

**TREHALASE INHIBITION IN *Aedes aegypti***

by

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B.Sc., Laurentian University, 2005

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## ABSTRACT

*Aedes aegypti* is the principal vector of Dengue virus, the most important mosquito-borne viral disease affecting humans. Infection of *Ae. aegypti* larvae by the trematode *Plagiorchis elegans* leads to a disruption of carbohydrate metabolism by preventing the conversion of trehalose to glucose, and the production of an oviposition deterrent compound. This thesis examines the dose-dependent effects of a trehalase inhibitor, Validoxylamine A (VAA), on *Ae. aegypti*. VAA had no noticeable effect on larval mortality but emerging adults were unable to fly. The hemolymph of VAA exposed larvae had increased trehalose levels, reduced glucose concentrations, and increased lipid content compared with controls. In addition, adult mosquitoes that ingested VAA laid significantly fewer eggs than controls. Parasitism by *P. elegans* did not reduce the energy reserves of larvae significantly, but influenced mosquito carbohydrate biochemistry. The concept of utilizing trehalase inhibitors for insect pest management is discussed.

**Keywords:** *Aedes aegypti*; *Plagiorchis elegans*; Validoxylamine A; Trehalase; Energy Reserves; Trehalose.

“Bugs are not going to inherit the earth. They own it now. So we might as well  
make peace with the landlord.”  
~T. Eisner

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# CHAPTER 1: INTRODUCTION

## 1.1 Mosquitoes, their importance, and their control

Mosquitoes transmit a number of parasites that cause significant human morbidity and mortality worldwide. The diseases caused by these parasites include Encephalitis, Malaria, and Dengue Fever among others. Due to their role in transmitting human pathogens, mosquitoes have been considered one of the most dangerous animals on earth. Malaria, caused by *Plasmodium* spp., is transmitted by anopheline mosquitoes and is responsible for over one million deaths per year, or one child every 30 seconds (WHO 2007), although the true numbers are probably much higher than reported (Greenwood et al. 2005). Malaria also causes an average loss of 1.3% annual economic growth (or US\$12 billion) due to worker illness in countries with intense transmission (Greenwood et al. 2005; WHO 2007). Currently there is no vaccine available, and plasmodia are becoming resistant to the drugs commonly used to treat Malaria (Greenwood et al. 2005). Two other major diseases, Dengue and Yellow Fever, transmitted by the Yellow Fever mosquito *Aedes aegypti*, affect approximately 250 million people each year (WHO 2001, 2002). The anthropophilic behaviour and cosmopolitan distribution of *Ae. aegypti* has put two fifths of the world's

population at risk of getting Dengue virus (WHO 2002; Gubler 2004). Dengue is caused by one of four closely related viruses, and although recovery provides immunity to one serotype, subsequent infections with a different serotype may result in more serious clinical symptoms (WHO 2002). There is a vaccine available for Yellow Fever, but it is not being used efficiently for primary protection (Gubler 2004). Between January and September 2008, there were five outbreaks of Yellow Fever in Africa and South America (WHO 2008). There is no remedial treatment available for Dengue or Yellow Fever (WHO 2001, 2002). Because of the problems associated with treating these major disease-causing-pathogens, much emphasis has been placed on eliminating the mosquito vectors.

Various programs have been developed to eliminate or reduce mosquito populations worldwide, from draining various wetlands in British Columbia (Belton 1983), to spraying insecticides in developing countries. The current trend is to use an integrated pest management (IPM) program that combines various strategies and methods in an effort to control mosquitoes without causing significant environmental damage. Tactics involve cultural methods, such as source reduction, combined with the use of synthetic and naturally derived insecticides when necessary. However, there are significant limitations to the IPM tactics currently available. There are very few commercially available biological control agents available for mosquito control and many of the possible agents are only effective under specific environmental conditions. Some of the older cultural practices, such as draining wetlands, are no longer acceptable,

while other forms of cultural control require the cooperation of the public, which often is difficult to maintain in the long term. During disease outbreaks, area-wide insecticide sprays may be necessary, but these are often met with public resistance, causing the government to reconsider its strategies. As well, the development of insecticide resistance by mosquitoes and changes to environmental legislation have resulted in the deregistration of many insecticides. Although this has prompted the development of novel insecticides that are more species specific and environmentally friendly, various issues, such as production costs and the actual cost of registering a pesticide, have limited the number of new insecticides being developed. All of these issues can limit the options available and thus the effectiveness of IPM programs.

### **Control methods available**

Many strategies have been used to reduce mosquito populations. A major tactic in mosquito control is source reduction (Eldridge 2005), which targets larval habitats and reduces emerging populations. Source reduction methods vary from emptying household water containers to draining wetlands (Eldridge 2005). Other tactics include the use of larvicides, such as the bacterium *Bacillus thuringiensis israelensis* (*Bti*) that forms pores in the mosquito larva's midgut causing a lethal septicemia, or applying oils that create a thin surface film that suffocates the larvae (Christophers 1960).

On a local scale, there are commercially available products to repel adults (repellents, mosquito coils), to prevent them from biting (bednets), or to kill them (insecticide impregnated bednets, insecticides, autocidal oviposition traps). On a community level, insecticides can be used in residual sprayings, coating surfaces where adult mosquitoes might land, such as house walls, and vegetable oils may be applied to water containers to eliminate larvae. On a larger scale, insecticides may be applied over large areas, usually using ultra low volume (ULV) technology (Eldridge 2005). The development of resistance to insecticides and our awareness of the effects of these compounds on non-target organisms and the environment have resulted in fewer insecticides available for spraying (Smith and Kennedy 2002; Eldridge 2005). A greater emphasis has been placed on the use of alternative methods, such as the use of biological control agents, to reduce mosquito populations.

### **Biological control**

Biological control refers to the reduction of a pest organism by purposely manipulating natural enemies in order to manage a pest species (Pedigo 2002). This involves the use of insect pathogens, natural predators, and in some cases toxins naturally produced by a microorganism (such as *Bti*), as well as the use of deterrent plants.

## **Biological control of the adult mosquito**

Most studies have examined the use of natural enemies, such as spiders, predacious insects such as dragonflies, and insectivorous birds and bats to reduce adult mosquito populations. However, all of these predators have a wide host range and their effectiveness in reducing mosquito populations is impractical and sporadic at best. Kahn and Offenhauser (1949) examined trapping males by sound, similarly to studies done with moths, but no significantly effective sound trap has been developed (Christophers 1960; Belton 1994). Currently there is no effective and reliable biological control agent available for adult mosquito control.

## **Biological control of mosquito larvae**

Due to their restriction to discrete aquatic habitats, the larvae and pupae have proven to be the most susceptible targets for biological control efforts. Many methods have been developed to treat larval habitats. One of the less commonly used tactics is the use of plants. Bladderwort and underwater sundews can passively trap and kill larvae, while duckweeds and water lettuce can cover the breeding area, passively killing larvae in a method similarly to oils (Christophers 1960). Although duckweed extracts have been shown to deter *Ae. aegypti* oviposition (Bentley and Day 1989), oviposition repellence using plants must be done with care, since other aquatic plant species, such as those of the genus *Chara*, have the opposite effect, promoting the growth of mosquito larvae by releasing oxygen into its environment (Christophers 1960).



Natural predators such as dystiscid beetles, backswimmers, and waterboatmen are often effective in the laboratory, but in the field, these predators will seek out other sources of food once mosquito populations are reduced. Various species of *Megarhinus (Toxorhynchites)* mosquito larvae have been found to be voracious eaters of mosquito larvae, but success using these predators has been variable (Christophers 1960). Aquatic birds and the small water tortoise, *Pelomedusa galeata*, feed on mosquito larvae, but not to the level required for population control (Christophers 1960). The mosquito fish, *Gambusia affinis*, has been used successfully to reduce larval populations; however the presence of the fish has been shown to deter female mosquitoes from ovipositing in sites containing these predators (Angelon and Petranka 2002). As a result of these limitations, most biological control programs involve the use of parasites, such as fungi, viruses, and bacteria, to kill mosquito larvae.

One of the more successful biological control agents developed to date for the control of mosquito larvae has been *Bti*. This product is applied to the larval habitat, where it is ingested by the mosquito larva. Once inside the larva, the endotoxin released under alkaline conditions binds to specific midgut receptors and causes a degradation of the midgut, a cessation of feeding, and a septicemia of the hemocoel. This bacterium is most effective against *Aedes* and *Culex* mosquito larvae, and can be used to control black fly larvae (Baumann et al. 1991).

Another control agent is *Bacillus sphaericus*. This bacterium, similar to *Bti*, produces endotoxins that also bind to the larval midgut receptors, causing a

degradation of the midgut, and septicaemia (Lacey 2007). Unlike *Bti*, *B. sphaericus* is only effective against mosquito larvae, especially those of the genera *Culex* and *Anopheles* (Baumann et al. 1991; Charles et al. 1996). *Bacillus sphaericus* can persist in polluted aquatic environments and can recycle in larval cadavers under certain circumstances (Baumann et al. 1991; Lacey 2007) thus requiring fewer applications than *Bti*.

Other potential agents also have been studied for the control of mosquito larvae, with mixed results. Although viruses can be an effective way to control lepidopteran larvae (Moscardi 1999), they have not been as successful in reducing mosquito populations (Woodring and Davidson 1996; Hemingway 2005). There are three types of viruses that affect mosquitoes: nuclear polyhedrosis virus (NPV), cytoplasmic polyhedrosis virus (CPV), and mosquito iridescent virus (MIV) (Woodring and Davidson 1996). All of these viruses require high doses in order to infect larvae (Woodring and Davidson 1996), which makes them costly for *in-vivo* production, and impractical for wide-scale use as effective biological control agents. Conversely, the ciliates *Tetrahymena pyriformis* and *Lambornella clarki* provide natural control of larvae that live in tree holes (Hemingway 2005). The microsporidian *Amblyospora* spp. is being evaluated as a biological control agent because it produces polymorphic spores, infects various mosquito species both orally and transovarially, causes high mortality, and persists in the environment (Woodring and Davidson 1996; Hemingway 2005). Although many *Amblyospora* species require an intermediate host, these microsporidia have been found to regulate mosquito populations in

nature, and therefore have the potential to be used as part of an IPM program (Woodring and Davidson 1996; Hemingway 2005). Despite the number of fungi that are able to infect mosquitoes, entomopathogenic fungi are not commonly used in mosquito control (Woodring and Davidson 1996; Hemingway 2005). One fungus, *Lagenidium giganteum*, has been approved for use in the United States (Woodring and Davidson 1996; Hemingway 2005). This fungus can be used in unpolluted waters, and can reduce high-density populations. Its oospore can last for 7 years so it can also persist long enough to control low density populations (Hemingway 2005). The fungus *Coelomomyces* spp. kills 90% of mosquito larvae (Hemingway 2005) but its complex lifecycle, temperature dependent activity, dosage requirement, and its low field persistence and recycling in nature have limited its effectiveness (Woodring and Davidson 1996). Of the many nematodes that attack insects, only one, *Romanomermis culicivorax*, was developed commercially for mosquito control. However, its unpredictable control, specific temperature requirements, inhibition by salt water, and a relatively long lifecycle (Woodring and Davidson 1996; Hemingway 2005) have restricted its use to specific geographical regions. This nematode is still use in parts of the world for small-scale mosquito control operations (Woodring and Davidson 1996).

Many parasites have been promoted as biological control agents to reduce mosquito populations. Some are host specific and persist naturally in the environment, while others must be re-applied as an insecticide. Viruses, fungi, nematodes, and other parasites appropriate metabolic resources from their hosts, and cause mechanical damage on entering or leaving the mosquito

(Hemingway 2005). Some parasites produce sub-lethal effects, resulting in deformities, small size, or reduced fecundity in their hosts. The appropriate stage of the parasite must coincide spatially and temporally with the susceptible stages of the mosquito, and must not affect beneficial non-target organisms. Specific mosquito parasites may be susceptible to environmental conditions such as water pH, salinity, temperature, and UV radiation, which affect the establishment and success of the parasite in the field (Hemingway 2005). Regardless of the biological control agent used, most efforts are directed towards the aquatic larval and pupal stages. These stages are restricted in their ability to disperse to new environments. The survival, development, and overall fitness of adult mosquitoes depends, to a large degree, on the quality of the larval habitat, its resources, and inter- and intra-specific competition for these resources. While the larvae have no control over the habitat in which they develop, gravid female mosquitoes determine the quality of the sites in which their offspring develop during oviposition site selection.

## **1.2 Mosquito oviposition ecology**

Mosquito oviposition is a very important stage in the mosquito life cycle and has become a target of many pest management programs. Lethal oviposition traps, used as part of control and monitoring programs around the world, have been developed to take advantage of the various host and oviposition site seeking cues used by females to select a suitable site in which to

lay their eggs (Becker et al. 2003). Identifying new attractants and repellent compounds will further help to increase the efficacy of monitoring programs and adult traps.

Typically, insects lay their eggs on, or near, an area where the larvae will have optimal resources for their development. Females that choose to lay their eggs in areas with good quality resources should have a high fitness advantage compared with conspecifics that oviposit in poor environments. The ability of a female to discriminate among discrete oviposition sites is based on the chemical and visual cues that through evolutionary time have permitted females to maximize their fitness (Resetarits 1996). Oviposition site selection is one of the few activities that a gravid female can control and actively use to increase the probability that her offspring will emerge as healthy adults.

Oviposition site selection is crucial because, as r-strategists (Smith and Smith 2001), mosquitoes do not provide parental-care, except for choosing the site in which the offspring will hatch and live. Some species, such as *Ae. aegypti*, are skip ovipositors and lay a few eggs in several oviposition sites (Christophers 1960), thus increasing the probability that at least a few offspring survive. In the initial stages, females respond to environmental cues, are attracted to potential oviposition sites, and then either accept or reject a site as suitable larval habitat (Clements 1999). Females assess a combination of physical and chemical cues (Bentley and Day 1989) including long-range visual or olfactory cues detected in flight including sun exposure, humidity, colour, temperature, and odour of a site, and surrounding vegetation (Bentley and Day

1989; Clements 1999). Subsequently females use shorter-range olfactory and gustatory cues such as volatile compounds near or within a site (Bentley and Day 1989). These cues may be considered as attractants if gravid females move towards their source or oviposition repellents if females move away (Bentley and Day 1989). In the case of oviposition deterrents, a female will move towards and may land on a potential oviposition site but will not lay eggs there due to the presence of specific compounds.

Chemical oviposition cues come from a number of sources in and around the potential site. Some of the more studied sources of these cues are those of larval origin (Bentley and Day 1989). Mosquitoes such as *Ae. aegypti*, *Ae. atropalpus*, *Ae. togoi*, and *Ae. triseriatus* oviposit preferentially in waters that have contained conspecific larvae (Bentley and Day 1989). However, waters that had contained larvae reared at high densities were found to repel gravid *Ae. aegypti*, and *Ae. atropalpus* females (Bentley and Day 1989). The concentration of this repellent compound subsequently is reduced as larvae pupate and eclose, reducing the larval population at the site. These studies suggest that chemical compounds of larval origin can influence the oviposition behaviour of conspecific, and possibly congeneric, adults. Environmental stresses, such as parasitism of *Ae. aegypti* larvae by the trematode *Plagiorchis elegans*, also leads to the production of an oviposition deterrent compound, putatively produced by infected larvae (Lowenberger and Rau 1994; Zahiri and Rau 1998). Waters containing, or which had previously contained, parasitized *Ae. aegypti* larvae were deterrent to gravid females compared with waters containing, or which had contained,

unparasitized larvae. A study examining hemolymph composition of the parasitized mosquito larvae suggested that the production of this deterrent compound might be related to parasite-induced alterations in carbohydrate metabolism (Zahiri et al. 1998).

### **1.3 Mosquito carbohydrate metabolism**

Insects use a number of different metabolic pathways to obtain the energy they require, such as glycogenolysis, glycolysis, and the pentose phosphate pathway. Readers are referred to Chefurka (1965), Chippendale (1978), and Kunieda et al. (2006) for more details on carbohydrate metabolism in insects.

Insects store carbohydrates in the form of glycogen (Chefurka 1965; Chippendale 1978; Friedman 1985), which is cleaved into glucose-1-phosphate by the enzyme glycogen phosphorylase before being converted into trehalose or undergoing glycolysis. Glycogen phosphorylase is controlled by the hyperglycemic hormone, which is released from the corpora cardiaca (Chippendale 1978). The insect hyperglycemic hormone is thought to act similarly to the mammalian glucagon (Candy 1981). There is also evidence of a hypoglycemic hormone, similar to mammalian insulin, but much research remains to be done in this area (Candy 1981).

The two major sugars found in mosquitoes are glucose and trehalose. Sucrose and fructose are both present in sugar feeding adults, however sucrose is quickly hydrolyzed into glucose soon after ingestion and is found only in trace

amounts (Van Handel and Day 1988). Glucose is the principal carbohydrate used by cells to obtain energy and is the only source of flight fuel available to most mosquitoes (Clements 1955). Unlike other insects, *Ae. aegypti* does not use lipids as a source of energy for flight and adults must constantly replenish their carbohydrate stores during extended periods of flight. Trehalose, the major carbohydrate found in the hemolymph, is composed of two glucose molecules joined by a 1,1-glycosidic bond (Becker et al. 1996). Trehalose concentrations in the hemolymph are thought to be regulated indirectly by the hyperglycemic hormone (Candy 1981). Trehalose concentrations often increase in times of stress partly due to the ability of trehalose to prevent protein denaturation (Elbein et al. 2003; Sampedro and Uribe 2004) and maintain internal homeostasis under hypertonic (Lamitina and Strange 2005), oxidative, and temperature stress (Elbein et al. 2003). Trehalose is hydrolyzed into two glucose molecules by trehalase, an enzyme present in the hemolymph and in the midgut, where it is thought to regulate the absorption of glucose (Sacktor 1968; Chippendale 1978). In insects that use carbohydrates for flight energy, trehalase has been found to be associated with mitochondria, and it is thought that the hydrolysis of trehalose occurs inside the flight muscle cell (Becker et al. 1996).

Inhibition of trehalase prevents the hydrolysis of trehalose into glucose. Trehalase inhibitors typically are composed of one D-glucose molecule joined to an aglycon moiety, giving them a structure similar to that of trehalose (Kobayashi 1999; Asano 2003; Fig. 1-1) and are thought to compete with trehalose for the active site of trehalase (Giebel and Domnas 1976; Gibson et al. 2007). These



trehalase inhibitors have insecticidal activity against the tobacco cutworm *Spodoptera litura*, the cabbage armyworm *Mamestra brassicae*, (Kono et al. 1994) and the silkworm *Bombyx mori* (Kono et al. 1993; Ando et al. 1995). Wegener et al. (2003) reported that trehalase inhibition causes hypoglycemia and gradual loss of movement followed by death in the locust, *Locusta migratoria*. Increased levels of trehalose and hypoglycemia were also recorded in three dipterans injected with the inhibitor Validoxylamine A, as well as a decrease in the flight activity of two species that use carbohydrates as their main source of fuel for flight (Takahashi et al. 1995). Despite being evaluated against terrestrial insects, few studies have examined the effects of trehalase inhibitors on aquatic insects. Due to the importance of trehalose and glucose to mosquitoes and other insects, compounds that interrupt carbohydrate metabolism may provide alternative sources of novel insecticides.

#### **1.4 Thesis objectives**

Previous studies demonstrated that parasitism of *Ae. aegypti* larvae by *P. elegans* metacercariae results in the production of an oviposition deterrent compound (Lowenberger and Rau 1994; Zahiri and Rau 1998). Parasitized larvae also have altered levels of hemolymph carbohydrates (Zahiri et al. 1998), and it has been proposed that the production of this oviposition deterrent may be a result of trehalase inhibition (Zahiri et al. 1998; Wallage et al. 2001). The purpose of this study is to determine whether manipulation of carbohydrate

metabolism in *Ae. aegypti* larvae results in the production of an oviposition deterrent, and if a change in the hemolymph carbohydrate ratio will affect larval development and metamorphosis as seen in *P. elegans*-parasitized *Ae. aegypti* larvae. I will use a synthetic trehalase inhibitor, Validoxylamine A (VAA), to prevent the hydrolysis of trehalose into glucose in the hemolymph, mimicking the putative effects of parasitism with *P. elegans*. The direct effects of VAA on *Ae. aegypti* larvae and pupae and the indirect effects on oviposition also will be examined.

## **The study organisms**

### ***Aedes aegypti***

The mosquito *Ae. aegypti*, is one of the primary vectors of human pathogens in the world, transmitting Yellow Fever and Dengue viruses in all tropical and subtropical regions worldwide. Currently over two-fifths of the world's population live in these areas and are at risk of infection (WHO 2002; Gubler 2004). An aggressive day-biting mosquito, *Ae. aegypti* preferentially feeds on humans and oviposits in man-made containers such as discarded tires or pots. Because of this anthropophilic behaviour, *Ae. aegypti* has become a ubiquitous peridomestic pest throughout the tropics and subtropics that thrives in urban environments and has a short dispersal range from its site of emergence (Muir and Kay 1998). This close association with humans ensures pathogen transmission to humans, increasing the potential of disease outbreaks. Many

programs have tried to eradicate *Ae. aegypti*. The Pan American Health Organization used a massive insecticide program in the 1950's and 1960's (Rigau-Perez et al. 1998; Speigel et al. 2005), and many countries were declared free of *Ae. aegypti*. This program was disbanded in the 1970's and *Ae. aegypti* re-established itself, either through re-invasions from other regions or from residual populations that were not eliminated. Because the incidence of arboviral diseases is increasing, there is a need for novel approaches towards mosquito control.

*Aedes aegypti* has four distinct life-stages: egg, larva, pupa, and adult or imago (Fig. 1-2). The adult is an active flying insect, while the larvae and the pupa breathe air but are strictly aquatic. Newly emerged adults feed on flower nectar and honeydew to increase their carbohydrate reserves, but females also feed on blood, which provides the proteins and lipids required to produce a clutch of eggs. Females lay small batches of eggs on wet substrates that eventually will become flooded. After hatching from an egg, the larvae go through four instars. The duration of their development depends on various environmental factors such as temperature, and food availability (Christophers 1960). After 6-8 days at 27 °C, the larvae metamorphose into non-feeding mobile pupae. The pupae remain in this stage for a period of 48 h, after which they emerge as adults. Typically, males emerge a day before females (Christophers 1960).

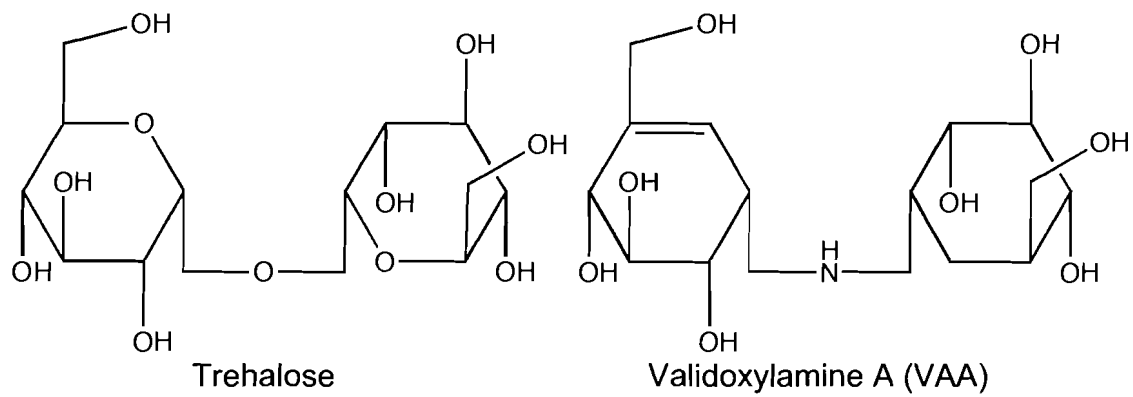
## ***Plagiorchis elegans***

A component of this thesis deals with the interactions between *Ae. aegypti* and the larval endoparasite *Plagiorchis elegans*. The definitive host of this generalist trematode *P. elegans* is a vertebrate with an internal body temperature of 37 °C (Lowenberger and Rau 1993a). Adult parasites reside in the small intestine, and forage on the intestinal mucosa and digested food. The eggs are released by the adult and pass through the intestines of its host. If the eggs are released into water, they may be ingested by an aquatic snail, where they hatch into a miracidium. The miracidium moves towards the hepatopancreas and produces a mother sporocyst, which in turn produces daughter sporocysts. This association results in the snail being chemically castrated. The daughter sporocysts produce cercariae, which emerge from the snail at night. The motile cercariae swim until they find their next host, an aquatic arthropod. The cercariae have approximately 12 h to find their host before their energy reserves are depleted, with peak infection occurring approximately 6-8 h after emergence (Lowenberger and Rau 1993b). Once the cercariae find their arthropod host, they attach to it with their oral sucker, use their stylet and hydrolytic enzymes to break through the host's cuticle, drop their tails, and enter the body cavity. Within the hemocoel, the cercariae transform into metacercariae, which are metabolically active, taking up resources as they develop (Lowenberger et al. 1994). Maturation to an infective parasite requires 72 h within their host (Lowenberger et al. 1994). Metacercariae can survive the metamorphosis of its insect host from larva to adult (C. Logan, personal observation). The cycle is

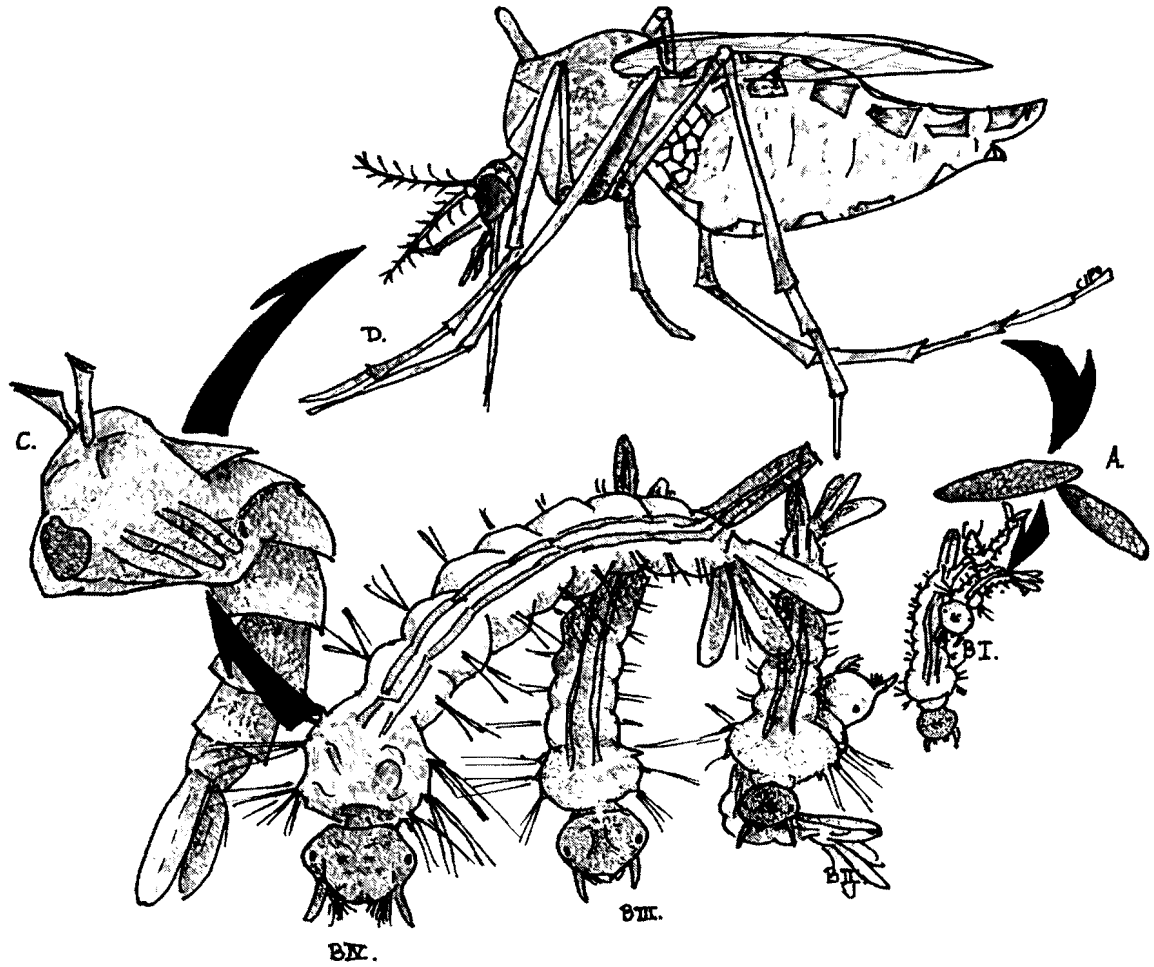
completed when the infected arthropod is eaten by the definitive host and the parasite excysts in the small intestine in the presence of bile salts (Bock 1984; Lowenberger and Rau 1993a) and attaches to the intestinal epithelium. The life cycle of *P. elegans* is shown in Figure 1-3.

*Plagiorchis elegans* has been considered as a biological control agent of mosquitoes in Canada (Popple 2003). In addition to directly killing or impairing the host species (Dempster et al. 1986; Nguyen et al. 2002; Schwab et al. 2003), infection of *Ae. aegypti* larvae by *P. elegans* induces the production of a compound that deters oviposition by conspecific adults (Lowenberger and Rau 1994; Zahiri and Rau 1998). The hemolymph of parasitized larvae contained significantly lower nutrient reserves compared with control larvae. The lone exception was trehalose, which was found in equal or greater amounts in parasitized larvae (Zahiri et al. 1998; Wallage et al. 2001). When glucose was given to *P. elegans*-parasitized larvae, the oviposition deterrent compound was no longer produced and the water became as attractive as the controls (Zahiri et al. 1998). This prompted the theory that the oviposition deterrent compound was produced by changes in the carbohydrate ratio in the larval hemolymph caused by the presence of the parasite. If this is true, then the use of a trehalase inhibitor should mimic the interruption in carbohydrate metabolism and should lead to the production of a larval-produced oviposition deterrent without the presence of a parasite.

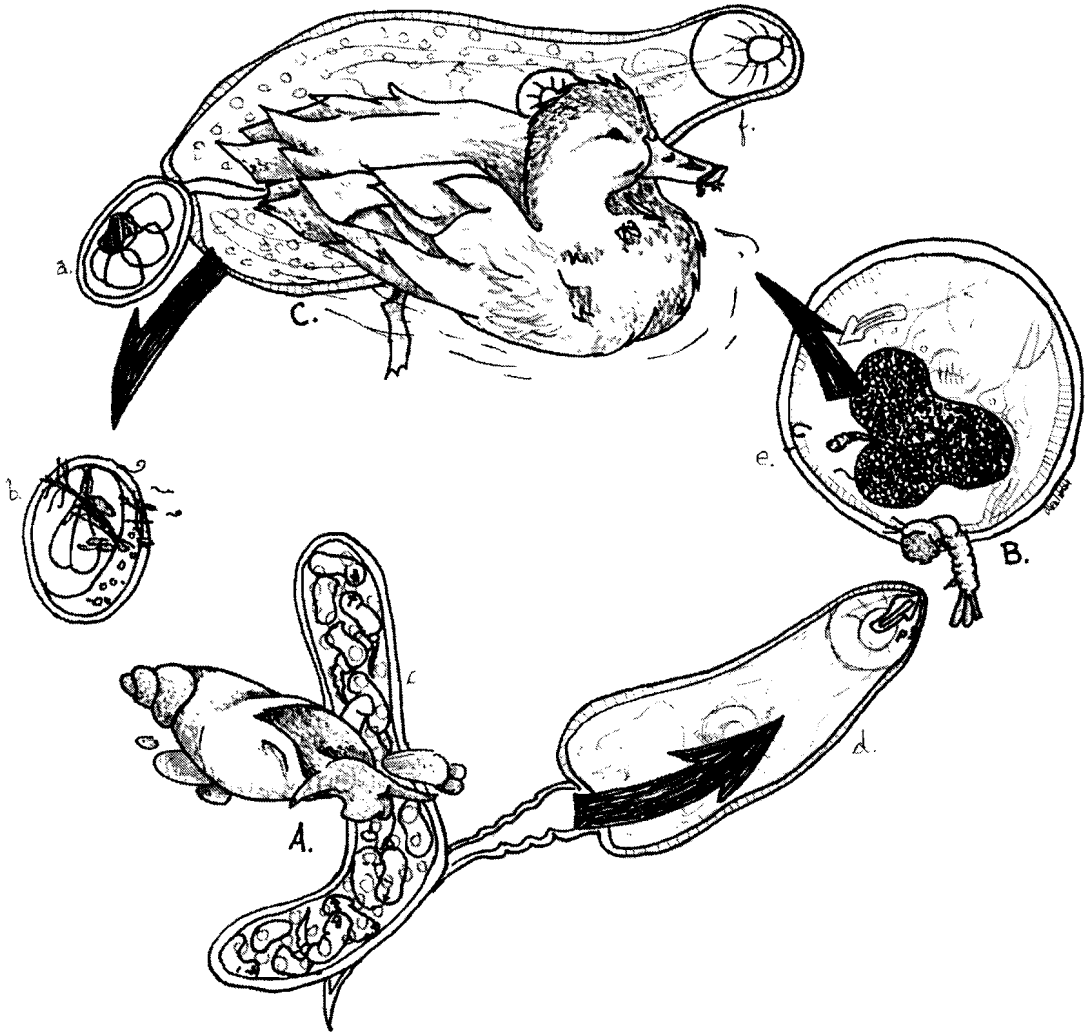
## 1.5 Figures



**Figure 1-1.** Structures of trehalose and Validoxylamine A. VAA structure from Asano (2003).



**Figure 1-2.** Life cycle of *Aedes aegypti* showing eggs (A), the four larval instars at various stages of development (B I-B IV), the mobile pupa (C), and the adult female taking a blood meal (D).



**Figure 1-3.** Life cycle of *Plagiorchis elegans* showing the first (A) and second (B) intermediate hosts, and the definitive host (C) in the fore-ground, and the egg (a), miracidium (b), daughter sporocysts (c), cercaria (d), metacercaria (e), and adult trematode (f) in the background.



## **CHAPTER 2: THE EFFECTS OF VALIDOXYLAMINE A ON *Aedes aegypti***

### **2.1 Introduction**

The mosquito *Aedes aegypti* is one of the principal vectors of arboviruses that cause human diseases. Yellow Fever causes over 20 000 deaths annually (WHO 2001, 2007), and there are 50-100 million cases of Dengue Fever each year. An estimated 2.5 billion people live in areas endemic for Dengue virus transmission (Solomon and Mallewa 2001; Gubler 2002; Guzman and Kouri 2003).

Mosquitoes have been controlled, and in some places eradicated, through the use of organochloride and organophosphate insecticides. However, due to environmental sensitivity and social legislation, the focus has shifted towards the development and use of biological controls or biorational insecticides that target insect specific biological processes. These new insecticides include glycosidase inhibitors that interrupt carbohydrate metabolism and inhibit the breakdown of specific sugars. Trehalase inhibitors might be used for insect control because they block an important insect metabolic process: the hydrolysis of trehalose to glucose.

Trehalose is one of the major circulating carbohydrates in insects, and comprises two glucose molecules joined by a 1,1-glycosidic bond (Fig. 1-1), allowing it to be present at high concentrations in the hemolymph without being toxic to the animal (Becker et al. 1996). Trehalose is released from stored glycogen into the hemolymph, where it is hydrolyzed by the enzyme trehalase. This is one of the fastest ways to liberate usable energy in insects, especially for insects that use glucose for flight, such as *Ae. aegypti* (Clements 1955; Nayar and Van Handel 1971) and bees (Suarez et al. 2005). Trehalose concentrations often increase in times of stress to prevent protein denaturation (Elbein et al. 2003; Sampedro and Uribe 2004), and to maintain internal homeostasis under hypertonic (Lamitina and Strange 2005) and oxidative conditions, and under temperature stress (Elbein et al. 2003).

Previously, Lowenberger and Rau (1994) and Zahiri and Rau (1998) found that *Ae. aegypti* larvae parasitized with the trematode *Plagiorchis elegans* produced a compound that discouraged conspecific females from ovipositing in water containing parasitized larvae. The identity and properties of this deterrent compound are currently being investigated. The production of this compound might be the result of an inhibition of trehalase in the mosquito larva, as shown by the high trehalose concentrations in parasitized larvae (Zahiri et al. 1998; Wallage et al. 2001).

Inhibiting the enzyme trehalase prevents the hydrolysis of trehalose into glucose, causing an accumulation of trehalose in the hemolymph and a shortage of free circulating hemolymph glucose. Although *P. elegans* can take up

glucosamine and leucine (Lowenberger et al. 1994) it is unclear if it has mechanisms to take up trehalose directly. Larvae parasitized by *P. elegans* show a decrease in hemolymph glucose, but trehalose levels are equal to or greater than those found in unparasitized larvae (Zahiri et al. 1998; Wallage et al. 2001). Other symptoms associated with parasitism by *P. elegans*, such as lethargy and convulsions before death, also have been associated with an inhibition of trehalase in insects (Kono et al. 1993; Takahashi et al. 1995; Wegener et al. 2003). To see if the physiological changes in parasitized larvae and the oviposition deterrence are the result of trehalase inhibition caused by *P. elegans*, I used a commercially available trehalase inhibitor.

Here, I present data on the effects of the trehalase inhibitor Validoxylamine A (VAA) on the mortality and viability of *Ae. aegypti*. I tested the physiological changes in the insects after exposure to VAA, and suggest possible explanations for these biological events, and I used oviposition bioassays to determine if the production of an oviposition deterrent is related to trehalose inhibition in larvae. My hypothesis was that VAA will interrupt carbohydrate metabolism by increasing trehalose and decreasing glucose levels.

## **2.2 Materials and methods**

### ***Aedes aegypti***

*Aedes aegypti* larvae were raised in an environmental chamber at 27±2 °C, with a 90-95% relative humidity, under a 14 h:10 h light:dark cycle. Larvae

were raised at a density of 100 larvae per litre of distilled water, unless otherwise stated, and were provided with finely ground tropical fish food (Hagen Nutrafin, Montreal, Quebec, Canada) *ad libitum*. Adult mosquitoes were kept in 300 mL paper cups covered with fine mesh in the same chamber, and fed 10% sucrose *ad libitum*.

### **VAA Trials**

A stock solution of VAA (5 mg/mL, Wako Chemicals, Richmond, Virginia, USA) was prepared using distilled water, and stored at  $-20^{\circ}\text{C}$ . Ten random two-day old mosquito larvae were placed in 20 mL glass vials containing 10 mL of a VAA solution at a concentration of 0, 0.1, 0.2, or 0.5 mg/mL, and maintained in the environmental chamber. Larvae were fed ground Nutrafin tropical fish food *ad libitum*.

The number of live larvae in each vial was recorded every 24 h post treatment for 5 days. The pupae were placed in 300 mL paper cups covered with fine mesh, and kept in the environmental chamber until all adults emerged. The first and last day of pupation, the total number of emerged adults, and the total number of adults that managed to fly (viable adults) were recorded at the time of collection. Adults were freeze-killed and sexed, and each wing-length was measured to the nearest 0.1 mm from the beginning of the anal vein to the apex of the wing excluding the fringe. Mosquitoes were stored individually in microcentrifuge tubes at  $-20^{\circ}\text{C}$ .

Because 0.2 mg/mL of VAA was the lowest concentration at which all adults emerged but were unable to fly, I raised larvae at this concentration for further studies. Ten random two-day old mosquito larvae were placed in a 20 mL glass vial containing 10 mL of a 0.2 mg/mL VAA solution. A control group was placed in a vial containing 10 mL of distilled water. All larvae were maintained and fed as described previously. After 72 h, the surviving larvae from each vial were rinsed with distilled water, and air-dried on a paper towel. This is the time that *P. elegans* requires to mature in the larva. Hemolymph was collected by placing the larva on a microscope slide, penetrating the fourth abdominal segment with a small sterilized needle, and collecting the pooled hemolymph from ~10 larvae in a microcentrifuge tube containing 10  $\mu$ L of 200  $\mu$ M phenylthiourea solution on ice to prevent melanization. The sample was then centrifuged at 8600 x g for one minute using an Eppendorf 5415D centrifuge, and the supernatant was collected and stored at  $-20$  °C.

### **Effects of VAA on adult fecundity**

I performed oviposition bioassays to determine if ingesting VAA affected the number of eggs laid by gravid females. Ten random female pupae and two male pupae were placed in a 300 mL paper cup containing a strip of paper towel, and covered with netting. Two days after emergence, the adults were exposed to cotton-balls soaked with 0.2 mg/mL of VAA. The adults were then blood-fed for 15 min and allowed to lay eggs for five days after the blood meal. The total number of eggs laid by the females was counted. Control bioassays were

performed simultaneously, using cotton-balls soaked with distilled water or 10% sucrose solution. The experiment was replicated five times.

### **Oviposition Bioassays**

I carried out oviposition bioassays to determine if gravid females would oviposit differentially in water that had held control larvae or larvae that had been exposed to VAA. Twenty 2-day old *Ae. aegypti* larvae were reared in 20 mL of 0.2 mg/mL VAA solution for 72 h, with finely ground Nutrafin fish food available *ad libitum*. Control larvae were raised under similar conditions without the VAA. After 72 h, when the larvae were in their fourth instar, the larval water was strained into a 20 mL glass vial through a generic, unbleached disposable coffee filter (#2 cone, Safeway, British Columbia, Canada), and stored at 4°C.

Larval waters (10 mL) were placed in 60 mm x 15 mm deep Petri dishes in a 20 L aquarium containing ten blood-fed adult *Ae. aegypti* females and two adult males. The two dishes were placed equidistant from each other and the sides of the aquarium and their orientation was determined randomly by a coin toss. Dishes were removed each day, the number of eggs oviposited in each dish was recorded, dishes were cleaned and fresh larval water solutions were added. The experiment was replicated four times. In a similar experiment, 10 mL of 0.2 mg/mL VAA solution was tested against 10 mL of distilled water. This experiment was repeated four times.

## **Extraction and Quantification of Proteins**

Ten fourth instar larvae that had been reared in 0.2 mg/mL of VAA for 72 h were removed and rinsed with distilled water. The larvae then were air-dried on a paper towel, transferred to individually labelled 1.5 mL microcentrifuge tubes, and stored at  $-20^{\circ}\text{C}$  until used. Soluble proteins were extracted by homogenizing the whole larva in 200  $\mu\text{L}$  of a Triton-X-sodium hydroxide solution (25  $\mu\text{L}$  of 0.1% Triton-X to 0.25 M sodium hydroxide to a final volume of 100 mL, modified from Vincent and Nadeau 1983). Soluble proteins were extracted from individual adult mosquitoes that, as larvae, were reared in water containing 0, 0.1, 0.2, and 0.5 mg/mL of VAA, by homogenizing the entire insect in 200  $\mu\text{L}$  of 1X phosphate buffered saline (PBS), pH 7.4. The homogenates were centrifuged for one min at 8600 x g at room temperature using an Eppendorf 5415D centrifuge. The supernatants were collected and stored individually at  $-20^{\circ}\text{C}$ . For hemolymph protein, 10  $\mu\text{L}$  of pooled hemolymph was mixed with 10  $\mu\text{L}$  of 1X PBS before being quantified. The amount of protein was determined as described by Bradford (1976). Ten microliters of the extracted protein homogenate were added to 10  $\mu\text{L}$  1X PBS, producing a 50% v/v solution. One milliliter of Bio-Rad Quick Start Bradford reagent (Bio-Rad Laboratories, Hercules, California, USA) was added to 20  $\mu\text{L}$  of the solution and incubated at room temperature for five min. Absorbance was measured at 595 nm using a Beckman DU-640 spectrophotometer. The amount of protein was extrapolated from a standard curve of bovine gamma-globulin solution (0-2 mg/mL).

## **Extraction of Lipids and Soluble Sugars**

Lipids and carbohydrates were extracted using a modified version of the protocols by Bligh and Dyer (Hamilton and Hamilton 1992) and Van Handel and Day (1988) respectively. Adults, whole larvae, or 10  $\mu$ L hemolymph samples were placed in 15 mL glass centrifuge tubes with 200  $\mu$ L of 2% sodium sulphate solution and were homogenized using a glass rod. Ten microliters of tripentadecanoin (C15:0) (Sigma-Aldrich, St. Louis, Missouri, USA) for adult samples, a mixture of 10  $\mu$ L of 1,3-dipentadecanoin (C15:0) (Sigma-Aldrich, St. Louis, Missouri, USA) and 10  $\mu$ L of tripentadecanoin (C15:0) for whole larvae samples (total 20  $\mu$ L), or 10  $\mu$ L of 1,3-dipentadecanoin (C15:0) for hemolymph samples were added to each tube as internal standards. All standard concentrations were at 1 mg/mL. I added 2.8 mL of chloroform:methanol (1:2) to each tube and mixed it with the sample before adding one mL of chloroform. The tubes were shaken by hand for two min and centrifuged at 1660 x g for 10 min at 25 °C in a Hermle Z 360 K centrifuge. The supernatant was transferred to a new tube, one mL of distilled water was added to each tube, and the tubes were shaken for two min and centrifuged at 1660 x g for 10 min. The upper methanol/distilled water phase containing soluble carbohydrates was removed using a Pasteur pipette and transferred to a new glass tube, concentrated to approximately 200  $\mu$ L in a boiling water bath, and stored at -20 °C. The lower chloroform phase containing lipids was transferred to a new glass tube and evaporated to dryness under nitrogen, before being stored at -20 °C.



Lipid fractions from whole larvae samples were reconstituted in chloroform and spotted on a labelled thin layer chromatography (TLC) plate along with diglyceride and triglyceride standards. The plate was placed vertically in a chamber lined with chromatography paper and filled with 152 mL of solvent (hexane:ether:acetic acid 45:30:1). The plate was removed from the chamber and lipids were resolved by UV light and 15 min in an iodine chamber. The diglyceride and triglyceride fractions for each sample were eluted using columns containing glass wool with chloroform into a seven mL glass vial. The solvent was evaporated with nitrogen gas and the samples were stored at  $-20^{\circ}\text{C}$ . Due to limited availability of material and the loss of material during TLC separation of whole larvae samples, the lipid samples from hemolymph and adults were not separated by TLC. All lipids from whole larvae, hemolymph, or adults were transmethylated as described by Morrison and Smith (1964), using boron-trifluoride in methanol (14%). The upper hexane layer containing the fatty acid methyl esters was transferred to a two mL glass vial and evaporated to dryness under nitrogen. The samples were reconstituted using 50  $\mu\text{L}$  of hexane for adult samples and 25  $\mu\text{L}$  of hexane for whole larvae and hemolymph samples. Samples were stored at  $-20^{\circ}\text{C}$ .

### **Quantification of Lipids by Gas Chromatography**

Lipids were quantified using an HP 5890 Gas Chromatography machine (GC) with a split injector and a flame ionization detector, containing an HP-Innowax column (30 m x 0.253 mm x 0.25  $\mu\text{m}$ ). The column was heated from

200 °C for one min to 260°C for nine min at a rate of 3 °C/min for total run time of 30 min. One µL of sample was injected into the GC with an injector temperature of 260 °C and a detector temperature of 280 °C. Data were collected with a Labview data collection program. Peak areas were analyzed using Labview or ImageJ (v. 1.37, National Institute of Health) when only the chromatogram was available. Lipids were identified using a fatty acid methyl ester standard mixture containing 1% myristic acid (C14:0), 4% palmitic acid (C16:0), 3% stearic acid (C18:0), 60% oleic acid (C18:1), 12% linoleic acid (C18:2), 5% linolenic acid (C18:3), 3% arachidic acid (C20:0), 3% eicosenoic acid (C20:1), 1% behenic acid (C22:0), 5% erucic acid (C22:1), and 3% lignoceric acid (C24:0).

### **Quantification of soluble sugars by ion-exchange chromatography**

Samples were analyzed using the Dionex ICS 3000 system (IC), with an autosampler, a gradient pump, and an electrochemical detector. I injected 2.5 µL of each sample into a Dionex CarboPac PA1 column (2 x 250 mm) with an AminoTrap guard column (3 x 30 mm). Carbohydrates were eluted using 0.25M sodium hydroxide at a rate of 0.25 mL/min. The isocratic program ran for 30 min, and the column was kept at 30 °C. Two hundred µL of double distilled water was added to each sample to bring the volume to at least 300 µL. Samples were analyzed using integrated amperometry, with the standard carbohydrate waveform provided by the program, and a silver/chloride reference electrode.

Because glycogen makes up a very small amount of the reserves of adult *Ae. aegypti* (Briegel 1990; Timmermann and Briegel 1999), and the nature of the

analytical column used, glycogen reserves were not measured in this study. However, changes to glycogen reserves can be deduced by looking at the soluble sugars, specifically trehalose, as the amount of trehalose increases directly at the expense of glycogen (Becker et al. 1996).

### **Statistical Analysis**

Statistical analyses were performed using JMP v.6.0 (SAS Institute 2005). Data were analyzed for normality using the Shapiro-Wilk W test. Survival of the larvae reared in different concentrations of VAA at 72 h was tested using the parametric survival test with an exponential distribution. Differences between the number of pupae and adults raised at different concentrations were evaluated using logistic regression. The wing lengths of the adults were used as a proxy measure of actual size. The wing lengths for each sex at different concentrations of VAA were compared using a one-way ANOVA. The mean wing length per adult per replicate was considered as one data point. For the fecundity bioassays, the total number of eggs laid on the paper towel strip per trial was normalized using an arcsine square root transformation, and analyzed using a one-way ANOVA. Differences were determined by the Tukey's honestly significant difference (HSD) test. For the oviposition bioassays, the number of eggs laid in each dish was normalized using an arcsine square root transformation, and analyzed using a repeated measures multi-way analysis of variance (MANOVA).

The protein data for whole larvae were log-transformed and analyzed for normality using the Shapiro-Wilk W test. The mean amount of protein from the surviving larvae in each trial vial was taken as one data point. The differences between total protein content of whole larvae and hemolymph in control larvae or larvae reared in 0.2 mg/mL of VAA were analyzed using Student's t-test. Differences between the amounts of adult protein per treatment were analyzed using a one-way ANOVA. The mean reserve per adult per replicate was considered as one data point.

Lipids were quantified using the ratio between the area of the known internal standard and the peak area of each free fatty acid methyl ester. Data were normalized using a log transformation. Differences between the total amount of lipid in control and larvae exposed for 72 h to 0.2 mg/mL of VAA were compared using Student's t-test. There was no observable effect of sex on the adult lipid data ( $P=0.31$ ; Standard Least Squares), therefore the data were combined. Adult lipids were analyzed using one-way ANOVA. The mean reserve per adult per replicate is considered as one data point. The experiment was repeated three times.

The amounts of glucose and trehalose were measured on the IC machine and quantified using a standard curve (0-20  $\mu\text{g}$ ). Differences between glucose and trehalose amounts in control larvae and larvae exposed for 72 h to 0.2 mg/mL of VAA were compared using the Wilcoxon Rank Sum test. Hemolymph trehalose data were normalized using a square-root transformation. Differences between amount of hemolymph glucose and trehalose in control and larvae

exposed for 72 h to 0.2 mg/mL of VAA were compared using the Student's t-test. No sex effect was found for adult trehalose or adult glucose data ( $P=0.23$ ; Standard Least Squares) therefore the respective data sets were combined. Differences among adults reared in the various treatments were analyzed separately for each carbohydrate using a one-way ANOVA. Differences among the treatment means were determined by Tukey's HSD test. The mean amount of reserve per adult per replicate was considered as one data point. All data were considered significantly different at  $P < 0.05$ .

## 2.3 Results

### VAA Trials

VAA had a dose-dependant effect on larvae (Fig. 2-1). The larvae showed no abnormal behaviours or deformities, other than an apparent reduction in food ingested that was not measured. There were no statistically significant differences in the survival of larvae reared in 0, 0.1, 0.2, or 0.5 mg/mL of VAA ( $P=0.92$ ; Fig. 2-1).

No obvious effects were seen in the development or duration of either the larval or pupal stages. The development of the larvae was not retarded, and pupation occurred at the same time as the control larvae. There was no significant difference in the number of surviving pupae ( $P=0.20$ ; Fig. 2-2), or the duration of pupation of insects reared in the four VAA concentrations (average 48 h) ( $P=0.77$ ; one-way ANOVA). Despite a trend for wing-length to decrease with

increasing VAA concentration, there was no significant difference between the wing-lengths of adult males (2.75-3.78 mm) ( $P=0.05$ ; Table 2-1) or adult females (3.13-4.75 mm) ( $P=0.07$ ; Table 2-1) reared in different VAA concentrations. However, there was a significant difference in the number of adults that eclosed ( $P=0.04$ ; Fig. 2-3), with more adults surviving in the control group compared with those that had been reared in any VAA concentration. There was also a significant difference between the numbers of adults reared in the various concentrations of VAA that flew ( $P<0.01$ ; Fig. 2-4). Very few adults reared at 0.1 mg/mL, and no adults reared at concentrations of 0.2 mg/mL and 0.5 mg/mL of VAA, could fly. In contrast, all emerged adults in the control group could fly.

### **The effect of VAA on adult fecundity**

There were significant differences in the number of eggs laid by *Ae. aegypti* females fed distilled water (317 eggs laid in total), 10 % sucrose solution (642 eggs laid in total), or 0.2 mg/mL of VAA solution for two days prior to blood-feeding (213 eggs laid in total) ( $P=0.03$ ; Fig. 2-5). Females fed sucrose laid approximately twice as many eggs as did females exposed to VAA.

### **Oviposition bioassay**

Significantly fewer eggs were laid in dishes containing the 0.2 mg/mL VAA solution (307 eggs laid in total) than the distilled water control (425 eggs laid in total) ( $P=0.03$ ). However, dishes containing water derived from larvae raised in

0.2 mg/mL of VAA for 72 h received significantly more eggs (589 eggs laid in total) than did the controls (204 eggs laid in total) ( $P<0.01$ ; Fig. 2-6).

## **Total protein**

### *Whole larvae proteins*

There was no significant difference in the mean amount of protein in larvae reared in 0.2 mg/mL of VAA for 72 h (90.66  $\mu\text{g}$ ) and larvae reared in only distilled water for the same period (76.22  $\mu\text{g}$ ) ( $P=0.46$ ; Table 2-2).

### *Larval hemolymph proteins*

There was no significant difference between the mean amount of hemolymph protein in larvae reared in 0.2 mg/mL of VAA for 72 h (0.47  $\mu\text{g}$ ) and larvae reared in only distilled water for the same amount of time (0.45  $\mu\text{g}$ ) ( $P=0.77$ ; Table 2-3).

### *Whole adult proteins*

There was no significant difference in the mean amount of protein in female adults (235.37-310.96  $\mu\text{g}$ ) ( $P=0.40$ ; Table 2-4) or male adults (136.45-158.14  $\mu\text{g}$ ) ( $P=0.67$ ; Table 2-4) that had been reared in different concentrations of VAA. Female pupae were found to contain significantly more protein than male pupae ( $P<0.05$ ; Standard Least Squares).

## **Total Lipids**

### *Whole larvae lipids*

The following free fatty acids were found in both control larvae and larvae reared in 0.2 mg/mL VAA: C14:0, C16:0, C18:0, C18:1, C18:2, C18:3, C20:0, C20:1, with C16:0, C18:0, and C18:1 being the most abundant. Overall, there were more triglycerides than diglycerides in both control larvae and VAA-treated larvae (Table 2-2). There was no significant difference between the mean amount of triglycerides (492.55-649.47  $\mu\text{g}$ ) ( $P=0.21$ ; Table 2-2) or diglycerides (58.20-61.31) ( $P=0.55$ ; Table 2-2) in control larvae and the larvae reared in 0.2 mg/mL of VAA.

### *Larval hemolymph lipids*

The following free fatty acids were found in both control larvae and larvae reared in 0.2 mg/mL VAA: C14:0, C16:0, C18:0, C18:1, C18:2, C18:3, and C20:1, with C16:0, C18:0, C18:1, and C20:1 being the most common. Larvae reared in 0.2 mg/mL of VAA for 72 h had significantly higher amounts of lipids in their hemolymph (274.67  $\mu\text{g}$ ) compared with control larvae (57.40  $\mu\text{g}$ ) ( $P=0.03$ ; Table 2-3).

### *Whole adult lipids*

The following free fatty acids were found in all adults: C14:0, C16:0, C18:0, C18:1, C18:2, C18:3, and C20:1, with C16:0, C18:0, C18:1, and C18:2



being the most common. No significant differences were found in the mean amount of lipid per adult exposed as larvae to different concentrations of VAA (3398.89-6179.28  $\mu\text{g}$ ) ( $P=0.58$ ; Table 2-4).

## **Soluble carbohydrates**

### *Whole larvae soluble carbohydrates*

Trehalose and glucose peaks on the IC chromatograph were obtained for all samples at 3.1 minutes and 3.7 minutes respectively. There was a significant difference in the mean amount of carbohydrates found in VAA treatment and control groups. Control larvae had significantly more glucose (6.45  $\mu\text{g}$ ) ( $P<0.01$ ; Fig. 2-7), but significantly less trehalose (0.18  $\mu\text{g}$ ) ( $P<0.01$ ; Fig. 2-7) than larvae reared in 0.2 mg/mL of VAA for 72 h (2.81  $\mu\text{g}$  and 4.27 mg respectively).

### *Larval hemolymph soluble carbohydrates*

Hemolymph trehalose peaks on the IC chromatograph were obtained for all 13 samples at 3.1 minutes. Glucose peaks were obtained at 3.7 minutes for three control samples and four treatment samples. There was significantly more trehalose in the hemolymph of larvae reared in 0.2 mg/mL of VAA for 72 h (0.48  $\mu\text{g}$ ) compared with control larvae (0.23  $\mu\text{g}$ ) ( $P=0.03$ ; Fig. 2-8). No difference was found in the mean amount of glucose in the hemolymph of control larvae and larvae reared in 0.2 mg/mL of VAA for 72h (0.003-0.01  $\mu\text{g}$ ) ( $P=0.08$ ; Fig. 2-8).

### *Whole adult soluble carbohydrates*

Treating the larvae with VAA increased trehalose and reduced glucose concentrations as adults (Table 2-5). There was no statistically significant difference in mean the amount of glucose between the different concentrations of VAA (1.96-5.43 µg) ( $P=0.10$ ; Table 2-5). However, there was a trend for the amounts of glucose to decrease with increasing VAA concentration. There was a significant difference in the mean amount of trehalose found in the different treatment groups (0.00-14.14 µg) ( $P<0.01$ ; Table 2-5), with adults that were reared in 0.2 mg/mL and 0.5 mg/mL of VAA having significantly more trehalose than controls. The effects of the trehalase inhibitor are dose dependent with respect to trehalose and glucose levels.

## **2.4 Discussion**

### **The effects of VAA**

The trehalase inhibitor VAA had a significant effect on the physiology of *Ae. aegypti*. Although VAA did not significantly affect larval mortality or their ability to pupate, it did have a noticeable and profound effect later in the mosquito's life history. The larvae reared in VAA solutions of 0.1, 0.2, and 0.5 mg/mL produced adults that were unable to fly despite appearing to develop normally. This observation is similar to results found by Takahashi et al. (1995), who recorded a decrease in the flight ability of the housefly, *Musca domestica*, and the fleshfly, *Boettcherisca peregrina*, after injection with VAA. These flies,

along with *Ae. aegypti* and many other Diptera (Steele 1981), depend on carbohydrates for flight (Takahashi et al. 1995), in contrast to orthopterans and lepidopterans, which initially use carbohydrates but later switch to lipids (Steele 1981).

Adult *Ae. aegypti* use carbohydrates as the principal source of energy for flight (Clements 1955; Nayar and Van Handel 1971). Interfering with their ability to fly would reduce the probability of survival, mating, escape from predators, blood-feeding, and oviposition, all of which would reduce their inclusive fitness. This reduction in fitness and population reduction suggest VAA, or compounds that function in a similar manner, might be used in mosquito control programs.

### **Oviposition bioassays**

One objective of this research was to determine if inhibition of trehalase would cause *Ae. aegypti* larvae to produce an oviposition deterrent as hypothesized by Zahiri et al. (1998) and Wallage et al. (2001). The bioassays performed here show that although VAA itself has a deterrent effect, water that contained larvae reared in VAA do not deter ovipositing females. Surprisingly, water that contained VAA-reared larvae are preferred by ovipositing females when compared with larval water obtained under normal rearing conditions. The effects of VAA, or the parasite *P. elegans*, may depend on the level of trehalase inhibition and the changes in the trehalose: glucose ratio in the hemolymph. A moderate inhibition may not cause significant change in the trehalose: glucose ratio, and therefore no oviposition deterrent effects. For these oviposition effects

to take place there might be a certain threshold of trehalase inhibition required that causes the larvae to switch metabolism pathways and generate the oviposition deterrent effects reported in the literature (Lowenberger and Rau 1994; Zahiri and Rau 1998; Zahiri et al. 1998).

### **The effects of VAA on total protein**

Exposure to VAA for 72 h had no significant effect on the amount of larval or adult protein. In late fourth instar larvae protein reserves are found mainly in the fat body. The levels of protein reserves may determine when moulting takes place and may contribute to determining the size of the larvae.

There was no significant difference in the amount of hemolymph proteins between control larvae and larvae exposed to VAA for 72h. The stability of hemolymph composition may reflect the various circulating hormones caused by the onset of metamorphosis. I may not have been able to measure small changes in hemolymph proteins caused by exposure to VAA.

### **The effects of VAA on lipids**

VAA did not significantly affect the total amount of lipids in whole larvae or adults. Insects exposed to VAA contained slightly more triglycerides compared with control larvae, but whether this is biologically significant is unknown. An increase in the total amount of lipids would be beneficial for a mosquito. Individuals that contain more lipid stores are better able to handle periods of

starvation, and an increase in lipids in females results in an increase in yolk for eggs. Changes in lipids are associated with sex, changes in diet, development, metamorphosis, and other metabolic and physiological demands (Downer and Matthews 1976). Because *Ae. aegypti* uses carbohydrates as its energy source for flight, changes in lipid stores are indicative of overall health.

The presence of the same kind of hemolymph lipids in both VAA-reared and control larvae are contrary to results obtained by Zahiri et al. (1998), who found a lack of C16:0 and phosphatidylethanolamine in the serum of stressed larvae as well as a lack of phosphatidylinositol in the serum of parasitized larvae. Since Wallage et al. (2001) found no difference in the fatty acid composition of *P. elegans*-parasitized and unparasitized larvae using gas chromatography, differences between this and the previous study by Zahiri et al. (1998) may be due to differences in techniques used to analyze lipids. Because lipids are transported in the hemolymph, variation in hemolymph lipid composition may indicate physiological changes in the insect (Downer and Matthews, 1976). A significant increase in diglycerides in the hemolymph of larvae exposed to VAA suggests a mobilization of lipid stores in these larvae.

### **The effects of VAA on soluble carbohydrates**

The high trehalose: glucose ratio found in whole larvae, larval hemolymph, and whole adults reared in VAA shows that trehalase inhibition increases trehalose and decreases circulating glucose.

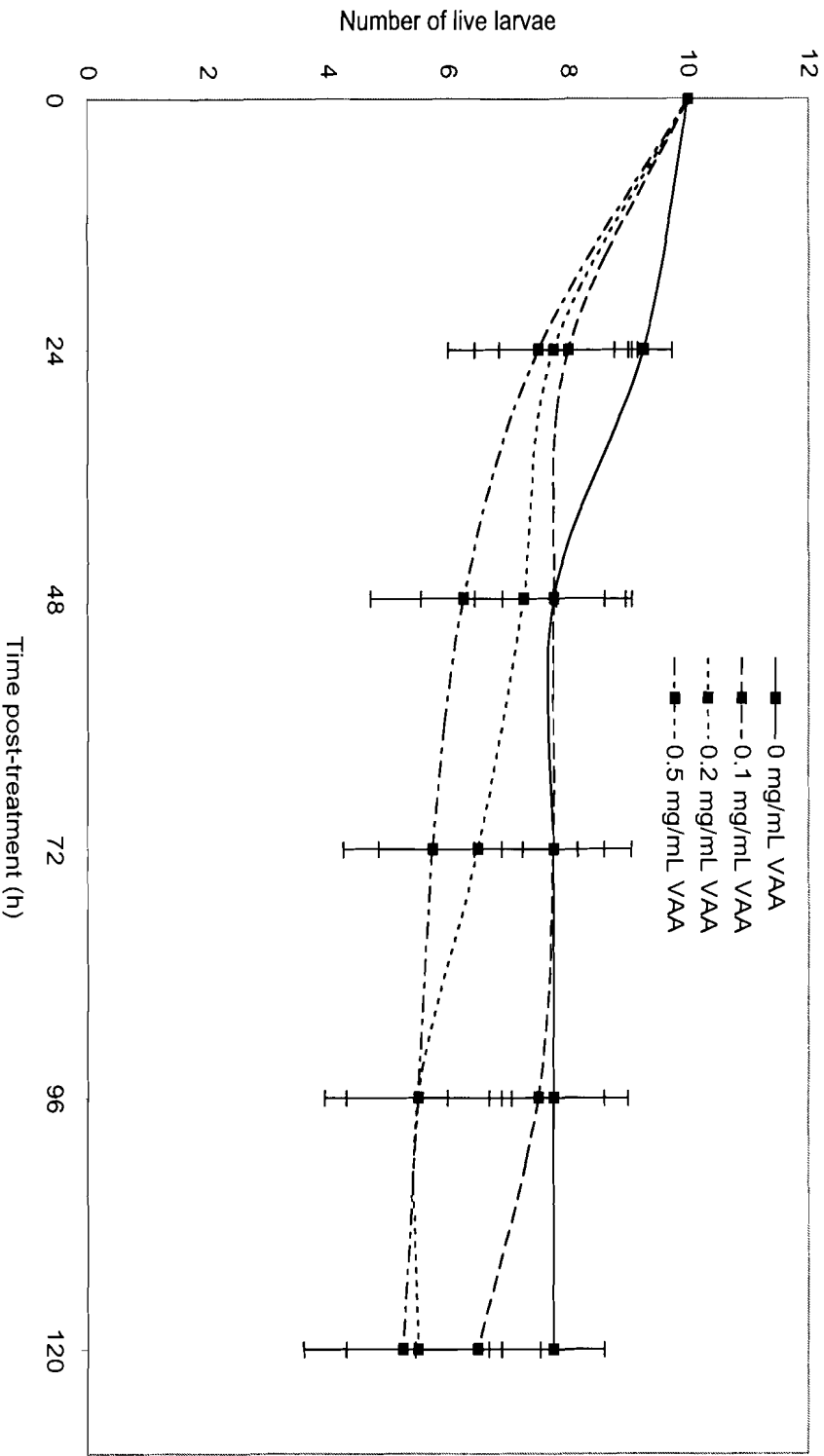
The fact that treated larvae maintain a certain level of circulating glucose in the hemolymph may have several explanations: the trehalase inhibitor did not inhibit all the enzyme produced by the larva; the glucose present was synthesized from glycogen stores through glycogenolysis; or VAA may have lead to the production of glucose through ketosis caused by hypoglycemia. During ketosis, the body is unable to utilize glucose for energy and oxaloacetate may be converted into glucose. This may reflect the importance of maintaining specific glucose levels in the hemolymph for homeostasis.

Although not statistically significant in males, there is a trend for glucose to decrease in a dose dependent manner in adults reared at different VAA concentrations. This lack of available glucose may limit the ability of adults to fly. The absence of trehalose in the adults reared at 0 mg/mL of VAA may be a result of the rapid hydrolysis of trehalose into glucose for flight energy.

The trehalase inhibitor VAA interrupted carbohydrate metabolism enough to mobilize lipid stores in *Ae. aegypti* as demonstrated by a significant increase in hemolymph lipids in larvae exposed to VAA. The use of lipids instead of carbohydrates for metabolic energy instead of carbohydrates may not affect larvae, but would limit the availability of carbohydrates for flight by adults. VAA is predicted to compete with trehalose for trehalase binding sites (Asano 2003). Because insects have other ways of obtaining metabolic energy, such as glycogenolysis and the fatty acid cycle, inhibition by VAA in otherwise unstressed *Ae. aegypti* larvae may cause sub-lethal effects that become apparent only in adulthood, through reduced energy for flight. Because trehalose accumulates in

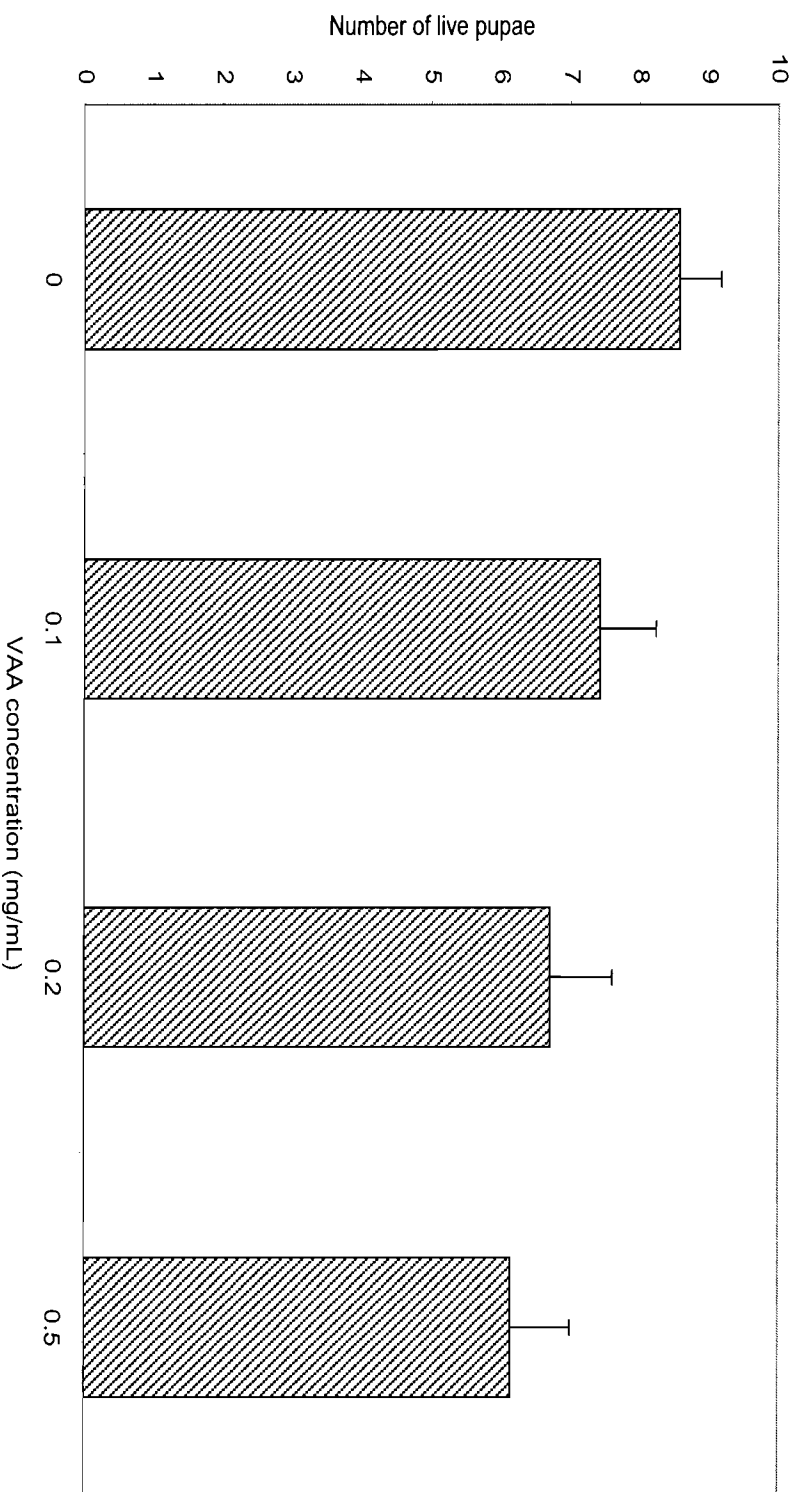
times of physiological stress (Elbein et al. 2003), the oviposition deterrent compound produced during parasitism by *P. elegans*, starvation, or overcrowding, may be a larval response to stress. This study is the first report of the effects of a trehalase inhibitor on aquatic insect larvae.

## 2.5 Figures

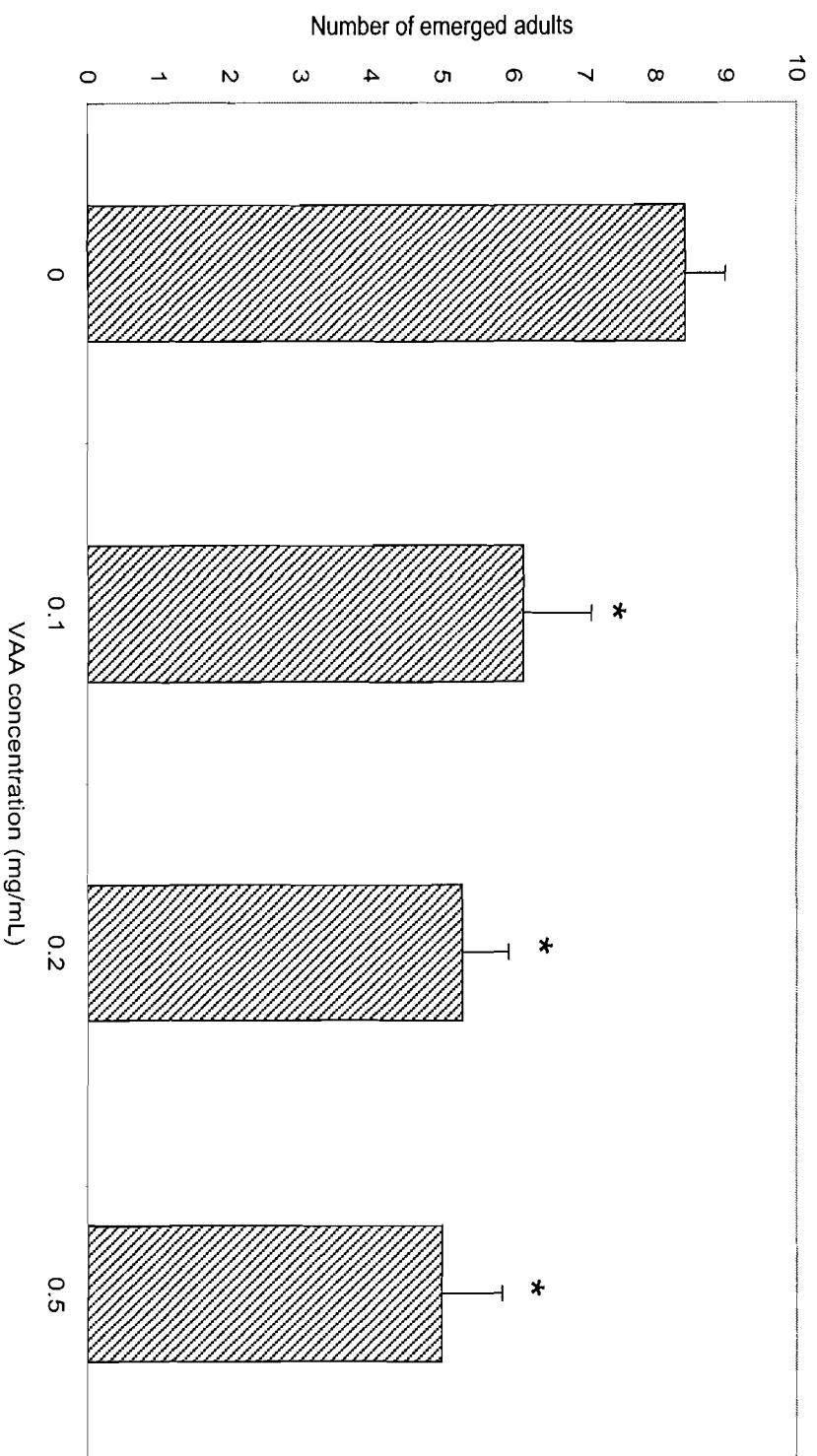


**Figure 2-1.** Mean  $\pm$  SE number of live *Aedes aegypti* larvae reared in 0, 0.1, 0.2, and 0.5 mg/mL solutions of the trehalase inhibitor VAA over time. Results represent the mean of seven independent trials.

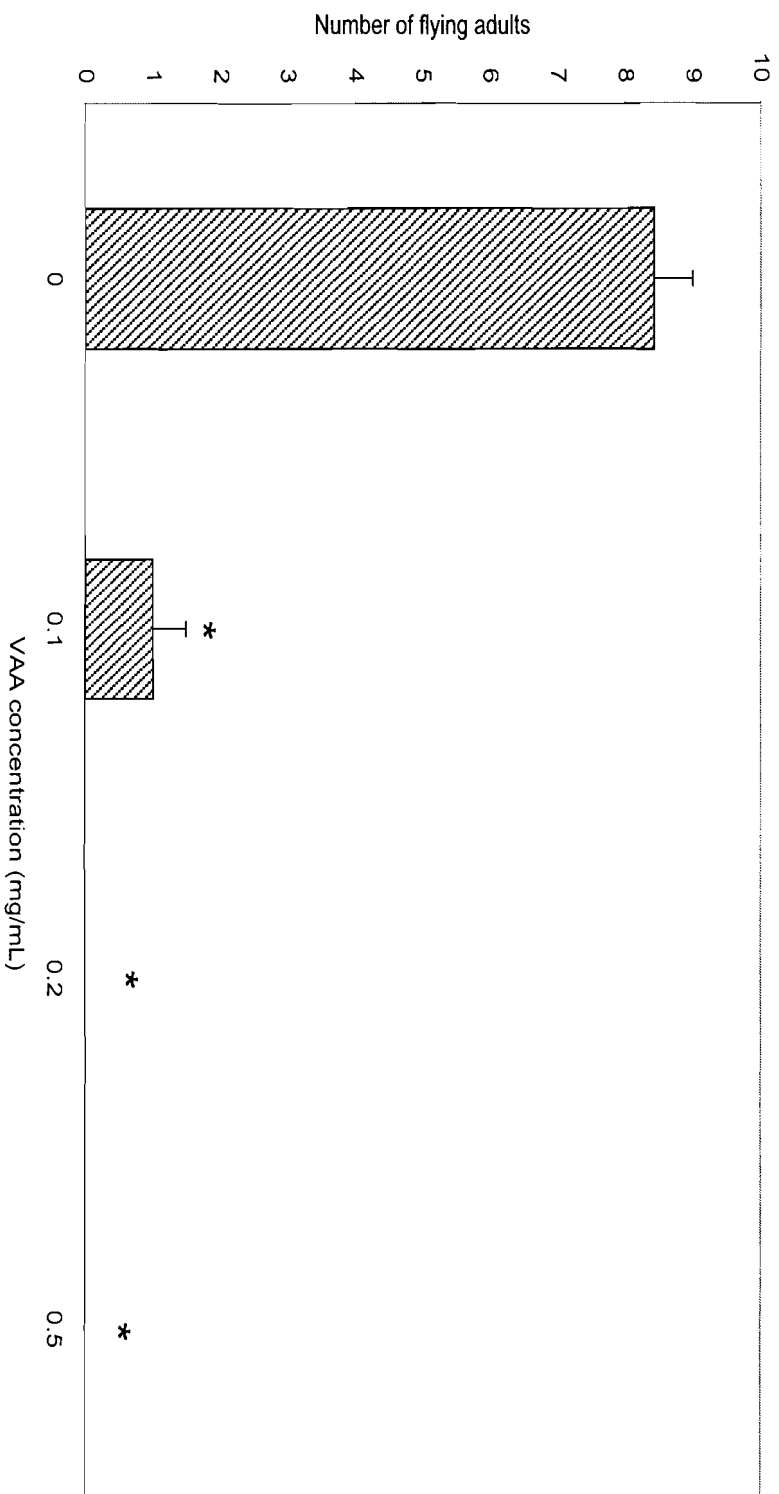




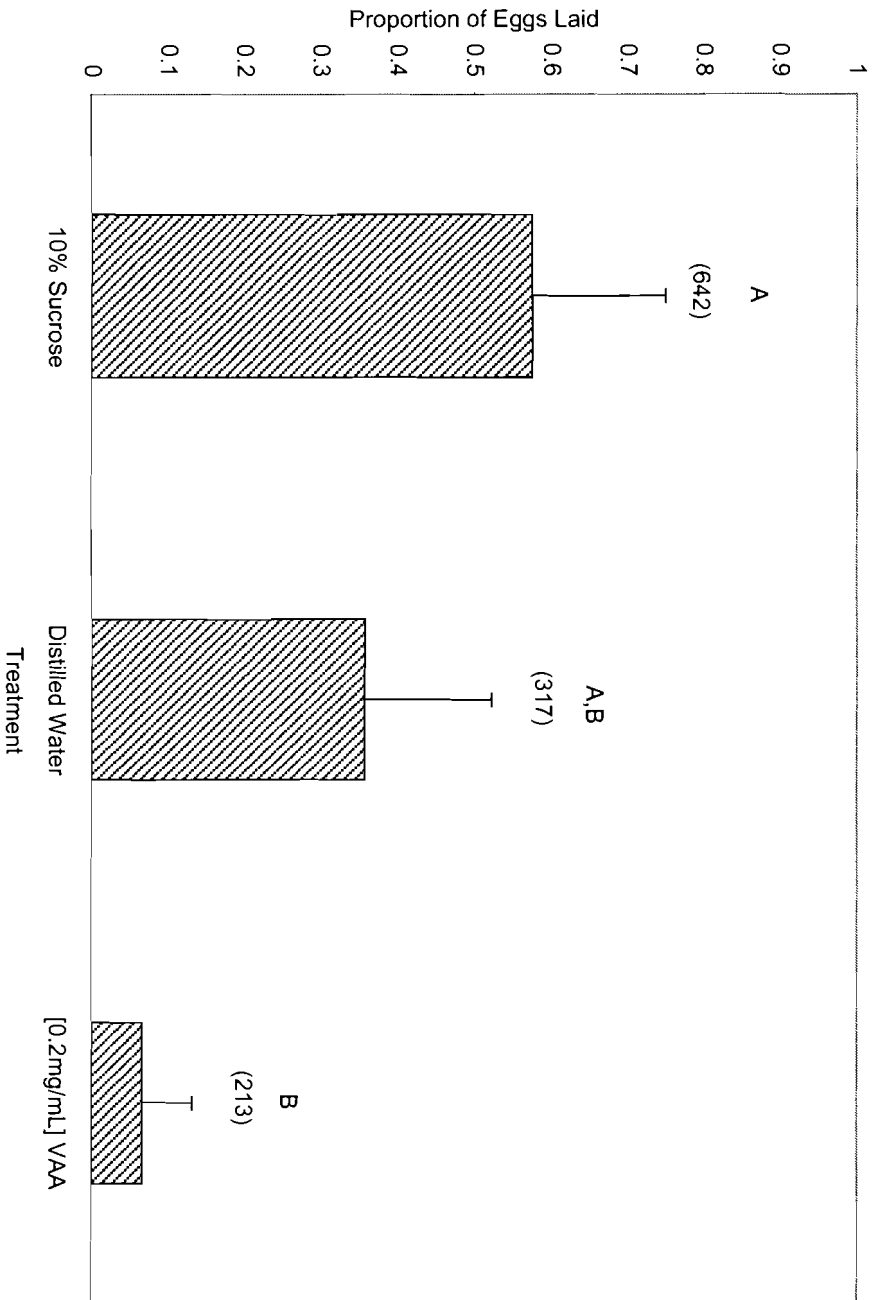
**Figure 2-2.** Mean + SE number of *Aedes aegypti* larvae incubated in solutions of different concentrations of the trehalase inhibitor VAA that pupated. Bars represent the results of seven independent trials.



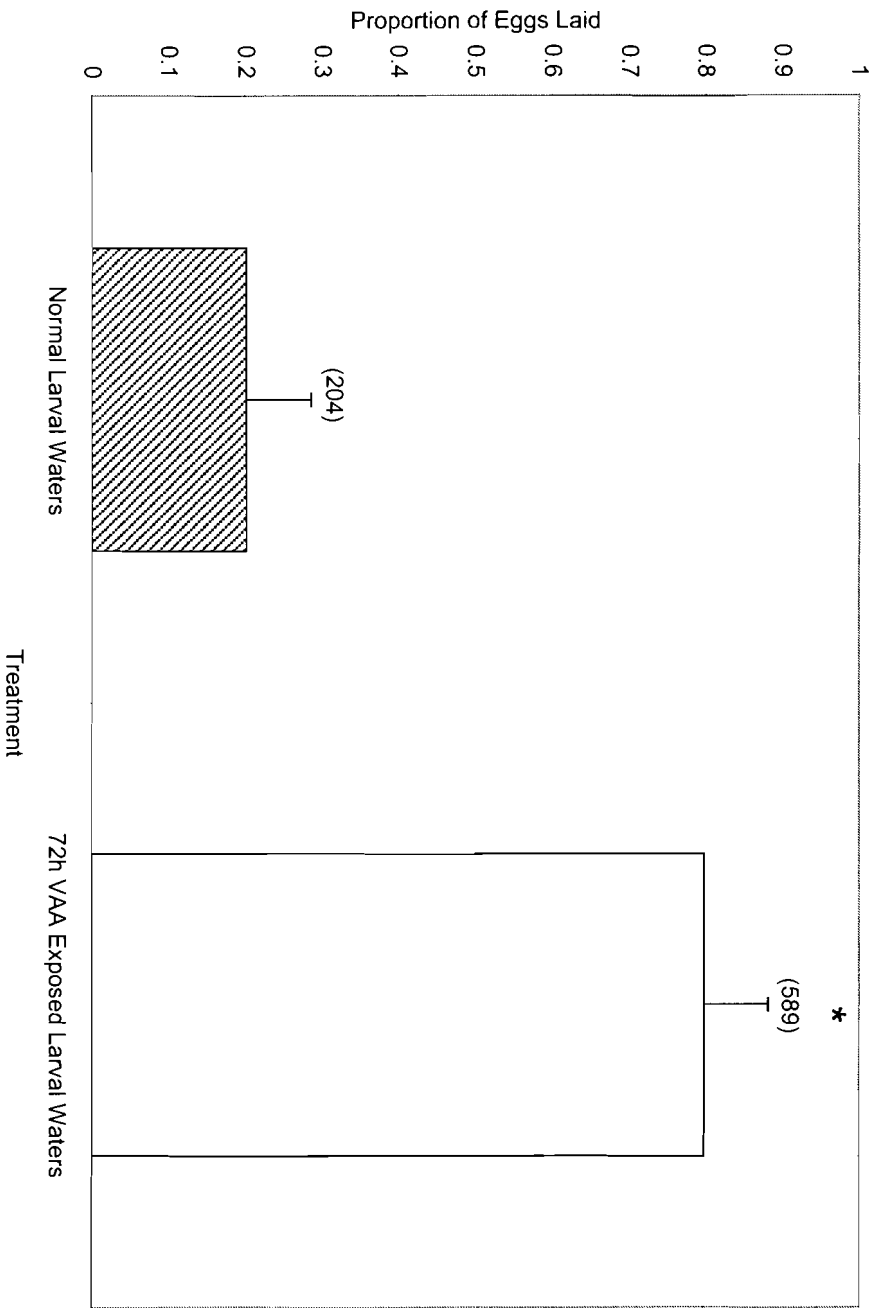
**Figure 2-3.** Mean + SE number of *Aedes aegypti* larvae incubated in solutions of different concentrations of the trehalase inhibitor VAA that pupated and eclosed. Bars represent the results of seven independent trials. \* denotes a significant difference at  $P < 0.05$ .



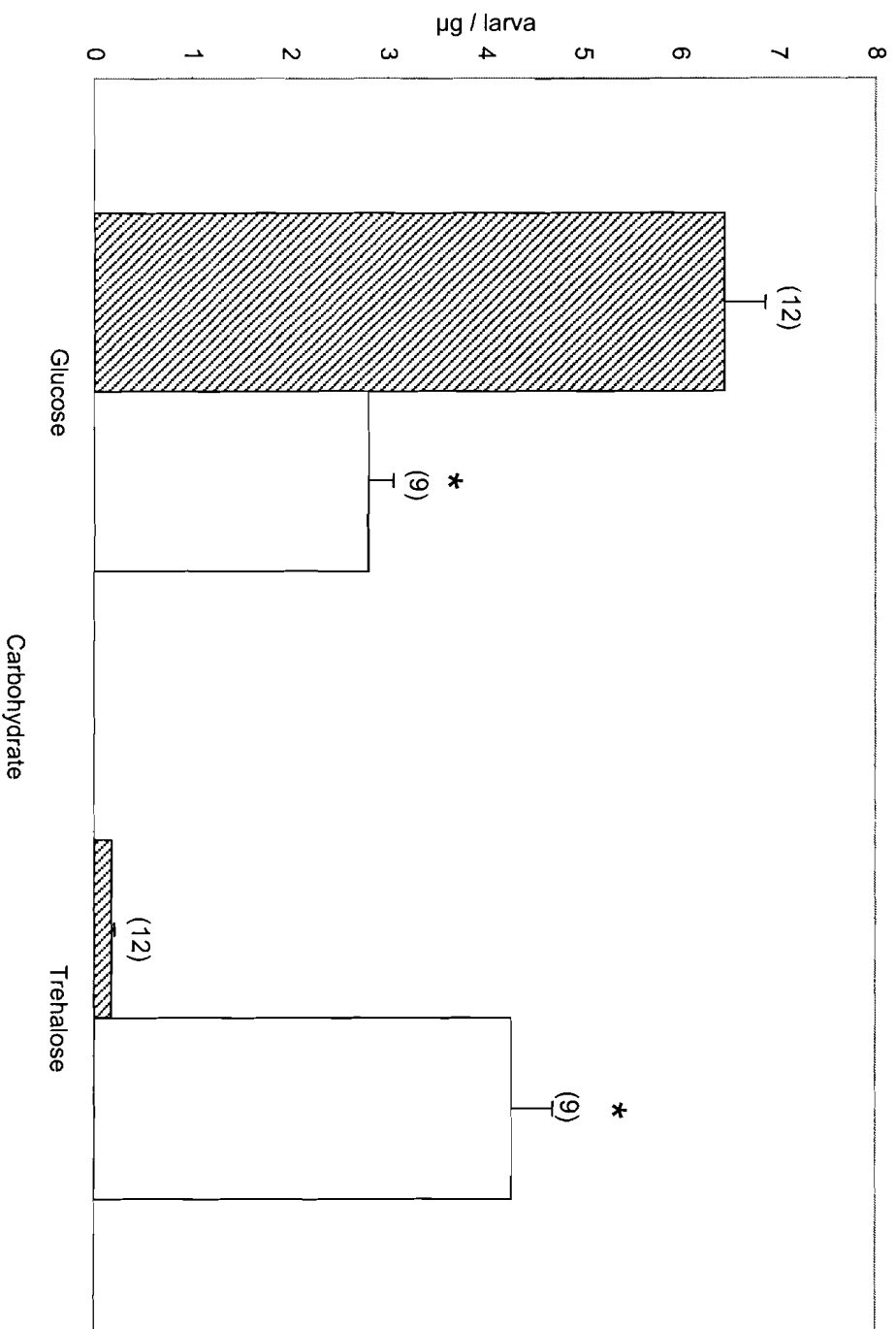
**Figure 2-4.** Mean + SE number of *Aedes aegypti* larvae incubated in solutions of different concentrations of the trehalase inhibitor VAA that pupated, broke the pupal casing, and flew. Bars represent the results of seven independent trials. \* denotes a significant difference at  $P < 0.05$ .



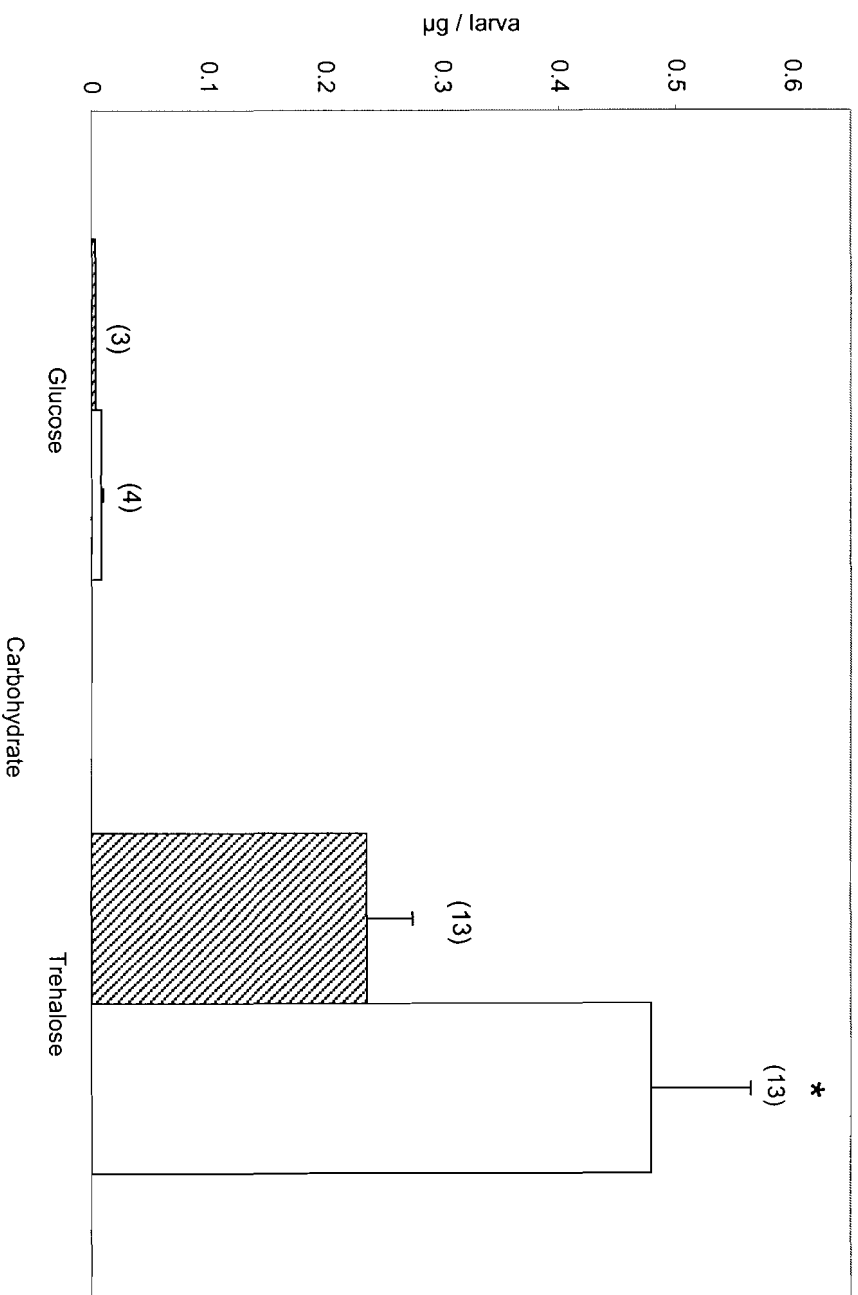
**Figure 2-5.** Mean + SE proportion of eggs laid by *Aedes aegypti* fed 10% sucrose solution, distilled water, or 0.2 mg/mL VAA solution for two days before a blood-meal. (N) = total number of eggs laid. The mean is for five independent trials. Bars with the same letter show no significant difference at  $P < 0.05$ .



**Figure 2-6.** Mean  $\pm$  SE proportion of eggs laid by *Aedes aegypti* in water from larvae reared in 0.2 mg/mL VAA solution (open bar) or larvae reared in distilled water (grey bar) over two days. (N) = total number of eggs laid. The mean is for four independent trials. \* denotes a significant difference at  $P < 0.05$ .



**Figure 2-7.** Quantification of the mean amounts of glucose and trehalose in whole control larvae (grey bars) and larvae reared in 0.2 mg/ml of VAA for 72 h (open bars). Bars represent the standard error. \* represents a significant difference at  $P < 0.05$ . (N) = number of replicates.



**Figure 2-8.** Quantification of the mean amounts of glucose and trehalose in the hemolymph of control larvae (grey bars) and larvae reared in 0.2 mg/ml of VAA for 72h (open bars). Bars represent the standard error. \* represents a significant difference at  $P < 0.05$ . (N) = number of replicates.

## 2.6 Tables

**Table 2-1.** Mean  $\pm$  SE wing-length of *Aedes aegypti* adults exposed as larvae to different concentrations of VAA. Data (in mm) represent the mean of four trials.

VAA Concentration	Wing-length	
	Female	Male
0.0 mg/mL	4.75 $\pm$ 0.05	3.78 $\pm$ 0.07
0.1 mg/mL	3.29 $\pm$ 1.11	3.46 $\pm$ 0.08
0.2 mg/mL	3.33 $\pm$ 1.11	3.64 $\pm$ 0.06
0.5 mg/mL	3.13 $\pm$ 1.05	2.75 $\pm$ 0.92
<i>P</i> -value <sup>a</sup>	0.07	0.05

<sup>a</sup> Results analyzed using one-way ANOVA.

**Table 2-2.** Mean  $\pm$  SE amount of protein and lipid per whole *Aedes aegypti* larva reared in distilled water (control) or 0.2 mg/mL VAA (treatment) for 72 h. Data (in  $\mu$ g) represent the mean of at least nine trials.

	Protein	Triglyceride	Diglyceride
Control	76.22 $\pm$ 8.84	492.55 $\pm$ 188.08	61.31 $\pm$ 9.23
VAA Treatment	90.66 $\pm$ 14.12	649.47 $\pm$ 217.31	58.20 $\pm$ 15.66
<i>P</i> -value <sup>a</sup>	0.46	0.23	0.53

<sup>a</sup> Results analyzed using Student t-test for all reserves.

**Table 2-3.** Mean  $\pm$  SE amount of hemolymph protein and lipid per *Aedes aegypti* larva reared in distilled water (control) or 0.2 mg/mL VAA (treatment) for 72 h. Data (in  $\mu$ g) represent the mean of nine trials.

	Protein	Lipid
Control	0.45 $\pm$ 0.06	57.40 $\pm$ 29.92
VAA Treatment	0.47 $\pm$ 0.05	274.67 $\pm$ 82.84
<i>P</i> -value <sup>a</sup>	0.77	<0.05*

<sup>a</sup> Results analyzed through Student t-test for all reserves. \* denotes a significant difference at  $P < 0.05$ .



**Table 2-4.** Mean  $\pm$  SE amount of protein and lipid in *Aedes aegypti* adults exposed as larvae to different concentrations of VAA. No significant differences were found in lipid or protein reserves in male or female adults that emerged from different VAA concentration regimes. (N) = number of independent replicates. Results analyzed using ANOVA. All quantities in  $\mu\text{g}$ .

VAA Concentration	Protein		Lipid	
	Female	Male	Female	Male
0.0 mg/mL	310.96 $\pm$ 32.2 (4)	158.14 $\pm$ 10.2 (4)	5517.31 $\pm$ 909.7 (3)	4347.75 $\pm$ 698.3 (3)
0.1 mg/mL	235.37 $\pm$ 46.6 (3)	136.45 $\pm$ 16.2 (4)	3398.89 $\pm$ 300.9 (2)	5431.07 $\pm$ 1124.5 (3)
0.2 mg/mL	280.96 $\pm$ 14.0 (3)	141.41 $\pm$ 14.7 (4)	6277.31 $\pm$ 1160.6 (3)	3919.38 $\pm$ 234.5 (3)
0.5 mg/mL	274.13 $\pm$ 7.1 (3)	145.33 $\pm$ 1.0 (3)	6179.28 $\pm$ 1218.2 (3)	5108.53 $\pm$ 397.3 (3)
P-value <sup>a</sup>	0.40	0.67	0.36	0.45

<sup>a</sup> Results were analyzed using ANOVA.

**Table 2-5.** Mean  $\pm$  SE amount of glucose or trehalose in *Aedes aegypti* adults exposed as larvae to different concentrations of VAA. Trehalose per adult increases significantly with increases in VAA concentration, while glucose decreases. Means are the average of 3 independent replicates. All quantities in  $\mu\text{g}$ .

VAA Concentration	Glucose		Trehalose	
	Female	Male	Female	Male
0.0 mg/mL	6.26 $\pm$ 0.97 <sup>1</sup>	4.62 $\pm$ 0.72 <sup>1</sup>	0.00 $\pm$ 0.00 <sup>1</sup>	0.00 $\pm$ 0.00 <sup>1</sup>
0.1 mg/mL	1.23 $\pm$ 0.21 <sup>2</sup>	3.18 $\pm$ 0.55 <sup>1</sup>	5.81 $\pm$ 0.08 <sup>2</sup>	6.92 $\pm$ 0.99 <sup>2</sup>
0.2 mg/mL	1.38 $\pm$ 1.21 <sup>2</sup>	1.97 $\pm$ 0.80 <sup>1</sup>	15.95 $\pm$ 6.22 <sup>3</sup>	8.65 $\pm$ 1.38 <sup>2</sup>
0.5 mg/mL	1.63 $\pm$ 0.73 <sup>2</sup>	2.30 $\pm$ 1.05 <sup>1</sup>	17.72 $\pm$ 5.13 <sup>3</sup>	10.58 $\pm$ 0.88 <sup>2</sup>
P-value <sup>a</sup>	<0.05	0.17	0.07	<0.01

<sup>a</sup> Results were analyzed using ANOVA. Means in columns with the same number are not significantly different at  $\alpha=0.05$  according to the Tukey's HSD test.

## **CHAPTER 3: ENERGY RESERVES IN PARASITIZED AND NON-PARASITIZED *Aedes aegypti* LARVAE.**

### **3.1 Introduction**

All animals require energy for the efficient functioning of the biological processes in life, such as movement to catch prey, and the work done by cells during respiration. The three major sources of energy are carbohydrates, lipids, and proteins. Carbohydrates, such as glucose and glycogen, are the main compounds used to produce energy quickly in animals. During periods of high-energy expenditure, many animals switch from a carbohydrate to a lipid source to generate energy (Stoker 2001). These may be stored as triglycerides, although other lipids are used for energy. The last major source of energy is proteins, which in periods of famine can be used to generate metabolic energy. The amount of carbohydrates, lipids, and proteins that are assimilated by an organism over time can have profound effects on its life history, including fecundity and survival.

Animals obtain and store energy for future use, allocating energy for various activities such as cell metabolism, heat production, growth through the

formation of new cells and tissues, and reproduction. In many insects, female fecundity depends on body size (Honek 1993), which depends on the amount of energy the insect acquires as a larva (Briegel 1990; Telang and Wells 2004; Rosenheim et al. 2007).

In *Aedes aegypti* and other mosquitoes, the amounts of energy reserves accumulated are proportional to the size of the mosquito larva (Briegel 1990; Timmermann and Briegel 1999). Larval size and growth rate are influenced by the amount of food the larva eats and stores, as well as by environmental factors, especially temperature (Clements 1963). The size of larvae, pupae, and subsequently adults, are all indirect predictors of fecundity (Briegel 1990; Foster 2008). Any event that delays the accumulation of reserves can extend the time the mosquito larva requires to develop, which increases the risk of predation, and the chance that the larval habitat will desiccate.

Overcrowding (Barbosa et al. 1972), starvation (Zahiri et al. 1998), and parasitic infection (Bailey and Gordon 1973; Weathersby and Noblet 1973; Mack et al. 1979; Schmidt and Platzer 1980; Zahiri et al. 1998) all influence the amount of energy reserves that larvae can assimilate and the rate of accumulation. Some parasites, such as the mermithid nematode *Romanomermis culicivorax*, always kill their mosquito hosts (Woodring and Davidson 1996; Hemingway 2005), whereas others use mosquitoes as an intermediate host in their life cycle. Here I present the effects of the trematode *Plagiorchis elegans* on the energy reserves of *Ae. aegypti* larvae as it develops.

In natural ecosystems, it is common for insects to be infected with a low number of *P. elegans* metacercariae (Janssen and Bock 1990). Heavy infections of more than two cysts per larva are rare, and extremely heavy parasite loads may be lethal, especially in small larvae (Schwab et al. 2003). Previous studies have shown that *P. elegans* metacercariae are metabolically active and will take up nutrients across the cyst wall (Lowenberger et al. 1994). An examination of the hemolymph of infected *Ae. aegypti* larvae has shown that *P. elegans* reduces total carbohydrate levels in the host hemolymph but has no significant effect on hemolymph trehalose (Zahiri et al. 1998; Wallage et al. 2001). In mosquito larvae, trehalose is the major carbohydrate in the hemolymph (Becker et al. 1996). In the previous chapter, I demonstrated the negative effects of inhibiting trehalose hydrolysis in *Ae. aegypti* (Fig. 3-1). In this chapter, I examine changes in the energy reserves of *Ae. aegypti* larvae during the development of *P. elegans* metacercariae. My hypothesis is that *Ae. aegypti* larvae parasitized by *P. elegans* metacercariae will have fewer available energy reserves than unparasitized larvae, and that the parasite will interrupt larval carbohydrate metabolism.

## 3.2 Materials and methods

### Larval and parasite rearing

*Aedes aegypti* larvae were raised as described in Chapter 2. *Plagiorchis elegans* was maintained in its snail intermediate hosts, *Stagnicola elodes* and *Lymnaea stagnalis*, held in aerated 37.8 L aquaria at room temperature with a 16 h:8 h light:dark cycle, and fed Nutrafin (Hagen Inc., Montreal, Canada) fish food and fresh Romaine lettuce *ad libitum* (Lowenberger and Rau 1993a/b; Zakikhani and Rau 1999). Snails were infected with the parasite by exposure to eggs collected from the faeces of an infected hamster, or were obtained as previously infected snails from M.E. Rau, McGill University. Infected snails were placed in individual cups containing 50 mL of water prior to scotophase and the ~2000-5000 cercariae that exited the snails were collected for infections 8 h later (Zakikhani and Rau 1999).

### Experimental design

Four days after hatching, 40 late third to early fourth instar larvae were randomly selected for analysis. Of these, 10 were used to determine the amount of lipid, glycogen, and soluble sugars per larva, 10 were used to determine the amount of protein per larva, and the remaining 20 were used to determine the amount of trehalose per larva. The larvae were rinsed with distilled water, air dried on a paper towel, and stored individually at  $-20^{\circ}\text{C}$  until needed. All experiments were replicated at least five times.

Another 40 larvae were rinsed using distilled water and placed in a glass dish. Water containing *P. elegans* cercariae collected as described above was placed in the same glass dish. Larvae were exposed to cercariae for 20 min, removed, and rinsed with distilled water. The larvae were placed in a 340 mL paper cup filled with 275 mL of distilled water containing finely ground Nutrafin tropical fish food, and maintained in the environmental chamber until needed. Two randomly selected *P. elegans*-parasitized and non-parasitized larvae were collected at 8, 12, 24, 48, and 72 h post exposure. These time points were chosen based on the development requirements of *P. elegans* while in a mosquito larva. The larvae were rinsed with distilled water and air dried on a paper towel before being stored individually at  $-20^{\circ}\text{C}$ . Parasitism was confirmed by examining larval and pupal cadavers, and by the presence of entry wounds on those larvae that were used for the assays (Fig. 3-2). This experiment was replicated at least twice. The variation in the number of replicates performed was caused by a low sample size as a result of high larval mortality.

Energy reserves were measured also in one-day old pupae. On the first day of pupation, 10 male pupae were randomly collected, rinsed with distilled water, placed in individual 0.8 mL centrifuge tubes, and stored at  $-20^{\circ}\text{C}$ . Ten female pupae from the same cohort were collected on the second day of pupation, as females generally begin to pupate a day after males (Christophers 1960). Sex was determined by size, females being significantly larger than the males (Christophers 1960). This experiment was replicated three times.

## **Extraction and determination of lipids and carbohydrates**

Lipids, glycogen, and soluble sugars were extracted using the methods outlined by Van Handel and Day (1988), except that larvae were individually homogenized in a 15 mL polypropylene centrifuge tube (Corning Inc., Corning, New York, USA). The centrifuged pellet, containing the glycogen fraction, was stored at room temperature until further analysis. The upper phase, containing soluble sugars, was concentrated to 0.1-0.2 mL in a boiling water bath. The lower phase, which contained lipids, was boiled to dryness. Samples for trehalose analysis were prepared from soluble sugar samples extracted as described, except that two larvae were used per 15 mL centrifuge tube.

The lipid samples were diluted 1:5 using vanillin reagent, and the lipid content determined using the sulpho-phospho-vanillin reaction (Van Handel 1985b). Total lipid amount was extrapolated from a standard curve of Mazola corn oil (ACH Food Companies Inc. Memphis, Tennessee, USA) (0 to 200  $\mu\text{g}$ ).

Glycogen, soluble sugar, and trehalose content were determined by the hot anthrone method outlined by Van Handel (1985a), except that the glycogen pellet was transferred to a glass tube before carrying out the assay. To determine trehalose concentrations, 50  $\mu\text{L}$  of 1M hydrochloric acid were added to the tubes, boiled in a hot water bath for 5 minutes, and cooled to room temperature. Subsequently 150  $\mu\text{L}$  of 1M sodium hydroxide were added, and the tubes were boiled, and cooled as described. These processes ensured that free glucose and fructose would not create an overestimation of trehalose concentrations (Wolfrom et al. 1948; MacLaurin and Green 1969). The amount

of soluble sugars and glycogen was extrapolated from a glucose standard curve (0-20  $\mu\text{g}$ ). The amount of trehalose was extrapolated from a standard curve (0-20  $\mu\text{g}$ ).

Ion-exchange chromatography was used to quantify more stringently the glucose and trehalose concentrations in parasitized and control larvae. Larvae were exposed to parasites and maintained as described above. At various time points three larvae were collected from each treatment group, the soluble carbohydrates were extracted as described previously, and double distilled water was added to each sample to bring the volume to 300  $\mu\text{L}$ . These samples were analyzed using the Dionex ICS 3000 system as previously described (p. 31).

### **Extraction and determination of proteins**

Proteins were extracted from individual whole larvae by homogenizing them in 200  $\mu\text{L}$  of 1X phosphate buffered saline (PBS), pH 7.4. The homogenates then were centrifuged for 1 min at 8600 x g at room temperature using an Eppendorf 5415D centrifuge. The supernatant was collected and stored at  $-20\text{ }^{\circ}\text{C}$ .

The amount of protein per larva was determined using the Bradford method (Bradford 1976). Ten microliters of the extracted protein homogenates were added to 10  $\mu\text{L}$  1X PBS, preparing a 50% solution (v/v). One milliliter of Bio-Rad Quick Start Bradford reagent (Bio-Rad Laboratories, Hercules, California, USA) was added to 20  $\mu\text{L}$  of the protein solution and the mixture was



incubated at room temperature for 5 minutes. Absorbance was measured at 595 nm using a Beckman DU-640 spectrophotometer. The amount of protein was extrapolated from a standard curve of bovine gamma-globulin solution (0-2 mg/mL).

### **Statistical analysis**

Statistical analyses were performed using JMP v.6.0 (SAS Institute 2005). For all experiments, the means of the reserves per replicate were considered as one data point, and data were tested for normality using the Shapiro-Wilk W test. Differences between male and female pupae reserves were analyzed using the Student's t-test. Values were considered significantly different at  $P < 0.05$ .

For the reserves of *P. elegans*-infected mosquito larvae, glycogen and trehalose data were normalized through a log transformation. Data were tested for normality using the Shapiro-Wilk W test. Differences in reserves at the different times for parasitized larvae were compared with data obtained for unparasitized larvae over time using Standard Least Square (SLS) analysis, and differences were analysed using Tukey's Honestly Significant Difference test. Values were considered significantly different at  $P < 0.05$ .

### **3.3 Results**

#### **Metabolic reserves in whole *Aedes aegypti* larvae at 4-days**

All larvae were at late third instar or early fourth instar at the start of the fourth day after hatching. The mean amounts of the various energy reserves at  $t=0$  h are presented in Figure 3-3. The majority of energy reserves in the larvae at this stage consisted of proteins and lipids. Larvae that were not collected and allowed to develop normally began pupation at six-days.

#### **Total reserves of whole larvae over time**

The mean energy reserves for non-parasitized larvae over time are presented in Figure 3-3 and Table A-1. The amount of protein per larva increased significantly over time ( $P<0.01$ ; SLS). The amount of lipids per larva also increased significantly over time ( $P=0.01$ ; SLS). Total soluble sugars were highly variable over time, and increased slightly over the course of the experiment. However, the amount of glucose per larva increased significantly over time (2.70-10.51  $\mu\text{g}$ ) ( $P<0.01$ ; SLS).

#### **Metabolic reserves in *Aedes aegypti* pupae**

No significant differences in the amount of lipids, glycogen, soluble sugars, or trehalose were observed between male and female pupae. However, female pupae had significantly more protein than male pupae (Fig. 3-3A).

### **Total reserves of parasitized larvae over time**

In this experiment, there was a mean intensity of 13.96 metacercariae/larva, and a mean prevalence of infection of 98.9%. The results for the differences between the energy reserves of parasitized and non-parasitized larvae are presented in Figure 3-3 and Table A-1. While not statistically significant, less protein (Fig. 3-3A), glycogen (Fig. 3-3C), and trehalose (Fig. 3-3E) was found in *P. elegans*-parasitized larvae when compared with unparasitized larvae. In contrast, the amounts of lipid (Fig. 3-3B) and soluble sugar (Fig. 3-3D) in parasitized larvae appear to increase in the presence of the parasite. However, analysis of the soluble sugars shows a significant decrease in the amount of glucose over time in *P. elegans*-parasitized larvae (2.57-6.70 µg) when compared with controls (2.70-10.51 µg) ( $P < 0.01$ ; Fig. 3-4). It was not possible to analyze changes in trehalose using ion-exchange chromatography due to an inability to obtain a measurable sample.

### **3.4 Discussion**

The data obtained for the amounts of metabolic reserves in *Ae. aegypti* larvae were similar to previous studies on basic nutritional reserves found in mosquito larvae (Appendix B; Van Handel 1988; Timmermann and Briegel 1993, 1999). Differences in the data generated in these studies may have been caused by differences in rearing conditions, such as diet and temperature, as these environmental factors can influence the amount of energy reserves

accumulated by larvae. Although the numbers are different between this study and that of Van Handel (1988), the patterns of change in protein, lipid, and carbohydrate levels as the insects develop are similar. The lone exception was glycogen (Appendix B).

I detected less overall protein in my analysis than what was reported by Van Handel (1988), although this difference was not significant (Table B-1A). Because the total amount of protein is correlated with larval size (Van Handel 1986), these differences may have been due to different rearing conditions, such as diet. The previous study by Van Handel (1986) determined the amount of protein per larva using the Lowry method whereas I used the Bradford assay. Differences caused by the technique used also may account for the slight differences in total protein between the experiments.

Lipid content was found to be slightly higher in my experiments when compared with a previous study by Van Handel (1988) (Table B-1B), but this may be a result of a difference in the food fed to the mosquito larvae. Lipids are a major component of *Ae. aegypti* larval reserves (Van Handel 1988; Timmermann and Briegel 1999), believed to facilitate larval survival during periods of starvation (Timmermann and Briegel 1999).

When compared with Van Handel's 1988 study, I found significantly lower glycogen levels in *Ae. aegypti* larvae (Table B-1C). However, because the critical amount of glycogen needed by a larva in order to pupate is approximately 0.07 calories or 17.5  $\mu\text{g}$  of glycogen (Chambers and Klowden 1990), the larvae could pupate normally (Fig. 3-3C). In contrast to glycogen, the amount of total

sugars or trehalose did not vary significantly compared with Van Handel's study (Table B-1D; Table B-1E). These values may reflect specific critical amounts of soluble carbohydrates that are needed and used by the larva for basic metabolism. Studies by Timmermann and Briegel (1993, 1999) and Briegel (1990) have found carbohydrate reserves to contribute very little to mosquito larval metabolism, to the point of being almost negligible. The actual amounts of carbohydrates obtained per larvae in the various experiments were very variable within each trial. This natural variability in carbohydrate reserves may account for the differences in amount of carbohydrates between replicates.

No significant difference was found between the metabolic reserves of male and female *Ae. aegypti* pupae, with the exception of protein reserves (Table 3-2). This sex difference in protein reserves may be a result of female pupae being larger than male pupae.

In contrast to previously published findings (Zahiri et al. 1998; Wallage et al. 2001), I found no statistically significant difference between the amount of most energy reserves found in normal and *P. elegans*-infected larvae. Also contradictory is the apparent decrease in trehalose reserves found in parasitized larvae (Fig. 3-3E), as the previous studies indicate no change or increase in trehalose reserves (Zahiri et al. 1998; Wallage et al. 2001). Parasitized larvae contained more lipids and total soluble sugars than control larvae, although these differences were not statistically significant. The increase in total soluble carbohydrates may be a product of glycogen break-down, as the amount of glycogen was lower in parasitized larvae than control larvae at 12, 48, and 72 h

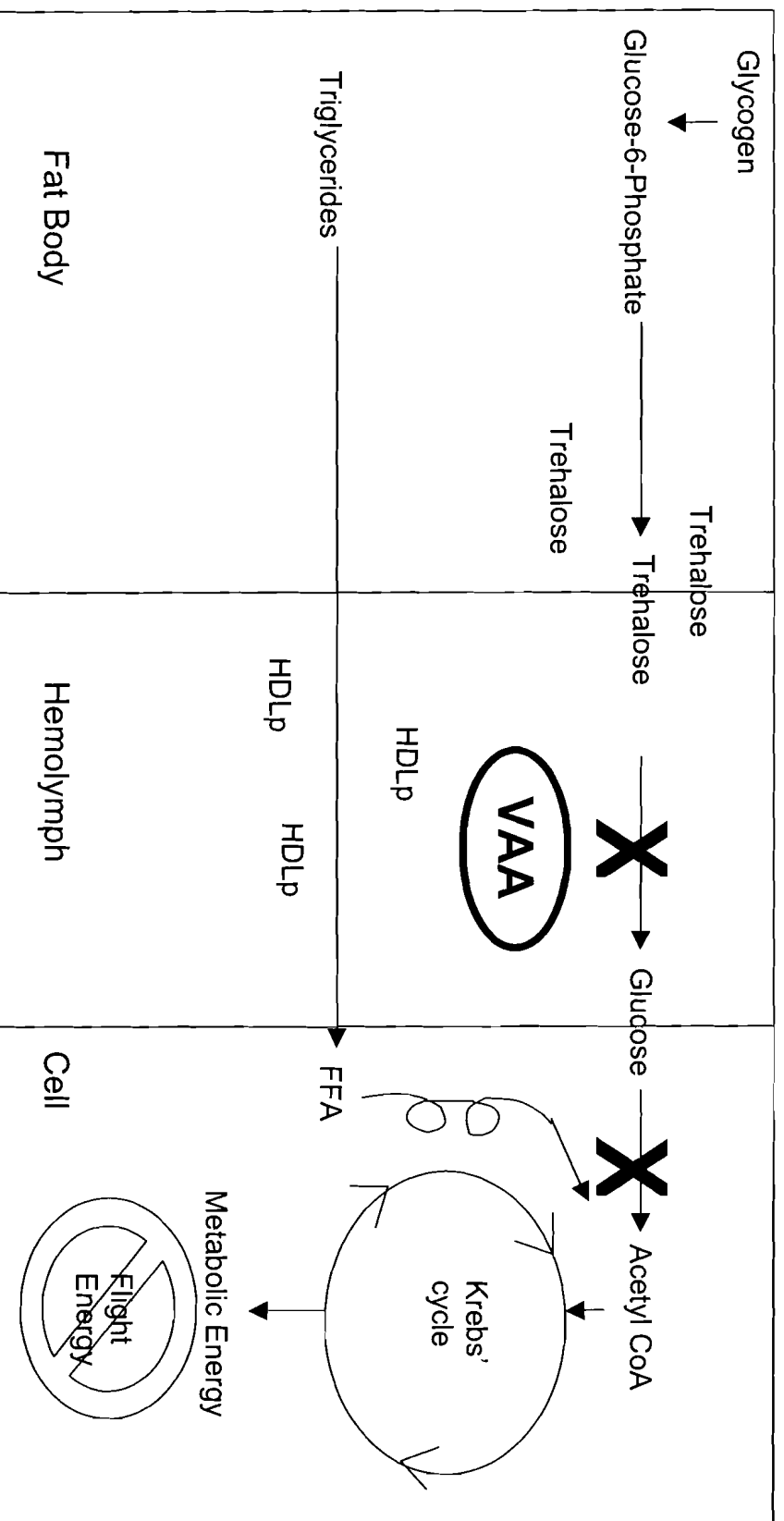
post-infection. If, as hypothesized, trehalose reserves in *Ae. aegypti* larvae are affected during parasitism by *P. elegans* (Zahiri et al. 1998; Wallage et al. 2001), the increase in total soluble sugars may be caused by the conversion of glycogen into trehalose, causing an artificial increase in the amount of trehalose, by either the larva or *P. elegans*. Because *P. elegans* is found within the insect hemolymph, we might assume that its main energy source would be the most abundant hemolymph carbohydrate, trehalose (Uglem et al. 1985; Bryant and Behm 1989). Alternatively, trehalose may be higher in parasitized insects because it is not taken up by the parasite and is allowed to accumulate. Trematodes that inhabit body cavities, tissues, and blood of their vertebrate hosts have greater access to simple carbohydrates than do trematodes that inhabit the intestine (Uglem et al. 1985; Bryant and Behm 1989). If trehalose were taken up by *P. elegans* metacercariae, it would be advantageous for this parasite to manipulate carbohydrate metabolism and increase the amount of trehalose freely available in the hemolymph. The decrease in trehalose I found over the first 48 h post-infection may reflect the parasite absorbing and metabolizing available hemolymph trehalose (Fig. 3-3E). Because previous experiments examining trehalose were done 72 h after infection (Zahiri et al. 1998), it is not known if *P. elegans* absorbs trehalose during its initial development from penetrating cercaria to infectious metacercariae. Parasite uptake of trehalose in the early stages of infection may result in the host larva hydrolyzing glycogen to restore losses. There is an increase, albeit not significant, in trehalose amounts 72 h after infection with the parasite. The metacercaria requires approximately 72 h in

the insect host to become a “mature” infectious metacercaria (Lowenberger and Rau 1993a). The increases over normal levels I measured at 72 h post infection may reflect the parasite no longer taking up trehalose and a delay in the time required for the host to stop producing trehalose.

My inability to detect significant differences in the concentrations of specific reserves between parasitized and non-parasitized *Ae. aegypti* larvae may have been due to the detection methods used. Subsequently, using the more sensitive ion-exchange chromatography, I measured a significant difference in glucose concentrations between these groups (Fig. 3-4).

It often is challenging to demonstrate specific detrimental effects of a parasite on its host. Although data obtained may not be statistically significant, they still may be biologically significant. Previous studies suggest that *P. elegans* causes an increase in hemolymph trehalose levels (Zahiri et al. 1998; Wallage et al. 2001), while the present experiments indicate that this increase may be a function of the host compensating for uptake of trehalose by the parasite or due to the parasite inhibiting the conversion of trehalose to glucose. Trying to differentiate among the different factors involved in the parasite-host relationship is one of the major challenges facing parasitologists today.

### 3.5 Figures

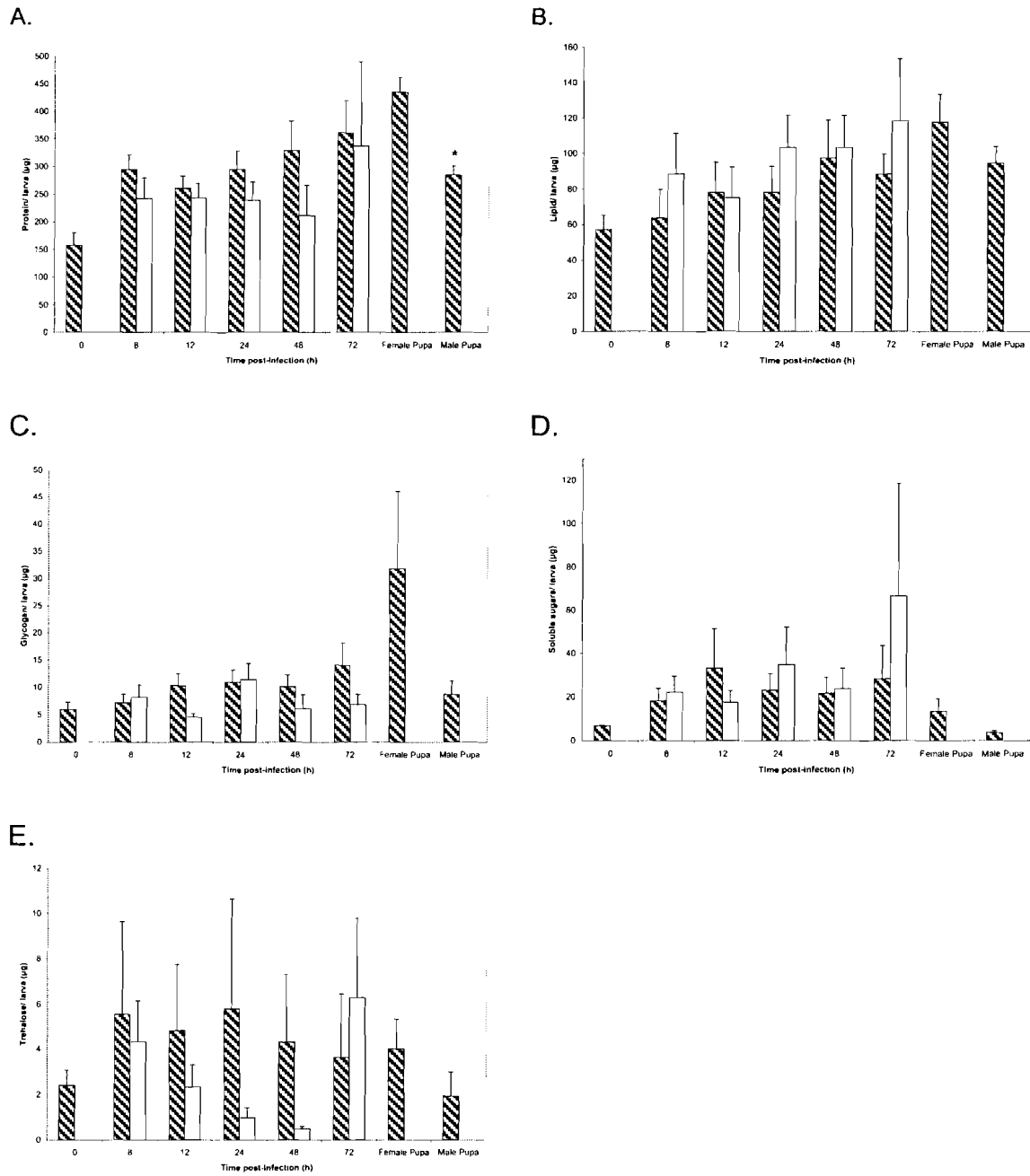


**Figure 3-1.** The effects of the trehalase inhibitor, Validoxylamine A (VAA), on *Aedes aegypti* larvae. The inhibition of trehalase prevents the hydrolysis of trehalose into glucose, causing an increase in trehalose and a decrease in available glucose. As a result, the larva is forced to utilize its lipid reserves for metabolic energy, and although the larva manages to pupate, adults cannot fly. HDLp = High-Density Lipoprotein; FFA = Free Fatty Acid.

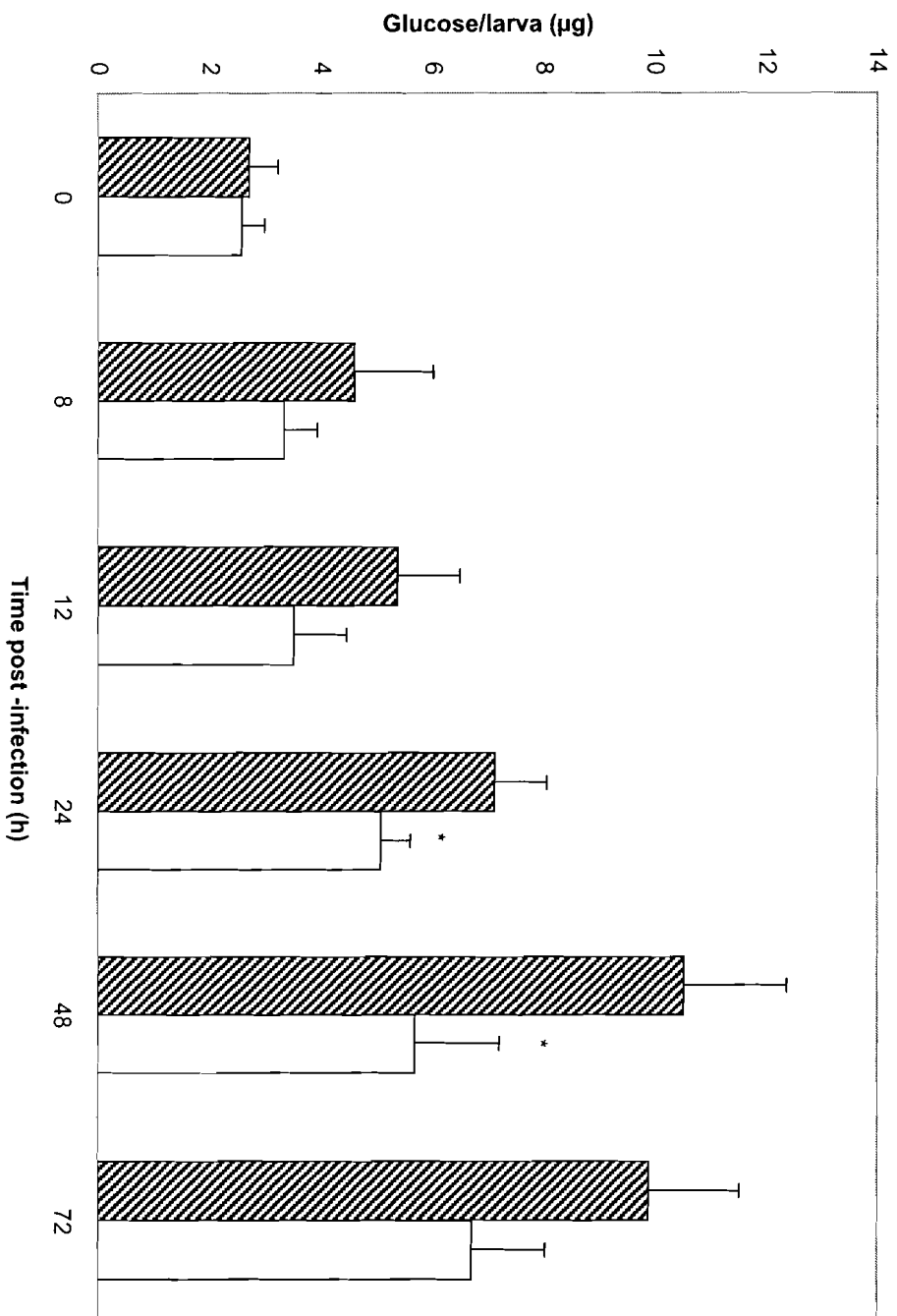




**Figure 3-2.** Parasitized fourth instar *Aedes aegypti* larva with an arrow pointing at an entry wound caused by a *Plagiorchis elegans* cercaria. The parasite uses its stylet to penetrate the cuticle of its arthropod host while releasing enzymes to help breakdown the cuticle. In response to the wound caused by the cercariae, the arthropod heals the area of entry by melanization, causing the formation of the dark spots on its cuticle.



**Figure 3-3.** The mean +SE amount of protein (A), lipid (B), glycogen (C), soluble sugars (D), and trehalose (E) in *Plagiorchis elegans*-infected (open bars) and -uninfected (grey bars) *Aedes aegypti* larvae and pupae. \* denotes a significant difference at  $P < 0.05$  between the sexes. Values in Table A-1.



**Figure 3-4.** The mean +SE amount of glucose reserves in *Plagiographis elegans*-infected (open bars) and -uninfected (grey bars) *Aedes aegypti* larvae. The mean is for three independent trials. \* denotes a significant difference at  $P < 0.05$  between treatments.

## CHAPTER 4: CONCLUSIONS

### 4.1 General Summary

In response to parasites, host organisms have developed mechanisms to counteract them, either directly through improved physiological defences, or indirectly by warning future generations. One common mechanism is the production of semiochemicals. Such chemicals may be produced by damaged plants to attract predators of the insects causing the initial damage (Takabayashi and Dicke 1996), or chemicals produced by mosquito larvae that warn conspecifics of a detrimental parasite in the habitat (Lowenberger and Rau 1994; Zahiri and Rau 1998). The purpose of this thesis was to determine if an oviposition deterrent produced by *Aedes aegypti* larvae parasitized by *Plagiorchis elegans* (Lowenberger and Rau 1994; Zahiri and Rau 1998) is related to trehalase inhibition in the mosquito larva (Zahiri et al. 1998; Wallage et al. 2001).

In the initial life-history bioassays with VAA, no significant differences in larval mortality were found over the course of the trials, although some larvae died with or without treatment. However, VAA had a significant effect on the number of adult mosquitoes that emerged and that were able to fly, with the

number of adults emerging and flying decreasing with increasing VAA concentration (Chapter 2). To find out whether physiological effects caused by VAA were comparable to results found by Zahiri et al. (1998), I examined the energy reserves in normal *Ae. aegypti* larvae and adults, and compared them to the energy reserves in mosquitoes reared with VAA. No significant differences were found between the amount of protein in mosquitoes reared with or without VAA. The total amount of lipids in whole larvae or adult mosquitoes was not significantly different with or without VAA. However there was a significant increase in hemolymph lipids in larvae exposed to VAA. I hypothesize that the inhibition of trehalase forces the mobilization of lipid reserves for metabolic energy (Chapter 2; Fig. 3-1). The trehalase inhibitor VAA was shown to significantly affect soluble carbohydrates in *Ae. aegypti* larvae and adults (Chapter 2), resulting in an increase in the amount of trehalose and a decrease in available glucose. The high amounts of trehalose and low amounts of glucose probably contribute to the absence of flight ability in VAA-exposed adults.

The water used to rear larvae exposed to 0.2 mg/mL of VAA for 72 h did not deter oviposition compared with water from larvae reared under standard laboratory conditions. On the contrary, water that had contained VAA-exposed larvae attracted adults (Chapter 2). This attraction must be the result of substances produced by the larvae rather than VAA, as control assays showed that VAA deters gravid females. Because of the imbalance between the use of carbohydrate and lipid metabolism caused by trehalase inhibition, the deterrent compound itself may be related to lipid metabolism, possibly the production of

ketone bodies. In vertebrates, exclusive use of lipid reserves to generate energy leads to ketosis, and the formation of ketone bodies that are excreted from the body (Stoker 2001). Similar processes may occur in mosquito larvae exposed to VAA. Preliminary data from another study in our laboratory has shown that the oviposition deterrent contains an ester group (K. Foster, personal communication). There are also patents examining the use of ketones and small carboxylic acids as mosquito repellents (Reifenrath 2001; Bernier et al. 2007).

With the exception of glycogen, my results produced data similar to those of Van Handel's 1988 study showing changes in the amounts of larval reserves over time (Appendix B). Differences between the amount of protein in whole larvae in Chapter 2 and Chapter 3 may have been caused by the different extraction reagents used, as both Triton-X and sodium hydroxide have been shown to interfere with the Bradford reagent at certain concentrations (Bio-Rad 2006). I was unable to replicate previous studies showing that parasitism by *P. elegans* reduces host hemolymph protein, lipid, and carbohydrate levels but causes an increase in the amount of hemolymph trehalose in *Ae. aegypti* larvae (Chapter 3). However, subsequent studies showed a clear but non-significant decrease in the amount of glucose in parasitized larvae when compared with control larvae, despite relatively high infection rates (Chapter 3; Fig. 3-4). Contrary to previous studies by Wallage et al. (2001), I found that larval trehalose decreased for 2 days post-infection with *P. elegans* (Chapter 3: Fig. 3-3E). This decrease in the amount of trehalose may reflect the parasite absorbing and metabolizing available hemolymph trehalose. The results of experiments in

Chapter 3 suggest that the parasite may be affecting the processing, uptake, or utilization of trehalose by *Ae. aegypti* larvae.

## **4.2 Future Directions**

### **Parasite ecology**

Unravelling the complex puzzle of host-parasite interactions makes the field of parasite ecology interesting and exciting. The ambiguity created by the experiments in Chapter 3 can be clarified by repeating the same experiments with a few modifications. In the experiments that measured reserves in *Ae. aegypti* larvae over time (Chapter 3) there were high levels of parasite-induced larval mortality that resulted in small sample sizes. Reducing the time of exposure of the larvae to *P. elegans* cercariae, or reducing the number of cercariae would allow for a lower mean intensity of infection, reducing larval mortality and giving a more realistic picture of what may be happening under natural settings.

### **Experimental Changes**

The use of plastic centrifuge tubes during the chloroform:methanol extractions (Chapter 3: Materials and Methods) may have released unwanted contaminating compounds into the lipid samples. This was later addressed with the use of glass centrifuge tubes in the experiments reported in Chapter 2; it

would be beneficial to use glass when repeating the Chapter 3 experiments. If the experiments in Chapter 3 are repeated, the data obtained are likely to provide a clearer picture of some of the interactions between a trematode parasite and its arthropod host.

### **Insect Physiology and Pest management**

I believe that VAA and other glycosidase inhibitors have the potential to be used in pest management. Chitinase inhibitors already are used for flea control in animals, and previous studies have shown that trehalase inhibitors may provide a novel method for dealing with specific insect pests. VAA had a negative impact on the development of *Ae. aegypti*, preventing the adults from flying (Chapter 2), and therefore future studies should look at developing this compound to control mosquitoes. Concentrations of 0.2 mg/mL of VAA attracted gravid females, and VAA solutions could be incorporated into oviposition traps in which ovipositing adults would be removed and any developing mosquitoes would be unable to fly.

The development of VAA as a mosquito control product would require efficacy trials, effects on vertebrates, determining LD<sub>50</sub>, and evaluating the effects of VAA on non-target organisms. The LD<sub>50</sub> may vary depending on the life-stage being targeted. The effects of trehalase inhibitors on non-target vertebrates and invertebrates, especially those that cohabit with mosquito larvae, should reflect



the anticipated mode of entry and concentrations to which the organisms would be exposed.

VAA is considered non-toxic to mammals because mammals generally do not utilize trehalose, although mammals still have trehalase present in their kidneys, and in their intestinal villi (Sacktor 1968). Trehalose is an important sugar for beneficial insects, such as bees, which exclusively use carbohydrates for flight energy (Suarez et al. 2005). This may limit the use of VAA as an insecticide to more discrete applications.

If required in large amounts, various trehalase inhibitors can be mass produced in fungi, such as *Micromonospora* sp. SANK 62390 (Ando et al. 1995) and *Streptomyces hygroscopicus* (Ando et al. 1995; Asano 2003).

The development of a synergist to increase activity, or absorption by the insect should also be investigated. A similar glycosidase inhibitor, Validamycin A (VMA), composed of VAA plus a  $\beta$ -D-glucopyranosyl is currently used to prevent the spread of fungal plant pathogens in Japan (Asano 2003).

Because of the interesting effects seen in the preceding chapters, the mechanism of VAA also warrants further study. VAA binds to trehalase through hydrogen bonds *in vitro* (Gibson et al. 2007), but how do trehalase inhibitors bind *in vivo*, and are they preferred by the enzyme over trehalose? Since trehalase can be found within cells (Becker et al. 1996), how are VAA and other trehalase inhibitors transported into the flight muscle cell or midgut cells? VAA might be transported into cells via the same mechanism as trehalose. The structure of VAA and trehalose is extremely similar (Fig. 1-1), and VAA may compete with

trehalose for the active site of the transport mechanism on the cell membrane. Preferential binding of the inhibitor may increase the amount of trehalase inhibitor transported into the cell and increase the probability of trehalase inhibition. The answers to these questions could have implications for the effects and applications of VAA and other trehalase inhibitors.

With regard to developing trehalase inhibitors as biorational insecticides for mosquito control, the future is unknown. In Canada, the major user of pesticides is the agriculture industry (PMRA 2006). Significantly fewer compounds are used in mosquito control. Developing countries often have the demand but lack the funds to carry out the research and development of mosquito insecticides. It is my sincere hope that research investigating alternative insect controls, be it through trehalase inhibition or another insect-critical biological mechanism, will continue, providing feasible alternatives to help control these troublesome global landlords.

I have shown in this thesis that:

1. *Plagiorchis elegans* does not significantly affect the amount of lipids, proteins, glycogen, trehalose, and soluble carbohydrates in *Aedes aegypti* larvae over time.
2. Contrary to previous studies, parasitism by *P. elegans* causes a clear but non-significant decrease in the amount of trehalose in *Ae. aegypti* during the first 48h.

3. The data support those of other studies showing changes in the amount of mosquito larval lipid and protein over time.
4. VAA does not significantly affect the development of *Ae. aegypti* larvae, but prevents the flight of exposed adults at emergence.
5. Ingestion of VAA by females leads to a decrease in the number of eggs laid when compared with females fed 10% sucrose.
6. Water containing only VAA deters gravid ovipositing females, but waters that held larvae exposed to VAA attract gravid females.
7. Exposure to VAA increases the amount of trehalose and hemolymph lipids in *Ae. aegypti* larvae, probably by blocking carbohydrate metabolism and mobilizing lipid stores. This imbalance in metabolism may lead to the production of ketone bodies.
8. VAA is taken up by *Ae. aegypti* larvae. This is the first known record of the effects of VAA on an aquatic insect.

## REFERENCES

- Ando O, Kifune M, and Nakajima M. 1995. Effects of trehalozin, a potent trehalase inhibitor, on *Bombyx mori* and plant pathogenic fungi. *Biosci. Biotech. Biochem.* 59: 711-712.
- Angelon KA, and Petranka JW. 2002. Chemicals of predatory mosquitofish (*Gambusia affinis*) influence selection of oviposition site by *Culex* mosquitoes. *J. Chem. Ecol.* 28: 797-806.
- Asano N. 2003. Glycosidase inhibitors: update and perspectives on practical use. *Glycobiology* 13: 93R-104R.
- Bailey CH, and Gordon R. 1973. Histopathology of *Aedes aegypti* (Diptera: Culicidae) larvae parasitized by *Reesemermis nielsenii* (Nematoda: Mermithidae). *J. Invertebr. Pathol.* 22: 435-441.
- Barbosa P, Peters TM, and Greenough NC. 1972. Overcrowding of mosquito populations: responses of larval *Aedes aegypti* to stress. *Environ. Entomol.* 1: 89-93.
- Baumann P, Clark MA, Baumann L, and Broadwell AH. 1991. *Bacillus sphaericus* as a mosquito pathogen: properties of the organism and its toxins. *Microbiol. Rev.* 55: 425-436.
- Becker A, Schlöder P, Steele JE, and Wegener G. 1996. The regulation of trehalose metabolism in insects. *Cell. Mol. Life Sci.* 52: 433-439.
- Becker N, Petric D, Zgomba M, Boase C, Dahl C, Lane J, and Kaiser A. 2003. Mosquitoes and their control. New York: Kluwer Academic/ Plenum Publishers.
- Belton P. 1983. British Columbia Provincial Museum, Handbook 41: The mosquitoes of British Columbia. Victoria, British Columbia: British Columbia Provincial Museum.
- Belton P. 1994. Attraction of male mosquitoes to sound. *J. Am. Mosq. Control Assoc.* 10: 297-301.
- Bentley MD, and Day JF. 1989. Chemical ecology and behavioral aspects of mosquito oviposition. *Ann. Rev. Entomol.* 34: 401-421.

Bernier UR, Kline DL, and Posey KH. 2007. Human emanations and related natural compounds that inhibit mosquito host-finding abilities. In: Debboun M, Frances SP, and Strickman D, editors. *Insect repellents: principles, methods, and uses*. New York: CRC Press.

Bio-Rad Laboratories (Bio-Rad). 2006. Quick start Bradford protein assay instruction manual. California, USA.

Bock D. 1984. The life cycle of *Plagiorchis* spec. 1, a species of the *Plagiorchis elegans* group (Trematoda: Plagiorchiidae). *Z. Parasitenkd* 70: 359-373.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-54.

Briegel H, 1990. Metabolic relationship between female body size, reserves, and fecundity in *Aedes aegypti*. *J. Insect Physiol.* 36: 165-172.

Bryant C, and Behm CA. 1989. *Biochemical adaptation in parasites*. New York: Chapman and Hall.

Candy DJ. 1981. Hormonal regulation of substrate transport and metabolism. In: Downer RGH, editor. *Energy metabolism in insects*. New York: Plenum Press. pp 19-52.

Chambers GM, and Klowden MJ. 1990. Correlation of nutritional reserves with a critical weight for pupation in larval *Aedes aegypti* mosquitoes. *J. Am. Mosq. Control Assoc.* 6: 394-399.

Charles J-F, Nielson-LeRoux C, and Delecluse A. 1996. *Bacillus sphaericus* toxins: molecular biology and mode of action. *Annu. Rev. Entomol.* 41: 451-472.

Chefurka W. 1965. Some comparative aspects of the metabolism of carbohydrates in insects. *Annu. Rev. Entomol.* 10: 345-382.

Chippendale GM. 1978. The functions of carbohydrates in insect life processes. In: Rockstein M, editor. *Biochemistry of insects*. New York: Academic Press. pp1-55.

Christophers SR. 1960. *Aedes aegypti* (L.) the Yellow Fever mosquito: its life history, bionomics and structure. Cambridge: Cambridge University Press. 739pp.

Clements AN. 1955. The source of energy for flight in mosquitoes. *J. Exp. Biol.* 32: 547-554.

Clements AN. 1963. The physiology of mosquitoes. New York: The Macmillan Company.

Clements AN. 1999. The biology of mosquitoes. Vol. II. Sensory reception and behaviour. Wallingford: Cabi Publishing.

Dempster SJ, Webber RA, Rau ME, and Lewis DJ. 1986. The effects of *Plagiorchis noblei* metacercariae on the development and survival of *Aedes aegypti* larvae in the laboratory. J. Parasitol. 72: 699-702.

Downer RGH, and Matthews JR. 1976. Patterns of lipid distribution and utilization in insects. Amer. Zool. 16: 733-745.

Elbein AD, Pan YT, Pastuszak I, and Carroll D. 2003. New insights on trehalose: a multifunctional molecule. Glycobiology 13: 17R-27R.

Eldridge BF. 2005. Mosquitoes, the Culicidae. In: Marquardt WC, Black WC, Freier JE, Hagedorn HH, Hemingway J, Higgs S, James AA, Kondratierff B, Moore CG, editors. Biology of disease vectors, 2nd ed. New York: Elsevier Academic Press. pp 95-111.

Foster K. 2008. Fitness consequences of oviposition site selection in *Aedes aegypti* [master's thesis]. Burnaby, British Columbia: Simon Fraser University.

Friedman S. 1985. Intermediary metabolism. In: Blum MS, editor. Fundamentals of insect physiology. New York: Wiley-Interscience Publication. pp 467-506.

Gibson RP, Gloster TM, Roberts S, Warren RAJ, Storch de Garcia I, García A, Chiara JL, and Davies GJ. 2007. Molecular basis for trehalase inhibition revealed by the structure of trehalase in complex with potent inhibitors. Angew. Chem. Int. Ed. 46: 4115-4119.

Giebel PE, and Domnas AJ. 1976. Soluble trehalases from larvae of the mosquito, *Culex pipiens*, and the fungal parasite, *Lagenidium giganteum*. Insect Biochem. 6: 303-311.

Greenwood BM, Bojang K, Whitty CJM, and Targett GA. 2005. Malaria. Lancet 365: 1487-1498.

Gubler DJ. 2002. Epidemic Dengue/Dengue hemorrhagic Fever as a public health, social and economic problem in the 21st century. Trends Microbiol. 10: 100-103.

Gubler DJ. 2004. The changing epidemiology of Yellow Fever and Dengue, 1900 to 2003: full circle? Comp. Immun. Microbiol. Infect. Dis. 27: 319-330.

Guzman MG, and Kouri G. 2003. Dengue and Dengue hemorrhagic Fever in the Americas: lessons and challenges. *J Clin Virol.* 27:1-13.

Hamilton RJ, and Hamilton S. 1992. *Lipid analysis: a practical approach.* Oxford: Oxford University Press. pp 13-64.

Hemingway J. 2005. Biological Control of Mosquitoes: In: Marquardt WC, Black WC, Freier JE, Hagedorn HH, Hemingway J, Higgs S, James AA, Kondratierff B, Moore CG, editors. *Biology of disease vectors*, 2nd ed. New York: Elsevier Academic Press. pp 649-660.

Honek A. 1993. Intraspecific variation in body size and fecundity in insects: a general relationship. *Oikos* 66: 483-492.

Janssen O, and Bock D. 1990. Identification and prevalence of two plagiorchiid trematodes from Hirundinidae in their intermediate and definitive hosts in the surroundings of Ulm, FRG. *Parasitol. Res.* 76: 451-453.

Kahn MC, and Offenhauser W. 1949. The first field test of recorded mosquito sounds used for mosquito destruction. *Am. J. Trop. Med.* s1-29: 811-825.

Kobayashi Y. 1999. The biology of trehalozins. *Carbohydr. Res.* 315: 3-15.

Kono Y, Takeda S, Kameda Y, Takahashi M, Matsushita K, Nishina M, and Hori E. 1993. Lethal activity of a trehalase inhibitor, validoxylamine A, and its influence on the blood sugar level in *Bombyx mori* (Lepidoptera: Bombycidae). *Appl. Entomol. Zool.* 28: 379-386.

Kono Y, Satoshi T, and Kameda Y. 1994. Lethal activity of a trehalase inhibitor, validoxylamine A, against *Mamestra brassicae* and *Spodoptera litura*. *J. Pesticide Sci.* 19: 39-42.

Kunieda T, Fujiyuki T, Kucharski R, Foret S, Ament SA, Toth AL, Ohashi K, Takeuchi H, Kamikouchi A, Kage E, et al. 2006. Carbohydrate metabolism genes and pathways in insects: insights from the honey bee genome. *Insect Mol. Biol.* 15: 563-576.

Lacey LA. 2007. *Bacillus thuringiensis* serovariety *israelensis* and *Bacillus sphaericus* for mosquito control. *J. Am. Mosq. Control. Assoc.* 23(sp2): 133-63.

Lamitina ST, and Strange K. 2005. Transcriptional targets of DAF-16 insulin signalling pathway protect *C. elegans* from extreme hypertonic stress. *Am. J. Physiol. Cell Physiol.* 288: C467-C474.

Lowenberger C, Chadee K, and Rau M. 1994. In vitro uptake and incorporation of [3H]glucosamine and [3H]leucine by *Plagiorchis elegans* metacercariae. J. Parasitol. 80: 363-370.

Lowenberger CA, and Rau ME. 1993a. *Plagiorchis elegans*: requirements for metacercarial development to infectivity, and conditions required for excystment. J. Helminthol. Soc. Wash. 60: 67-71.

Lowenberger CA, and Rau ME. 1993b. *Plagiorchis elegans*: emergence, longevity and infectivity of cercariae, and host behavioural modifications during cercarial emergence. Parasitology 109: 65-72.

Lowenberger CA, and Rau ME. 1994. Selective oviposition by *Aedes aegypti* (Diptera: Culicidae) in response to a larval parasite, *Plagiorchis elegans* (Trematoda: Plagiorchiidae). Environ. Entomol. 23: 1269-1276.

Mack SR, Samuels S, and Vanderberg JP. 1979. Hemolymph of *Anopheles stephensi* from noninfected and *Plasmodium berghei*-infected mosquitoes. 3. Carbohydrates. J. Parasitol. 65: 217-221.

MacLaurin DJ, and Green JW. 1969. Carbohydrates in alkaline systems. I. Kinetics of the transformation and degradation of D-glucose, D-fructose, and D-mannose in 1 M sodium hydroxide at 22°C. Can. J. Chem. 47: 3947-3955.

Morrison WR, and Smith LM. 1964. Preparation of fatty acid methyl esters and dimethylacetals with boron fluoride-methanol. J. Lipid Res. 5: 600-608.

Moscardi F. 1999. Assessment of the application of baculoviruses for control of lepidoptera. Annu. Rev. Entomol. 44: 257-289.

Muir LE, and Kay BH. 1998. *Aedes aegypti* survival and dispersal estimated by mark-release-recapture in northern Australia. Am. J. Trop. Med. Hyg., 58: 277-282.

Nayar JK, and Van Handel E. 1971. The fuel for sustained mosquito flight. J. Insect Physiol. 17: 471-481.

Nguyen D, Dutilleul P, and Rau ME. 2002. Impact of nutrition and exposure to the parasite *Plagiorchis elegans* (Trematoda: Plagiorchiidae) on the development of *Aedes aegypti* (Diptera: Culicidae): analysis by time-dependent transition probabilities. Environ. Entomol. 31: 54-64.

Pedigo LP. 2002. Entomology and pest management. 4th ed. Upper Saddle River, New Jersey: Prentice Hall.



Pest Management Regulatory Agency (PMRA). 2006. Information note: Pesticide application methods and areas of use. Canada.

Popple I. 2003, August 9. It's a snail parasite vs. the West Nile mosquito. The Globe & Mail. pF7

Reifenrath WG. 2001. Natural insect and arthropod repellent. United States Patent No. 6306415.

Resetarits WJ Jr., 1996. Oviposition site choice and life history evolution. Amer. Zool. 36: 205-215.

Rigau-Pérez JG, Clark GG, Gubler DJ, Reiter P, Sanders EJ, and Vorndam AV. 1998. Dengue and Dengue Haemorrhagic Fever. Lancet 352: 971-977.

Rosenheim JA, Jepsen SJ, Matthews CE, Solance Smith D, and Rosenheim MR. 2007. Portrait of an ephemeral adult stage: egg maturation, oviposition, and longevity of the gall midge *Rhopalomyia californica* (Diptera: Cecidomyiidae). Ann. Entomol. Soc. Am. 100: 549-561.

Sacktor B. 1968. Trehalase and the transport of glucose in the mammalian kidney and intestine. Biochemistry 60: 1007-1014.

Sampedro JG, and Uribe S. 2004. Trehalose-enzyme interactions results in structure stabilization and activity inhibition. The role of viscosity. Mol. Cell. Biochem. 256/257: 319-327.

SAS Institute Inc. 2005. JMP. Version 6.0. Cary, NC.

Schmidt SP, and Platzer EG. 1980. Changes in body tissues and hemolymph composition of *Culex pipiens* in response to infection by *Romanomermis culcivorax*. J. Invertebr. Pathol. 36: 240-254.

Schwab A, Lewis DJ, and Rau ME. 2003. The impact of selective oviposition and infection with *Plagiorchis elegans* on *Aedes aegypti* pre-imago population dynamics at optimal food availability. J. Med. Entomol. 40: 830-840.

Smith EH, and Kennedy GG. 2002. History of pesticides. In: Pimentel D, editor. Encyclopedia of pest management. New York: Marcel Dekker. pp. 376-380.

Smith RL, and Smith TM. 2001. Ecology and field biology. 6th ed. New York: Benjamin Cummings.

Solomon T, and Mallewa M. 2001. Dengue and other emerging flaviviruses. J Infect. 42: 104-115.

Spiegel J, Bennett S, Hattersley L, Hayden MH, Kittayapong P, Nalim S, Wang DNC, Zielinski-Gutierrez E, and Gubler D. 2005. Barriers and Bridges to Prevention and Control of Dengue: The Need for a Social–Ecological Approach. *EcoHealth* 2: 273–290.

Steele JE. 1981. The role of carbohydrate metabolism in physiological function. In: Downer RGH, editor. *Energy metabolism in insects*. New York: Plenum Press. pp 101-133.

Stoker HS. 2001. *General, organic, and biological chemistry*, 2nd edn. Boston: Houghton Mifflin Company.

Suarez RK, Darveau C-A, Welch KC Jr., O'Brien DM, Roubik DW, and Hochachka PW. 2005. Energy metabolism in orchid bee flight muscles: carbohydrate fuels all. *J. Exp. Biol.* 208: 3573-3579.

Takabayashi J, and Dicke M. 1996. Plant-carnivore mutualism through herbivore-induced carnivore attractants. *Trends Plant Sci.* 1: 109-113.

Takahashi M, Kono Y, Kurahashi H, Matsushita K, Nishina M, and Kameda Y. 1995. Effect of a trehalase inhibitor, Validoxylamine A, on three species of flies. *Appl. Entomol. Zool.* 30: 231-239.

Telang A, and Wells MA. 2004. The effect of larval and adult nutrition on successful autogenous egg production by a mosquito. *J. Insect. Phys.* 50: 677-685.

Timmermann SE, and Briegel H. 1993. Water depth and larval density affect development and accumulation of reserves in laboratory populations of mosquitoes. *Bull. Soc. Vector Ecol.* 18: 174-187.

Timmermann SE, and Briegel H. 1999. Larval growth and biosynthesis of reserves in mosquitoes. *J. Insect Physiol.* 45: 461-470.

Uglen GL, Lewis MC, and Larson OR. 1985. Niche segregation and sugar transport capacity of the tegument in digenean flukes. *Parasitology* 91: 121-127.

Van Handel E. 1985a. Rapid determination of glycogen and sugars in mosquitoes. *J. Am. Mosq. Control Assoc.* 1: 299-301.

Van Handel E. 1985b. Rapid determination of total lipids in mosquitoes. *J. Am. Mosq. Control Assoc.* 1: 302-304.

Van Handel E. 1986. Growth of three mosquitoes on two larval diets measured by protein accumulation. *J. Am. Mosq. Control Assoc.* 2: 289-291.

Van Handel E. 1988. Nutrient accumulation in three mosquitoes during larval development and its effect on young adults. *J. Am. Mosq. Control Assoc.* 4: 374-376.

Van Handel E, and Day JF. 1988. Assay of lipids, glycogen and sugars in individual mosquitoes: correlations with wing length in field-collected *Aedes vexans*. *J. Am. Mosq. Control Assoc.* 4: 549-550.

Vincent R, and Nadeau D. 1983. A micromethod for the quantitation of cellular proteins in Percoll with the Coomassie brilliant blue dye-binding assay. *Anal. Biochem.* 135: 355-362.

Wallage HR, Niven DF, and Rau ME. 2001. Effects of *Plagiorchis elegans* (Trematoda: Plagiorchiidae) infection on the carbohydrate metabolism of fourth-instar *Aedes aegypti* (Diptera: Culicidae). *J. Med. Entomol.* 38: 312-317.

Weathersby AB, and Noblet R. 1973. *Plasmodium Gallinaceum*: Development in *Aedes aegypti* maintained on various carbohydrate diets. *Exp. Parasitol.* 34: 426-431.

Wegener G, Tschiedel V, Schlölder P, and Ando O. 2003. The toxic and lethal effects of the trehalase inhibitor trehalozin in locusts are caused by hyperglycemia. *J. Exp. Biol.* 206: 1233-1240.

Wolfrom ML, Schuetz RD, and Cavalieri LF. 1948. Chemical interaction of amino compounds and sugars. III. The conversion of D-glucose to 5-(hydroxymethyl)-2-furaldehyde. *J. Am. Chem. Soc.* 70: 514-517.

Woodring J, and Davidson EW. 1996. Biological control of mosquitoes. In: Beaty BJ, and Marquardt WC, editors. *The biology of disease vectors*, 1st ed. Niwot, Colorado: University Press of Colorado.

World Health Organization (WHO). 2001. Fact sheet N°100: Yellow Fever. [Internet]. Available from: <<http://www.who.int/mediacentre/factsheets/fs100/en/>> Accessed 2007

World Health Organization (WHO). 2002. Fact sheet N°117: Dengue and Dengue Haemorrhagic Fever. [Internet]. Available from: <<http://www.who.int/mediacentre/factsheets/fs117/en/>> Accessed 2007 September 10.

World Health Organization (WHO). 2007. Fact sheet N°94: Malaria. [Internet]. Available from: <<http://www.who.int/mediacentre/factsheets/fs94/en/>> Accessed 2008 January 7.

World Health Organization (WHO). 2008. Epidemic and pandemic alert and response: Yellow Fever. [Internet]. Available from: <[http://www.who.int/csr/don/archive/disease/yellow\\_fever/en/](http://www.who.int/csr/don/archive/disease/yellow_fever/en/)> Accessed 2008 September 11.

Zahiri N, and Rau ME. 1998. Oviposition attraction and repellency of *Aedes aegypti* (Diptera: Culicidae) to waters from conspecific larvae subjected to crowding, confinement, starvation, or infection. J. Med. Entomol. 35: 782-787.

Zahiri N, Dunphy GB, and Rau ME. 1998. Serum composition of *Aedes aegypti* (Diptera: Culicidae) larvae and the production of an oviposition repellent are influenced by infection with the entomopathogenic digean *Plagiorchis elegans* (Trematoda: Plagiorchiidae), starvation and crowding. J. Med. Entomol. 35: 162-168.

Zakikhani M, and Rau ME. 1999. *Plagiorchis elegans* (Digenea: Plagiorchiidae) infections in *Stagnicoles elodes* (Pulmonata: Lymnaeidae): host susceptibility, growth, reproduction, mortality, and cercarial production. J. Parasitol. 85: 454-46.

## APPENDIX A: ENERGY RESERVES IN CONTROL AND *PLAGIORCHIS ELEGANS*-INFECTED *AEDES AEGYPTI* LARVAE OVER TIME.

**Table A-1.** Mean  $\pm$  SE analyses of whole *Aedes aegypti* larvae or larvae parasitized with the trematode *Plagiorchis elegans* over time. Means of at least 2 replicates. Data shown in Figure 3-3. All quantities in  $\mu\text{g}$ .

Time <sup>a</sup>	Group	Protein	Lipid	Glycogen	Soluble sugars	Trehalose
8	Control	294.68 $\pm 64.88$	67.72 $\pm 39.59$	7.22 $\pm 3.87$	18.41 $\pm 12.77$	5.56 $\pm 7.09$
	Parasitized	242.29 $\pm 82.84$	88.55 $\pm 50.86$	8.20 $\pm 5.05$	22.24 $\pm 16.32$	4.32 $\pm 3.16$
12	Control	260.51 $\pm 53.69$	78.05 $\pm 41.99$	10.31 $\pm 5.63$	33.47 $\pm 44.20$	4.84 $\pm 5.00$
	Parasitized	243.05 $\pm 59.15$	75.21 $\pm 38.86$	4.55 $\pm 1.41$	17.65 $\pm 12.36$	2.35 $\pm 1.68$
24	Control	295.21 $\pm 81.23$	78.09 $\pm 39.59$	10.97 $\pm 5.43$	23.21 $\pm 18.34$	5.79 $\pm 9.73$
	Parasitized	239.14 $\pm 73.94$	94.54 $\pm 42.24$	11.41 $\pm 6.60$	35.03 $\pm 38.61$	0.97 $\pm 0.77$
48	Control	321.94 $\pm 145.64$	94.27 $\pm 57.69$	10.20 $\pm 5.31$	21.92 $\pm 18.04$	4.32 $\pm 5.96$
	Parasitized	211.32 $\pm 95.49$	103.44 $\pm 40.49$	6.13 $\pm 5.70$	23.94 $\pm 20.89$	0.50 $\pm 0.14$
72	Control	329.42 $\pm 131.55$	87.60 $\pm 28.93$	14.07 $\pm 9.24$	28.66 $\pm 33.86$	3.64 $\pm 3.97$
	Parasitized	337.64 $\pm 215.01$	146.51 $\pm 51.47$	6.86 $\pm 3.30$	66.46 $\pm 90.49$	6.28 $\pm 4.98$
<i>P</i> -value <sup>b</sup>		0.13	0.38	0.19	0.71	0.45

<sup>a</sup> Time post-infection with *Plagiorchis elegans* in h.

<sup>b</sup> Results were analyzed for all energy reserves using a Standard Least Square (SLS) analysis.

## APPENDIX B: COMPARISON OF AMOUNTS OF LARVAL RESERVES OVER TIME

**Table B-1.** Mean  $\pm$ SE amount of protein (A), lipid (B), glycogen (C), total sugars (D), and trehalose (E) found in *Aedes aegypti* larvae at different times after hatching. The values are compared with those of Van Handel (1988). N = number of replicates. All quantities in  $\mu$ g.

A.

	Protein/larva	N	Van Handel (1988)
Day 4	277.59 $\pm$ 59.52	12	250
Day 5	295.21 $\pm$ 81.23	5	400
Day 6	329.42 $\pm$ 131.55	5	350

B.

	Lipid/larva	N	Van Handel (1988)
Day 4	70.62 $\pm$ 39.62	12	50
Day 5	78.09 $\pm$ 39.59	5	85
Day 6	94.27 $\pm$ 57.69	5	75

C.

	Glycogen/larva	N	Van Handel (1988)
Day 4	8.76 $\pm$ 4.88	12	20
Day 5	10.97 $\pm$ 5.43	6	40
Day 6	10.20 $\pm$ 5.31	6	55

D.

	Total sugars/larva	N	Van Handel (1988)
Day 4	17.62 $\pm$ 15.34	10	20
Day 5	23.21 $\pm$ 18.34	6	23
Day 6	21.92 $\pm$ 18.04	6	11

E.

	Trehalose/larva	N	Van Handel (1988)
Day 4	4.57 $\pm$ 5.29	7	7
Day 5	5.79 $\pm$ 9.73	4	8
Day 6	4.32 $\pm$ 5.96	4	5