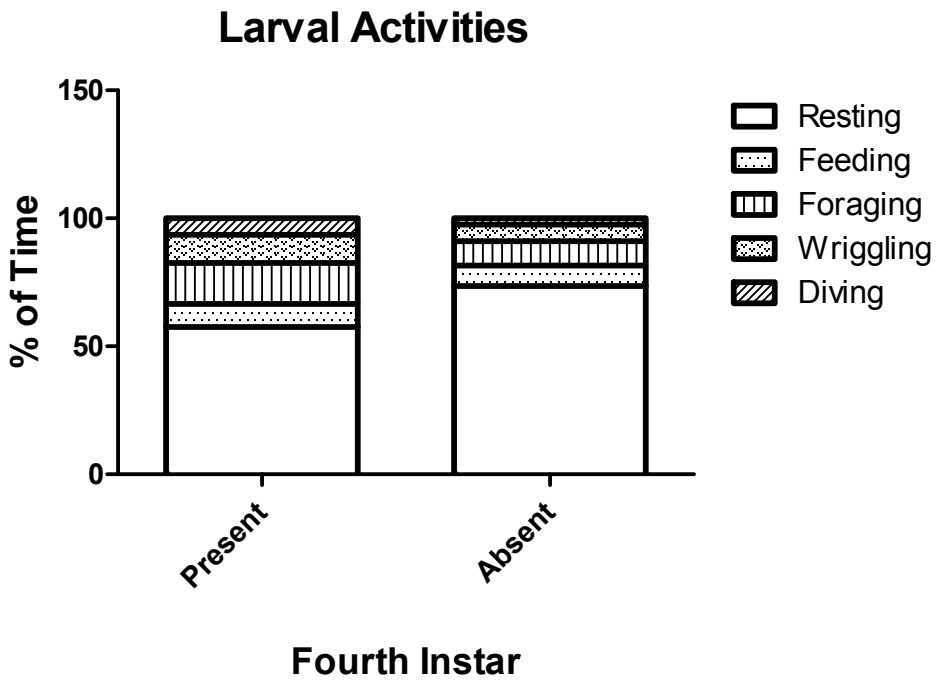
Larval Activities
150
100
50
0
% of Time
Present
Absent
Fourth Instar
Resting
Feeding
Foraging
Wriggling
Diving

Figure 4-5

CHAPTER 5: SUMMARY OF MAJOR FINDINGS AND SUGGESTED FUTURE STUDIES

In this study I attempted to determine the effect of various larval environments on adult life. I asked three major questions:

(i) How does larval density affect larval life and adult life?

(ii) Do adults deriving from different nutritional environments differ in terms of larval development, which could affect body reserves, and body size?

(iii) What effects do older cannibalistic conspecifics have on the life history parameters of younger instar larvae of *Anopheles gambiae s.s.*?

To answer these questions, I conducted a series of experiments that evaluated the impact of the above manipulations on some life history parameters, such as larval mortality, mean age at pupation, larval activity, somatic reserves etc. of *An. gambiae s.s*.

5.1 SUMMARY OF MAJOR FINDINGS

5.1.1 Chapter 2

This study had three sets of experiments with varying larval density, amount of food and tub size. In first set, larval density and tub (i.e. nursery site) size were fixed while food amount was variable. In the second experiment, larval density varied with fixed amount of food and tub size. In the third experiment, food amount and density were fixed within a large container.

In experiments 2 and 3, larval mortality increased with larval density. For experiment 2 where container size was small, larval density had negative effect on mortality but positive effect on the length of larval period suggesting that lower density slowed down the developing time of the larvae. Female biased sex ratio in low density treatments was also observed. In the third experiment, adults from low density had shorter larval period than the high density.

5.1.2 Chapter 3

This study presented the effect of two kinds of diets on larval development. Nutritional composition of adults was measured from larvae subjected to different diet treatments. Algae *Chaetophora* sp. was used against Nutrafin fish food. The combination of algae and fish food diet performed better in case of most of the

life history attributes than the above single food diets. Larval death occurred mostly in the algae treatment compared to the fish food and the combination food. Insufficient amount of food might be one of the reasons of the high larval death in algae treated larvae. Proportion of cannibalism, however was higher in control treatment.

The combination food had lower mortality and mean age at pupation probably because this complex diet provided both protein and carbohydrates. Regarding body size, adults from algae fed larvae grew significantly bigger than the combination food treatment and the control treatment. Body reserves analysis showed high sugar and lipid reserves at eclosion in algae fed larvae, which supports the positive correlation between sugar based diet and body size.

5.1.3 Chapter 4

This study had two sets of treatments, one set had a fourth instar larva separated inside a mesh tube as a possible threat to the first instar larvae in the experiment tubs and the other set had no fourth instar inside the mesh tube. Larval mortality and mean age at pupation were significantly lower in the treatments with the predator that was likely due to cannibalism pressure on the younger instar larvae. Pupa and adults from the predator treatments were also significantly larger than the control treatment. Larval activity such as feeding, foraging, filtering, wriggling,

etc. also increased with the older instar present in the experiment. From this result, I concluded that younger instars obtained some kind of cue of possible danger, which drove them out of the threatened environment quicker by growing faster with increased activity and body size.

In conclusion, it can be said that, density, nutrition and the presence of cannibalistic predators had significant effects on larval and subsequent adult life. Although in field condition the whole scenario could be different.

5.2 SUGGESTED FUTURE STUDIES

In this study, I focused on larval habitat questions where *Anopheles gambiae s.s.* was the focal species; factors like types of food, larval density, larval interactions, predation etc. appear to act in concert with the habitat's physical properties such as, size, water volume etc. to produce poor or good quality adults. Hence, the complex dynamics of larval interactions in the environment and with conspecifics leads to many questions regarding vector compatibility of subsequent emerging adults. Some suggested future studies are as follows:

- This experiment was conducted by using larvae of *Anopheles gambiae s.s.* alone. Interactions with other mosquito species at the larval stage could be

conducted in future to see the impact of multiple species interactions on the life history parameters of both larvae and adults.

- Gut content analysis could reveal the proportion of cannibalism occurring among the conspecifics on a per instar basis. For instance, fourth instar larvae presumably are more cannibalistic than the second instar. Such information would be useful for larval control measure of the field population where a habitat contains larvae of different instars and different species.

- Fitness of adults that emerged from different larval habitats containing different kinds of food can be evaluated by comparing the survivorship and fecundity of the females. Large or small body size, biting persistence and biting frequencies of females will determine if it is a good or poor disease transmitter.

- Male competence from low and high larval density and from algae and non-algae food sources could be determined at by examining number of successful inseminations of newly emerged male adults. The experiment could be done by placing individual males from the different treatment classes in cages with females of same age. These females will be dissected after one day exposure to the males. The number of females with sperm would be counted and compared between treatments.

- Future studies should also measure somatic energy reserves (Sugar, glycogen, lipid and protein) for all the four larval instars. Comparison of body reserves between the instars could be done to see the pattern of changing the body reserve over the instars depending on the type of food larvae consumed from the habitats and the type of body reserve needed for larvae over the instars (more protein required in later instars). For example, algae-fed larvae had more sugar and lipid reserves than fish food fed larvae in this study. These data would provide information on which nutritional stress causes the high mortality or which nutritional reserves vary the reproductive ability of females and survival for both sexes.

GLOSSARY

Anthropophilic

Bloodsucking arthropods, such as, mosquitoes that has strict preference to human blood than other animals.

Cannibalism

Cannibalism is a unique class of interaction between individual of a species where one individual get killed and eaten by its conspecific, often in an environment where resources are limited.

Conspecific

When two or more individual organisms belong to the same species they are conspecific to each other. Conspecific organisms share resources in one ecological niche.

Fecundity

The reproductive rate of an organism is termed as its fecundity. It is more equivalent to fertility.

Holometabolism

Holometabolism is complete metamorphism. This is a term applied to insect groups to describe the specific kind of insect development which includes four life stages - as an egg, a larva, a pupa and an adult. This type of development gives the insects the unique advantage of being able to inhabit different ecological niches because of the morphological differences in the different stages of their life cycle.

Instar

This is a larval developmental stage of arthropods between each moult or ecdysis, until sexual maturity is reached.

Oviposition

Oviposition is the process of laying eggs by oviparous organisms.

Predation

Predation describes a biological interaction where a predator of one species feeds on its prey of another species. Predators may or may not kill their prey prior to feeding on them, but the act of predation always results in the death of the prey, and is never to its benefit. The key characteristic of predation is the predator's direct impact on the prey population.

Protandry

In protandous insects, males precede females in emergence into a seasonal breeding population, males are generally smaller than females, the former sex trading body mass for the sake of earlier eclosion. Protandry is a reproductive strategy of males resulting from competitions for mates.

Scramble competition

A type of competition where the resource is inadequate for individuals to fit their needs for growth and reproduction.

Teneral reserve

A short maturation period following emergence from the pupal casing, usually lasting less than 12 hours. Physiological and behavioral changes such as, hardening of the cuticle, degeneration of larval tissues, increase in size of flight muscles, sexual maturation of males, and development of female preference for human odours.

Vectorial capacity

The potential of a mosquito population to transmit the pathogen. It can be described mathematically by the equation: $C = ma^2p^n / -\log p$, where m is the number of female mosquito per night, n is the incubation period of pathogen in the mosquito, and p is the daily survival rate of mosquitoes.

Vector competence

Vector competence refers to the ability of arthropods (e.g. mosquitoes) to acquire, maintain and transmit microbial pathogen. The pathogen must survive or even develop in arthropod tissues, such as hemolymph, muscles or the reproductive system; and finally, the pathogen must penetrate the salivary glands for injection into a new host.

APPENDIX

Biochemical Analysis of Teneral Reserve (Protein, Lipid, Glycogen and Carbohydrate)

Part 1: Protein extraction and quantification by using Bradford Assay

Dried mosquitoes of each treatment were crushed in centrifuge tubes containing 100 µl phosphate-buffered saline (PBS) solution. One mosquito per centrifuge tube was crushed with a glass rod/pestle to the finest possible. The pestle was then rinsed with 100 µl PBS solution into the same centrifuge tube that gave a total volume of 200 µl of solution in total. Then the solution was centrifuged for 1 minute at 8.6 × g. 30 µl of supernatant was transferred to a clean, labeled, 16× 125 mm glass test tube. The remaining supernatant and pellet was stored in -80˚c freezer for Anthrone and Vanillin assay.

Bradford reagent was warmed in room temperature for about 30 minutes. A Quick-Start™ Bradford Protein Assay Kit from Bio-Rad Laboratories was used for determining the amount of protein in each sample. A standard curve was prepared from bovine gamma globulin, with concentrations of 0, 1.5, 2.5, 5, 10, 15, 20, 30 and 40 µg/ml. 1 ml of Bradford reagent was added to each tube both

sample and standard. All the tubes were put into a test tube holder rack and vortexed briefly and incubated at room temperature for 5 minutes.

Absorbance of both standard and sample were read on a Beckman Du 640 spectrophotometer at a wavelength of 595nm. A standard curve was made from the standards by plotting µg of protein vs. absorbance. The amount of protein in µg contained in each 30 µl sample was calculated from the standard curve. The value was multiplied by 6.667 to calculate the amount of protein in µg contained in the whole mosquito. This correction was required because only 30 µl of the original 200 µl was used for the Bradford assay.

Part 2: Carbohydrate and lipid extraction

The remaining 170 µl of stored samples from Bradford assay were thawed at room temperature to extract and quantify the amount of carbohydrates (glycogen and sugars) and lipids from each mosquito. The procedures were described by Van Handel (1985a, 1985b) and Van Handel and Day (1988). Thawed samples were vortexed again to resuspend the pellet, and then transferred to a 15 ml centrifuge tube. 200 µl of 2% Na_2SO_4 (w/v, in dH_2O) was pipetted into the 15 ml centrifuge tube. Then the sample (supernatant and pellet) was transferred to the centrifuge tube using a Pasteur pipette and vortexed. Pasteur pipettes are 5 ¾´´. Mixing with the sodium sulfate solution dissolved all the carbohydrates in the sample (Van Handel and Day 1988). A solution of 1:1 chloroform: MeOH (using

the solution to rinse the microcentrifuge tube that contained the sample) was added to each sample to give a total volume of 3 ml and the samples were vortexed. Adding chloroform-methanol mixture to the sample dissolved lipids and precipitated glycogen, while leaving other carbohydrates (sugars) in solution (Van Handel and Day 1988). Samples were then centrifuged at 6000 rpm for 10 min (make sure deceleration on centrifuge is at 1). The pellet contains glycogen (and carcass), the supernatant contains lipids and the remaining carbohydrates. As the pellet is very fragile the samples were carefully handled. The supernatant, containing lipids and sugars was then transferred to a new centrifuge tube. The tube containing the pellet (glycogen) was set aside for analysis using the hot anthrone assay. The tube containing the supernatant was topped up with distilled water to a total volume of 5 ml. After adding the dH_2O two layers were formed; the top layer and the precipitate between the two layers contained the remaining carbohydrates dissolved in methanol and water and the bottom layer had lipids dissolved in chloroform (Van Handel and Day 1988). All samples were then vortexed and centrifuged at 3000 rpm for 10 minutes. The top layer (carbohydrates) and precipitate (white and cloudy layer) was transferred to a clean, labeled 16×125 mm glass test tube. The test tubes were placed in a hot water bath at 100˚c and evaporated to dryness. Once evaporation was complete the samples were removed from the hot water bath and set aside for analysis using the vanillin assay. The bottom layer was transferred to a different clean, labeled glass tube.

Part 3: Anthrone assay (quantification of glycogen and carbohydrates)

Glass test tubes containing carbohydrates were placed in a hot water bath at100ºC and evaporated until ~ 200 µl is left. Anthrone reagent was prepared as described by Van Handel (1985a). Anthrone reagent changes color from yellow to green when added to samples containing sugars and can be used in conjunction with a standard curve to quantify the amount of sugar and glycogen in a mosquito (Van Handel 1985a). A standard curve for glucose was prepared by placing 10, 25, 50, 100, 150 and 200 µl of 1mg /ml glucose (in 25% ethanol) into 16×125 mm glass test tubes. A blank was also prepared by pipetting 200 µl of 25% ethanol into a test tube. Anthrone reagent was added to each samples, carbs in the glass tubes and glycogen in the centrifuge tubes, and each standard (including the blank) to give a total volume of 5ml. All samples and standards were vortexed and then incubated in a water bath at 100 ºC for 17 minutes. Samples and standards were placed on ice immediately following incubation to stop the reaction and allowed to cool on ice.

Absorbance of the blank, standards and samples were read on a Beckman Du 640 spectrophotometer at a wavelength of 625 nm. A standard curve was generated from the blank and standards by plotting µg of glucose vs. absorbance. The amount of sugars or glycogen in µg contained in each sample

was then calculated from the standard curve. This value was then multiplied by 1.176 to calculate the amount of sugars or glycogen contained in the whole mosquito. This correction was required because only 170 µl of the original 200 µl was used for the hot anthrone assay.

Part 4: Vanillin assay (quantification of lipids)

Glass tubes containing lipids were placed in a hot water bath at 100 ºC and evaporated the solvent to dryness. Vanillin reagent was prepared as described by Van Handel (1985b). This reagent changes color from colorless to pink when added to samples containing lipids and can be used in conjunction with a standard curve to quantify the amount of lipid in a mosquito (Van Handel 1985b). A standard curve for lipids was prepared by placing 10, 25, 50, 100, 150 and 200 µl of 1mg/ml lipid (vegetable oil in chloroform) into 16× 125 mm labeled glass test tubes. A blank was also prepared by pipetting 200 µl of chloroform into a test tube. The blank and standards were then evaporated to dryness in a hot water bath at 100 ºC. 200 µl of concentrated sulphuric acid H_2SO_4 was added to each tube (blank, samples and standards). Heat all these tubes in hot water bath at 100ºC for 10 minutes. All tubes were then cooled for a few minutes at room temperature. 4.8ml of vanillin reagent (total volume of 5 ml) was added to each tube (blank, sample and standard). The tubes were vortexed. The samples were incubated at room temperature for 5 minutes.

Absorbance of the blank, standards and samples were read on a Beckman Du 640 spectrophotometer at a wavelength of 525 nm. A standard curve was generated from the blank and standards by plotting µg of lipids vs. absorbance. The amount of lipids in µg contained in each sample was then calculated from the standard curve. This value was then multiplied by 1.176 to calculate the amount of lipids contained in the whole mosquito. This correction was necessary because only 170 µl of the original 200 µl was used for the vanillin assay.

Preparation of the Reagents

Anthrone reagent :

- Measure 150 ml of dH_2O into a 2l flask and place flask on ice (in the fumehood)
- Slowly add 380 ml of concentrated sulfuric acid, swirling and allowing solution to cool, add about 10 ml at a time until half of the sulfuric acid is added, then add about 20-30 ml at a time (always add acid to water, never add water to acid)
- Add 750mg of anthrone to the diluted sulfuric acid, swirl to mix
- Store in amber bottle in fridge (light and temperature sensitive)
- Stable for a few weeks

Vanillin reagent :

- Add 600 mg of vanillin to 100 ml hot dH_2O, swirl to mix
- Add 400 ml of 85% phosphoric acid, swirl to mix
- Store in amber bottle (light sensitive)
- Stable for a few months

I mg/ml Glucose:

- I mg/ml glucose in 25% EtOH

1 mg/ml Lipid:

- 1 mg/ml of vegetable oil in chloroform
- Store in amber bottle (light sensitive)

2% Na_2SO_4 :

- 2 g in Na_2SO_4 100 ml dH_2O

Chloroform : MeOH (1:1)

- Mix 100 ml chloroform and 100 ml dH_2O
- Store in amber bottle (light sensitive)