FITNESS CONSEQUENCES OF OVIPOSITION BEHAVIOUR IN *AEDES AEGYPTI*

by

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ABSTRACT

Aedes aegypti is the main vector of Dengue fever, the most important mosquito-borne viral disease affecting humans. Oviposition site selection has direct effects on vector fitness and population numbers. Female *Ae. aegypti* that emerged from poor habitats with high larval densities and low food availability were smaller, had lower teneral reserves, and laid smaller, fewer, and less viable eggs than did females raised in good habitats. Females that were raised under good conditions but were infected with a trematode parasite were smaller, laid smaller eggs, and fewer parasitized females oviposited than control females. Females should evaluate site quality and avoid ovipositing in poor habitats. We have identified putative oviposition deterrent compounds, possibly of larval origin, in waters containing parasitized or stressed larvae. Females that recognize these compounds may reject these oviposition sites and search for sites that will allow them to maximize their individual fitness.

Keywords: *Aedes aegypti*, oviposition, *Plagiorchis elegans*

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DEDICATION

This thesis is for my parents, Bryan and Karen Foster, who have always been pillars of strength and who have given me undying support throughout my life. In undertaking any endeavour, confidence and belief in oneself are the most important allies in the trudge toward completion and success. I am very lucky to have parents who, through their own sincere belief in me and through endless encouragement, have ingrained in me the ideas that anything is possible and that my future is not only bright, but brilliant. I owe my success in this degree to the values and character they have instilled in me, and it is with immeasurable gratitude and love that I dedicate this thesis and my work to them.

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CHAPTER 1: INTRODUCTION TO AEDES AEGYPTI AND OVIPOSITION

Infectious and arthropod-borne diseases

Infectious diseases are a leading cause of mortality and major health problems worldwide (Gubler 1998a, DaSilva and Iaccarino 1999, Gratz 1999, Gubler 2002b, Mackenzie et al. 2004). Despite the available treatments and prevention measures for many bacterial, viral and parasitic diseases, 15 million deaths annually can be attributed directly to infectious disease (Schrag and Wiener 1995, Morens et al. 2004). Arthropod-transmitted pathogens cause significant human mortality and morbidity throughout the world. This is especially true in developing countries where vector-borne diseases place an increasing burden on the health care systems. Worldwide, there are 500 million cases of malaria annually and 1-3 million deaths annually are directly attributable to the disease (Sachs and Malaney 2002, Greenwood et al. 2005). Malaria costs Africa US$12 billion each year and severely impedes economic growth through loss of productivity due to worker illness, absenteeism and death (Gallup and Sachs 2001, Greenwood et al. 2005). Dengue and yellow fever, transmitted by the yellow fever mosquito, *Aedes aegypti*, affect over 100 million people each year (Gubler 1998b, Monath 2001, Gubler 2002a), and more than half of the world's population is at risk of contracting dengue virus (Mairuhu et al. 2004).

In addition to these major diseases, arthropods transmit the parasites and pathogens that cause bubonic plague, Lyme disease, trench fever, onchocerciasis, African trypanosomiasis (African sleeping sickness), leishmaniasis, and American trypanosomiasis (Chagas' disease) among others.

Mosquito vectors and mosquito-borne diseases

Of particular note, mosquitoes, due to their role in transmitting many parasites, pathogens and viruses to humans and domestic animals, are considered the most medically important vectors of human disease. Mosquitoes serve as vectors for malaria, dengue fever, dengue haemorrhagic fever, yellow fever, filariasis, West Nile virus, Japanese encephalitis virus, Rift valley fever, St. Louis encephalitis, O'nyong nyong virus, Western and Eastern equine encephalitis virus among others. West Nile virus and dengue haemorrhagic fever are considered emerging diseases, they have appeared recently in novel host populations and/or geographic regions (Schrag and Wiener 1995, DaSilva and Iaccarino 1999, Gratz 1999). Yellow fever and dengue fever are re-emerging diseases, they have re-appeared in host populations and/or geographic regions after a period of absence and/or they are present at new epidemic levels within host populations levels rather than endemic levels (Gubler 1998a, Gratz 1999, Gubler 2002b, Mackenzie et al. 2004).

Public health interventions, chemotherapy, and vector control programs have been used effectively to reduce the spread of these diseases.

Unfortunately, these interventions and their benefits often have been short-lived. The resurgence of many vector-borne diseases is a result of a dependence on single short-term solutions. As a consequence, parasite, pathogen and vector populations have developed drug and insecticide resistance respectively. The changing of public health policies and the deprioritizing of prevention all have contributed to the current situation (Gubler 1998a).

The transmission of vector-borne diseases depends to a large extent on vector population levels and distributions. High or increasing vector densities within a geographic region can create epidemic transmission of new or previously low-level endemic diseases in the susceptible human population (Gratz 1999). Human activities, such as uncontrolled population growth, rapid and unplanned urbanization and changes in agriculture and forestry practices often create ideal conditions for vector-borne disease transmission. Man-made vector habitats close to human housing (Schrag and Wiener 1995, Gubler 1998a, 2002b, Mackenzie et al. 2004) allow vectors to increase their populations exponentially and exploit humans as a blood source.

A change or shift in vector or host ecology can significantly increase or decrease transmission of vector-borne disease (Schrag and Wiener 1995). Researchers have long warned that global warming could change the geographical distribution of vectors, introducing pathogens into naïve host populations (Gubler 1998a, Patz et al. 1998, Gratz 1999, Forget and Lebel 2001,

Solomon and Mallewa 2001, Sachs and Malaney 2002). Patz et al. (1998) predict that increasing seasonal temperatures will increase the epidemic potential for Dengue fever in geographic regions where cases are currently low or absent. Higher ambient temperatures may increase vector populations, decrease generation times, and expedite viral infection dissemination in mosquito vectors (Patz et al. 1998).

Dengue Fever

Dengue fever, caused by any of the four serotypes of the Dengue virus (DENv), is the most important and rapidly spreading mosquito-borne viral disease affecting humans (WHO 2006). There are 50-100 million cases of Dengue fever (DF) annually and an estimated 2.5 billion people live in areas endemic for Dengue virus transmission (Solomon and Mallewa 2001, Gubler 2002a, Guzman and Kouri 2003). The major vector of Dengue viruses is the yellow fever mosquito, *Aedes aegypti.* An expansion of the range of this vector and of the four serotypes of DENv have created hyperendemic regions in which multiple Dengue strains are circulating (Gubler 1998b). Dengue fever is a self-limiting febrile illness whose symptoms include myalgia, headache and retro-orbital pain and is treated with anti-pyretics and oral hydration (Mairuhu et al. 2004). Dengue Haemorrhagic Fever (DHF) is a more serious form of DF with haemorrhagic manifestations, a decrease in blood platelets and increased vascular permeability resulting in loss of plasma (Mairuhu et al. 2004). Dengue Shock Syndrome (DSS) occurs when sufficient plasma is leaked into the extravascular space to cause shock (Mairuhu et al. 2004). DHF and DSS are caused by sequential infections

with different serotypes of Dengue virus. Therefore, hyperendemicity has facilitated the emergence of DHF in new geographic regions. Currently, there are 250,000- 500,000 cases of DHF annually (Guzman and Kouri 2003). The global burden of Dengue virus has increased at least four-fold in the past three decades and over 2.5 billion people are estimated to be at risk for Dengue transmission (WHO 2006). The 2005 Revision of the International Health Regulations by the World Health Assembly included Dengue as a disease that may be a public health emergency of international concern (WHO 2006).

Because there is no vaccine for DENv, the main method of intervention is mosquito control (Rigau-Pérez et al. 1998, Solomon and Mallewa 2001) to interrupt transmission of the virus and the spread of DF/DHF in human populations. *Aedes aegypti,* the principal vector of DF, is a highly anthropophilic mosquito species distributed 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).

Mosquito control and Public Health

Mosquito control programs coordinated by the Pan American Health Organization through the 1950s and 1960s focused mainly on large scale insecticide-based strategies (Spiegel et al. 2005). This top-down approach employed on a massive scale was initially very successful (Rigau-Pérez et al. 1998), and many countries in the Americas were declared free of *Ae. aegypti*. Due in part to its success, this regional program was disbanded in the 1970s and

Ae. aegypti re-established itself in the Americas. Whether this was due to a re-invasion from other regions or from residual populations that re-emerged after control programs were stopped is unknown. Regardless, the situation has returned to pre eradication levels and there is a need for new approaches toward mosquito control.

Large-scale vertical insecticide-based programs that are not integrated with other disease prevention efforts are not feasible long-term solutions (Spiegel et al. 2005). Programs focused on the elimination of both immature and adult stages of mosquito vectors in a sustainable way with community involvement are now considered to be the best approaches. *Aedes aegypti* thrives in peridomestic environments; human hosts are readily available and man-made containers provide larval habitat (Christophers 1960, Clements 1999) (Figure 1). Targeting immature stages of mosquito vectors and eliminating oviposition habitat are the mainstays of these programs and cleaning up domestic refuse and eliminating sources of standing water in and around homes are central to these efforts. Methods for identifying the most productive larval habitats and focusing control efforts at these sources are needed to improve efficacy, sustainability and cost-effectiveness of vector control programs (WHO 2006). Coordinating these control efforts to maximize the reduction of vector populations and risk of DENv transmission in a cost effective manner requires intimate knowledge of the biology, physiology and ecology of the mosquito vectors.

Aedes aegypti lifecycle

Within the life cycle of *Ae. aegypti*, bloodfeeding and oviposition are two major activities that affect changes in population numbers and directly affect pathogen transmission (Figure 2). Males feed exclusively on sugar whereas females feed on both sugar and blood (Foster 1995, Costero et al. 1998, Briegel et al. 2001). Females require a bloodmeal as a source of lipids and protein to produce eggs (Klowden 1990) which are laid on, or near, free standing water. Eggs hatch after submersion in water and four subsequent free-swimming larval stages develop in the aquatic environment (Figure 2). Adults eclose from pupae and both male and female adults disperse from the larval habitat. Bloodfeeding frequency therefore affects vector population levels as well as the transmission frequency of Dengue virus (Canyon et al. 1999). Oviposition site selection can affect the number of offspring that emerge as healthy adults, thus affecting dynamics of vector populations and virus transmission. *Aedes aegypti* is a proficient vector of DENv partially because it preferentially feeds on human hosts (Harrington et al. 2001) and exploits human-made habitats, creating and reinforcing the human-mosquito-human transmission cycle (Mackenzie et al. 2004).

Chemoreception

Both bloodfeeding and oviposition depend on the perception of environmental stimuli. Female mosquitoes use physical and chemical environmental cues to find hosts and oviposition substrates. Sensillae are the basic sense organs of insects and are composed of three parts: a cuticular

interface with the environment, sensory neurons and associated cells (Ryan 2002). Sensory neurons translate environmental cues into electrical impulses that cause behavioural or physiological responses (Jacquin-Joly and Merlin 2004). There are three types of sensillae: aporous, uniporous and multiporous. Aporous sensillae usually are responsive to mechanical stimulation or to temperature, moisture or texture. Both uni- and mulitporous sensillae are chemoreceptive sensillae that respond to chemical stimuli. Uniporous sensillae, those having only one pore or cuticular breach, are classed as gustatory or tactile sensillae (Ryan 2002). Mosquito gustatory sensillae are located on the mouthparts and tarsi and are reactive upon contact with liquids (Clements 1999). Multiporous sensillae, those having many pores, are olfactory sensillae that are responsive to volatile or airborne chemical stimuli (Ryan 2002). In the mosquito these are located on the antennae and maxillary palps (Clements 1999).

Female mosquitoes respond to different cues to find suitable hosts and take a bloodmeal: carbon dioxide, heat, rough visual stimuli, moisture and chemicals (or specific mixes of chemicals) in host emissions and odours (Bosch et al. 2000, Bernier et al. 2003, Zwiebel and Takken 2004). Carbon dioxide is especially important in sensitizing *Ae. aegypti* females to other host odours in the host-seeking process (Dekker et al. 2005).

Female *Ae. aegypti* are fertilized within hours of emergence and begin host-seeking behaviour within 24 hours of eclosion (Christophers 1960, Klowden

1990). The sperm from their first mate is used to fertilize their eggs; matrone, a proteinaceous male accessory gland excretion, prevents fertilization of eggs after the first copulation event (Hiss and Fuchs 1972). Detailed reviews of the physiology of, behavioural aspects of, and events leading to the chemoreception of host cues, the identification of suitable vertebrate hosts, the processes associated with bloodfeeding, and the chemical ecology of bloodfeeding events can be found elsewhere (Allan et al. 1987, Bowen 1991, Zwiebel and Takken 2004). Once a full bloodmeal is ingested, the distension of the female mosquito's abdomen inhibits further host-seeking behaviour (Klowden and Lea 1978, 1979, Klowden 1990). This abdominal-distension inhibition is coupled with an oocyte-development inhibition of host-seeking behaviour that continues through egg development and ends with oviposition (Klowden 1990).

Oviposition

Female *Ae. aegypti* oviposit approximately 48 to 72 hours after ingesting a bloodmeal (Christophers 1960, Clements 1999). This species exhibits skip-oviposition, a process in which females deposit eggs from one egg batch in multiple oviposition sites (Corbet and Chadee 1993, Colton et al. 2003, Trexler et al. 2003). Eggs are laid in small temporary sources of clean water; commonly in man-made containers such as tires, domestic refuse, domestic water storage containers, flower vases and plant pots (Christophers 1960, Clements 1999). As opposed to other genera, eggs of *Aedes* sp. are often laid on the sides of containers or temporary pools either at the water line or on the moist area just above it (Christophers 1960, Clements 1999). Many oviposition sites are

transient and larvae feed on organic material usually on the bottom or sides of the containers they inhabit. Therefore females should lay their eggs in a site containing sufficient water and resources to sustain larval populations (Christophers 1960, Bentley and Day 1989, Muir and Kay 1998, Clements 1999).

Oviposition site-seeking behaviour begins after the digestion of a bloodmeal (Klowden and Blackmer 1987). With the ingestion and digestion of a bloodmeal, neurons used to detect host cues become inactive and neurons used to detect oviposition-site cues are activated (Bentley and Day 1989). Gravid females are sensitive to environmental cues indicative of oviposition sites rather than of host-types. There is a physiological switch in chemoreception during oogenesis that stimulates a female to find appropriate larval habitat for her progeny (Klowden and Blackmer 1987). The process of oviposition can be broken down into two discrete behavioural phases: pre-oviposition and oviposition. Pre-oviposition constitutes all behaviours associated with locating and selecting an oviposition site, whereas oviposition is the actual laying of eggs (Klowden and Blackmer 1987, Bentley and Day 1989).

In pre-oviposition, gravid *Ae. aegypti* use both physical and chemical environmental cues to locate and ultimately accept or reject an oviposition site (Bentley and Day 1989, Clements 1999).

Females respond to environmental cues and use different sensory organs to evaluate site quality and accept or reject that site (Clements 1999). Generally, eyes, and aporous and olfactory sensillae are used in the attraction/location phase whereas aporous, olfactory and gustatory sensillae are used in the acceptance/rejection phase. The initial phase of pre-oviposition involves long-range visual or olfactory cues detected in flight. Sun exposure, humidity, colour, temperature, and odour of a site and vegetation surrounding it are well established as important long-range cues (Bentley and Day 1989, Clements 1999). The acceptance/rejection phase of pre-oviposition involves shorter-range olfactory and gustatory cues such as volatile compounds emanating from the site or chemicals in the water of the site (Bentley and Day 1989). Chemical cues are classified according to the behavioural responses they elicit from gravid females in pre-oviposition. A substance is considered an oviposition attractant if gravid females show directed movement toward its source. If gravid females actively direct themselves and move away from its source the substance is considered an oviposition repellent. In the case of oviposition deterrents, a female may move toward a cue source, will land upon a site, but will lay few or no eggs (Bentley and Day 1989, Clements 1999).

Oviposition site selection, larval performance and maternal fitness

The survival, growth and fecundity of organisms with low parental investment and immature dispersal depend greatly on the quality of the habitat selected by the female (Heard 1994). In selecting an oviposition site, females choose the habitat for their offspring. This habitat choice affects overall maternal

fitness by affecting the performance of progeny developing in that habitat (Heard 1994, Mokany and Shine 2003). Female mosquitoes preferentially oviposit in waters that contain healthy conspecific larvae (Kalpage and Brust 1973, Bentley and Day 1989, Lowenberger and Rau 1994, Zahiri et al. 1997a). Gravid females selectively lay eggs in sites containing oviposition-attractant compounds, and heneicosane has been promoted as an oviposition-attractant compound of larval origin responsible for this phenomenon in *Ae*. *aegypti* (Mendki et al. 2000). The ability to assess site quality and select sites that provide sufficient resources to support larval development is crucial in situations where subsequent parental investment is minimal (Kalpage and Brust 1973). Decaying, fermenting or dissolved organic material and bacteria are good food sources for mosquito larvae; thus bacteria and their metabolites can act as oviposition attractants (Laird 1988, Allan and Kline 1995, Navarro et al. 2003, Trexler et al. 2003). Gravid females are attracted to good quality sites containing conspecific or congeneric larvae but if larvae are stressed, in poor quality environments or are exposed to unfavourable conditions they may produce substances that deter oviposition (Zahiri and Rau 1998).

Ovipositing in a site where progeny would suffer high mortality due to high competition, parasitism or predation should not be favoured by natural selection (Petranka and Fakhoury 1991, Stav et al. 1999). Density-dependent competition for resources, both intraspecific and interspecific, increases larval mortality and delays larval development within a site (Agnew et al. 2000, Gleiser et al. 2000).

With all other variables controlled, larvae with access to sufficient quantities of food develop faster, are larger, and become larger adults than those that do not (Hawley 1985, Briegel 1990b, a, Lowenberger and Rau 1994, Tun-Lin et al. 2000, Nguyen et al. 2002). Larger females live longer, have higher fecundity and can complete a gonotrophic cycle faster than smaller females (Hawley 1985, Briegel 1990b). There is a distinct fitness disadvantage to larvae developing in environments with high density-dependent competition, and *Ae. aegypti* females are deterred from ovipositing in waters containing crowded and/or starved conspecific larvae (Zahiri et al. 1997b, Zahiri and Rau 1998). The presence of accumulated waste products, bacterial metabolites, and allelopathic substances produced under crowded conditions may all be factors that contribute to a female deciding not to oviposit in crowded sites (Bédhomme et al. 2005). Some compounds associated with crowding have been identified and shown to be oviposition repellents (Hwang et al. 1974a, b, Hwang et al. 1976b, Hwang et al. 1976a, Hwang et al. 1978).

Oviposition in larval habitats with high risk for predation also should be avoided (Stav et al. 1999). The overall fitness of ovipositing females decreases as larval mortality due to predation increases. Gravid females show reduced oviposition in larval habitats that contain predators or cues associated with predators of mosquito larvae (Stav et al. 1999, Mokany and Shine 2003, Blaustein et al. 2004, Eitam and Blaustein 2004). Thus, the presence of predators in an otherwise acceptable site should deter or repel females from

ovipositing. This same logic extends to parasites that impact the survival and development time of mosquito larvae and the fitness of adults emerging from an infested site (Haq et al. 1981, Galloway and Brust 1985, Rao et al. 1985, Dempster et al. 1986). The trematode parasite *Plagiorchis elegans* can use mosquito larvae as second intermediate hosts. Parasitic xiphidiocercariae emerge from their snail intermediate host, penetrate the cuticle of mosquito larvae, encyst in the insect haemocoel, absorb nutrients from their host and develop into infective metacercariae (Lowenberger and Rau 1993, Lowenberger et al. 1994). *Aedes aegypti* is repelled/deterred from ovipositing in waters containing conspecific larvae infected by *P. elegans* metacercariae (Lowenberger and Rau 1994).

Infection with high numbers of *P. elegans* metacercariae can increase larval mortality, prolong larval development and decrease adult emergence in *Ae. aegypti* (Dempster et al. 1986, Nguyen et al. 2002, Schwab et al. 2003). This oviposition repellent/deterrent compound is suspected to be of larval origin (Lowenberger and Rau 1994). Whether infected or moribund larvae produce a compound to communicate poor habitat conditions to gravid females or whether this is a simple by-product of parasite/host metabolism is unknown (Hilker and Meiners 2002). Because *Ae. aegypti* females typically disperse less than 500m from their site of emergence, and exhibit skip oviposition, there is a high likelihood that *Ae. aegypti* larvae at a given site are highly related to ovipositing females (Apostol et al. 1993, Corbet and Chadee 1993, Apostol et al. 1996, Muir and Kay 1998, Colton et al. 2003, Trexler et al. 2003) and the concept of

oviposition deterrent compounds being components of kin selection have been proposed (Lowenberger and Rau 1994, Hilker and Meiners 2002).

Females that make poor choices and oviposit in sites that will not support the development and survival of their progeny risk losing their maternal investment and thus decreasing their fitness (Zahiri and Rau 1998). Aside from the more obvious lethal effects on the overall fitness of females, there are also sub-lethal effects. The main objectives of this thesis are to elucidate the sub-lethal fitness effects of poor oviposition decisions and explore the links between these fitness effects and the chemical ecology of oviposition behaviour in *Ae. aegypti*. If a female incurs detrimental fitness effects by ovipositing in poor habitats, can poor decisions be avoided? If gravid *Ae. aegypti* females can distinguish between healthy or parasitized larvae in good or poor habitats through chemoreception, what are the semiochemicals involved, and how, when, and by whom are they produced?

Investigations

We investigated the sub-lethal effects on fitness parameters of *Ae. aegypti* in response to crowding and starvation and parasitic infection by *P. elegans*. Three larval habitats were simulated under laboratory conditions: (1) control mosquito larvae (CM) where larvae were reared at a low density and with ample food, (2) density and food stress (DFS) where larvae were reared under crowded and starvation conditions, and (3) *P. elegans* parasitized (PEL) where larvae

were infected with *P. elegans* but otherwise held under control conditions. The fitness parameters of adult mosquitoes eclosed from each larval treatment regime were measured and compared to approximate the sub-lethal effects on fitness of each treatment. Adult size, fecundity, egg size, egg viability were used as indicators of fitness and the nutrient reserves of protein, carbohydrates and lipids of both pupae and eggs laid were used as indicators of performance.

The selective oviposition of *Ae. aegypti* to waters containing stressed conspecific larvae was also investigated. Six larval stress treatments were used to attempt to induce the expression of deterrent compounds by larvae including activating the immune system, mimicking the penetration process of parasites, exposing larvae to *P. elegans* or to a nematode endoparasite, and exposing larvae to a larvicide.

We also investigated the differential presence of specific compounds in larval waters that were attractive or deterrent to gravid females using various solvents and a comparison of chemical profiles. Solvent extracts were analyzed by coupled gas chromatographic-electroantennographic detection (GC-EAD) and GC-mass spectrometry to identify biologically active molecules. The fatty acid profiles of waters that were attractive and repellent also were compared.

Until drugs or vaccines become available, inexpensive, and easily implemented, programs to reduce diseases associated with *Ae. aegypti* will be based on reducing vector populations. The experiments described in this thesis evaluate the consequences of making poor decisions in selecting a suitable oviposition site. To minimize the negative consequences, females should respond to cues, both positive and negative, to evaluate site quality. Understanding the chemical ecology of mosquito oviposition and the chemicals that elicit a response might lead towards understanding how insect stages in different media (aquatic larvae and terrestrial adults) produce and perceive cues, and the specific molecules responsible for changes in oviposition behaviour. These molecules then might be developed and incorporated into long-term strategies and sustainable approaches to manipulate natural larval habitats, to disrupt normal mosquito behaviour, and for monitoring the presence, or changes in population numbers, of this important vector species.

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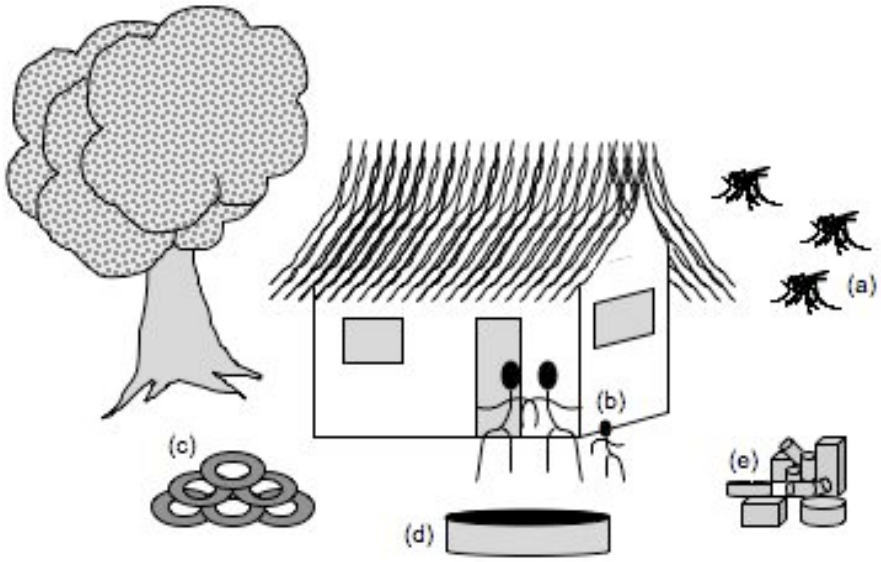

Figure 1 - Peridomestic environment with larval habitat and human hosts. *Aedes aegypti* and dengue virus transmission thrive in peridomestic environments where adults (a) have access to human hosts (b) and can use man-made containers as larval habitat: tires (c), water storage tanks (d) and domestic refuse (e) are all commonly used as larval habitat.

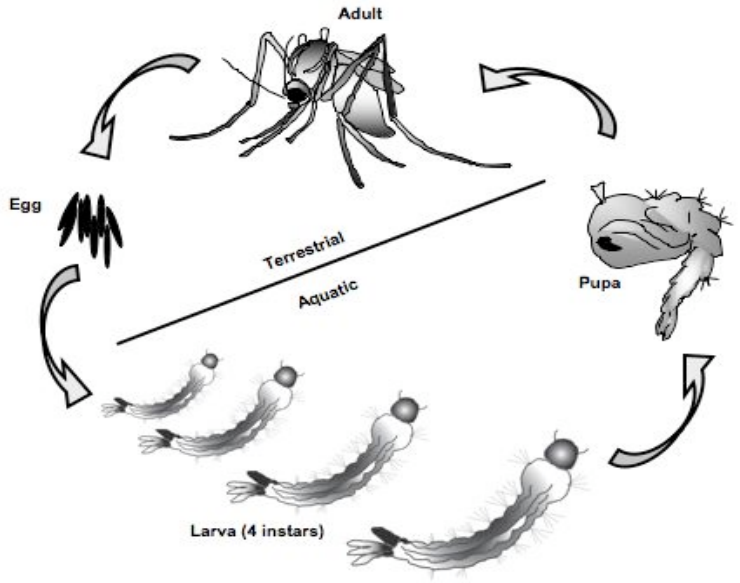

Figure 2 - Life cycle of *Aedes aegypti* consisting of the following stages: egg, four larval instars, pupa and adult.

CONNECTING STATEMENT

In the previous chapter we discussed the concept that female mosquitoes should discriminate between potential oviposition sites to select those sites that would best support the development and growth of her offspring. This would imply that females can use physical and chemical cues to evaluate the quality of potential sites. More importantly, this concept implies that there are negative consequences of not being choosy in selecting where to lay her eggs. In the next chapter we manipulate the conditions in which larvae hatch, grow, and emerge to determine if, indeed, there are consequences of laying eggs in different sites on the success of those offspring.

CHAPTER 2: FITNESS CONSEQUENCES OF LARVAL HABITAT IN *AEDES AEGYPTI*

Introduction

Dengue fever is the most important and rapidly spreading mosquito-borne viral disease affecting humans (WHO 2006). An estimated 2.5 billion people live in areas endemic for Dengue virus transmission resulting in 50-100 million cases of Dengue fever annually (Guzman and Kouri 2003). The principal vector of Dengue viruses around the world is *Aedes aegypti* (L.), the yellow fever mosquito. This species has become associated with humans to a point where it feeds almost exclusively on human blood and resides within and around human habitation. After the successful *Ae. aegypti* eradication program in the Americas was disbanded in the 1970s, this species has re-invaded most countries in the region (Gubler 2002). This successful recolonization was due in part to the ability of this species to exploit environmental conditions and optimize its fitness parameters; bloodfeeding, fecundity and survival.

Among the life history processes carried out by *Ae. aegypti*, bloodfeeding and oviposition are potentially the most dangerous. Therefore "decisions" made during bloodfeeding and oviposition site selection should be made to ensure survival while optimizing fecundity. The quality of sites in which eggs are laid has significant implications for the nutrition, size, survival and the

probability of offspring emerging as viable adults (Nasci 1986, Briegel 1990a, b, Lowenberger and Rau 1994b, Blaustein et al. 2004, Telang and Wells 2004). The importance of these parameters suggests that females should, and can, discriminate between different substrates and evaluate the quality and suitability of specific sites for their offspring prior to laying eggs.

Our research addresses oviposition site selection and the consequences of site selection decisions made by gravid *Ae. aegypti* for her offspring. If larval habitat can significantly affect larval fitness, then oviposition site selection decisions made by gravid females can significantly impact their own overall fitness. Natural selection should strongly favour adult behaviours that increase larval survival such as avoiding oviposition in aquatic habitats where offspring are likely to suffer high mortality due to predators and parasites or reduced fitness due to high inter- and intraspecific competition for resources (Petranka and Fakhoury 1991). Behavioural traits that increase the longevity and reproductive success of offspring should be selected for and retained. For example, *Ae*. *aegypti* avoids ovipositing in sites containing high densities of conspecific larvae, where food is limited, or in waters that previously contained starved larvae (Bentley and Day 1989, Lowenberger and Rau 1994a, Zahiri et al. 1998, Zahiri and Rau 1998). Similarly, females avoid ovipositing in waters containing, or which previously contained, parasitized larvae (Bentley and Day 1989, Lowenberger and Rau 1994a, Zahiri et al. 1998, Zahiri and Rau 1998) and preferentially oviposit in waters containing, or which had contained, healthy

conspecifics (Lowenberger and Rau 1994a, Zahiri and Rau 1998, Tilak et al. 2005). Survival of larvae, adult size, longevity, fecundity and egg size of offspring are integral to overall reproductive success of the female.

The quality of the larval habitat can affect the physical attributes and overall performance of emerging adults. In *Ae. aegypti*, larval food availability significantly impacts teneral reserves, adult size, volume of bloodmeals that can be ingested and the allocation of reserves and bloodmeal nutrients to reproduction (Steinwascher 1984, Briegel 1990b, Briegel et al. 2002, Zhou et al. 2004). Survivorship and fecundity often are related to adult size (Steinwascher 1982, Begon et al. 1986); larger females have greater teneral reserves (Van Handel and Day 1988, Briegel 1990b) and greater flight potential (Nasci 1986) and fecundity (Briegel 1990b). Winglength, a proxy measure of overall adult size, correlates well with both potential and actual fecundity (Packer and Corbet 1989, Armbruster and Hutchinson 2002).

In addition to egg numbers, the size of eggs also may affect performance. While there is some variation in egg size between female *Ae. aegypti* eclosed from a single larval habitat, in general, under identical rearing conditions, females that arise from larger eggs become larger, grow faster, can ingest larger blood meals and lay more and larger eggs than females that originated from small eggs (Steinwascher 1984). Egg size, used commonly as a proxy for egg quality, does not account for differential provisioning of the eggs by

females. Maternal investment of protein, carbohydrates and lipids to her eggs may fluctuate based on female age (Briegel et al. 2002), larval nutrient reserves (Telang and Wells 2004) and adult nutrient intake. Eggs laid by large and small females may be provisioned with the same number of crude calories (Briegel 1990b) but the composition of those calories may differ, which could be as important as egg size to the fitness of offspring (McIntyre and Gooding 2000). Theoretically, there should be a minimum egg size and egg provisioning required to produce a viable egg and excessive provisioning of individual eggs may detract significantly from the total number of eggs laid. There must be some balance to optimize fecundity.

Parasitic infection also can reduce survival and impede or delay development of affected individuals (Zahiri et al. 1997, Platzer 2007). Endoparasites compete with their hosts for nutrients and may damage or kill their hosts. There may be similarities between the effects of parasitism or starvation on larval development based on reduced nutrient reserves available to the larvae.

Briegel (1990b) demonstrated some consequences of overcrowding in the development of *Ae. aegypti*. We report similar consequences to the overall fitness of *Ae. aegypti* ovipositing in good or poor larval habitats but also in 'good' habitats that contain larval parasites. Similar to adults emerging from poor habitats, adults raised in 'good' habitats but parasitized by the trematode *Plagiorchis elegans* (Rudolphi) were significantly smaller, and laid

fewer, smaller, and less viable eggs than those from females emerging from good habitats. There was no significant difference between the numbers of eggs laid by parasitized females and females eclosed from good habitats. Parasite load negatively affected winglength, egg size and proportion of females that oviposited. Eggs from all females contained similar amounts of total protein, lipid and carbohydrates. The selection of optimal larval habitat through oviposition site acceptance or rejection may be a pivotal contributor to the overall fitness of female mosquitoes.

Materials and Methods

Organisms

A laboratory colony of *Ae. aegypti* (LVP strain) was maintained at 27°C and 80% relative humidity with a 14:10 (L:D) light regime (Lowenberger et al. 1999). Adult mosquitoes were fed 10% sucrose solution *ad libitum*.

Larval Rearing

We reared larvae in three different laboratory-created larval habitats: Control mosquito larvae (CM) were reared at densities of ~100/L of distilled water and fed ~ 10 ml/week of a fish food and water slurry (15 ml ground Nutrafin™ (Hagen Inc., Montreal, Canada) fish food in 30 ml of distilled water) to produce large, healthy adult mosquitoes. CM larval habitat controlled for environmental, density-dependent and parasite-dependent fitness effects. Density and food stressed mosquito larvae (DFS) were reared at densities of ~ 600 larvae/L of distilled water and fed 1 ml of fish food slurry per week to produce small adult

mosquitoes. Larvae for the *Plagiorchis elegans* parasitism fitness experiment (PEL) were reared at densities of ~100/L of distilled water and fed ~ 10 ml/week of a fish food and water slurry. Mosquito larvae were exposed individually for 5 hours on day 3 of their development to five 6-8 hour-old *P. elegans* xiphidiocercariae in ~0.5 ml of distilled water in 96 well plates (Lowenberger and Rau 1994b). The parasites are maintained in their snail intermediate hosts, *Stagnicola elodes* (Say) and *Lymnaea stagnalis* (L.) in aerated 10 gallon aquaria at room temperature with a 16:8 (L:D) light regime, and fed Nutrafin ™(Hagen Inc., Montreal, Canada) fish food and fresh Romaine lettuce *ad libitum* (Lowenberger and Rau 1993, Zakikhani and Rau 1999). To obtain cecrariae, snails were placed in clear plastic cups containing 20 ml of water prior to scotophase, when cercariae emerge.

Density & Food Stress Fitness Experiment

Larvae reared in the high density/low food habitat (DFS) and control larvae (CM) were allowed to develop to the pupal stage at which time they were transferred to holding containers. The emergent adults were combined into mating pairs: CM♀ x CM♂, CM ♀ x DFS ♂, DFS ♀ x CM ♂ and DFS ♀ x DFS ♂. We used these mating pairs to investigate both paternal and maternal fitness effects on the F1 generation. We hypothesized that high density and low food availability in the larval environment would affect larval development and accumulated reserves which would have negative effects on

the size and fitness of the resulting adults. For each mating cross, 15 pairs were isolated per replicate and maintained in 12 oz. Sweetheart® food cups (Solo Cup Company, Highland Park, USA) with screened lids and access to 10% sucrose *ad libitum*. Females were bloodfed to repletion 3 days after eclosion and provided with an oviposition substrate of a moistened paper towel 72 hours after the first bloodmeal. Oviposition substrates were remoistened every 24 hours then removed from the cup 6 days after feeding and stored individually in zipper-locked plastic bags until processed. Eggs were counted and measured using an ocular micrometer in a binocular dissecting microscope. Females were bloodfed to repletion a second time and the oviposition protocol was repeated. After the second gonotrophic cycle was completed the females were sacrificed and their winglengths were measured using an ocular micrometer in a binocular dissection microscope. The number of eggs laid and egg size were recorded for each female for each gonotrophic cycle. Each experiment was replicated three times.

Plagiorchis elegans Parasitism Fitness Experiment

Control larvae (CM) and larvae exposed to *P. elegans* (PEL) were allowed to develop to the pupal stage at which time they were transferred to holding containers. The emergent adults were combined into mating pairs: CM♀ x CM♂ and PEL♀ x PEL♂ to investigate the effects of parasitism of female adults on various fitness parameters. We hypothesized that infection with *P. elegans* during the larval stage would produce adults that were less fit than their uninfected controls. For each mating pair type, 23 pairs were isolated per

replicate. Inter-treatment mating pairs were not used in PEL experiments due to data indicating that male size had no significant effects on egg number or egg size. Adult maintenance, bloodfeeding, oviposition and parameter measurement protocols were carried out as described above. Parasite load was determined by microscopic examination of adult females after the second gonotrophic cycle. Each PEL experiment was replicated three times.

Egg hatching

Fifteen samples of 10 eggs each were taken from 15 different females from each treatment (CM, DFS and PEL) and placed in covered 60 x100 plastic Petri plates containing 10 ml of autoclaved double-distilled water with one drop of Nutrafin™ (Hagen Inc., Montreal, Canada) fish food slurry. The Petri plates were kept at 27° C, 80% humidity and 14:10 L:D regime for 48 hours to allow eggs to hatch and larvae to develop. The number of first instar larvae that emerged was counted and the proportion of eggs that hatched was calculated. This experiment was replicated for each gonotrophic cycle.

Quantification of reserves in pupae and eggs

Pupal protein reserves

Protein was extracted from 20 pupae reared using CM larval conditions, 10 using DFS conditions and 20 using PEL conditions. Individual pupae were homogenized in 200 μl of Triton-X-NaOH in a 1.5 ml microcentrifuge tube and

centrifuged at 9600 rpm at room temperature for 1 minute. Then 10 μl of the supernatant was added to 500 μl of Bio-rad Quick Start Bradford Reagent (Bio-Rad Laboratories, Hercules, California, USA) (Bradford 1976) in a clean microcentrifuge tube and incubated at room temperature for 5 minutes. Samples were mixed by pipette to ensure homogeneity of colour and the absorbance was measured at 595 nm using an Eppendorf Biophotometer (Eppendorf, Hamburg, Germany).

Egg protein

Ten samples of 10 eggs from different females of each regime (CM, DFS and PEL) were homogenized in 100 μl of Triton-X-NaOH in a 1.5 ml microcentrifuge tube and centrifuged at 9600 rpm at room temperature for 1 minute. Twenty μl of the supernatant were added to 500 μl Bradford reagent (Bio-Rad Laboratories, Hercules, California, USA) and the absorbance measured at 595 nm as described above. The protein content for all samples was estimated by extrapolation to a standard curve made using Bio-Rad bovine gamma-globulin protein standards of 12.5 μg, 25 μg and 50 μg total protein content (Bio-Rad Laboratories, Hercules, California, USA).

Carbohydrates

Carbohydrates were extracted from 20 pupae from both the CM and PEL larval treatments, and 10 from DFS. Individual pupae or 10 samples of 15 eggs from each of the CM, PEL and DFS larval treatments were homogenized in 5 ml of anthrone reagent (Van Handel 1985a) in a clean glass culture tube using a

glass rod. Samples were placed in a boiling water bath for 17 minutes and allowed to cool to room temperature. Samples were mixed by inversion to ensure homogeneity of colour and the absorbance was measured at 625 nm in a Beckman DU® 640 spectrophotometer (Beckman Coulter, Inc., Fullerton, California, USA). Carbohydrate content for all samples was estimated by extrapolation to a standard curve using glucose standards of 10 μg, 25 μg and 50 μg total carbohydrate content (Van Handel 1985a).

Lipids

Lipids were extracted from 10 pupae and ten egg samples from each larval treatment regime. Individual pupae or 15 eggs from each of the CM, PEL and DFS larval treatments were homogenized in 500 μl of 1:1 chloroform:methanol in a clean glass culture tube. Samples were evaporated to dryness in a boiling water bath. Then 200 μl of sulfuric acid (H_2SO_4) was added to each sample before heating again in a boiling water bath for 10 minutes. Samples were allowed to cool to room temperature and 5 ml of vanillin reagent (Van Handel 1985b) was added and colour allowed to develop for 5 minutes. Samples were mixed by inversion to ensure homogeneity of colour and the absorbance was measured at 525 nm with a Beckman DU® 640 spectrophotometer. If absorbance at 525 nm was above 1.0, samples were read at 490 nm or diluted 5x with fresh vanillin reagent and reread at 525 nm. Lipid content for all samples was estimated by extrapolation to a standard curve using lipid standards of 10 μg, 25 μg and 50 μg total lipid content (Van Handel 1985b).

We expected that DFS and PEL pupae and eggs would contain less lipid than those of CM.

Statistical analyses

All analyses were done using JMP statistical software (SAS Institute, Inc., Cary, NC). Winglength, egg number and egg size data were compared by least squared means analysis for the DFS fitness experiment. Winglength and data for the PEL fitness experiment were compared by least squared means analysis, proportion of females laying eggs data were compared by Cochran-Mantel-Haenszel Chi-Square tests and egg number for ovipositing female data were analysed by least square means analysis. Proportion of eggs hatched for CM, DFS and PEL treatments were compared by one-way ANOVA and Tukey-Kramer pairwise analyses. Gonotrophic cycle analyses were done using least squared means analysis. Total protein, carbohydrate and lipid quantities from CM, DFS and PEL pupae and egg samples were analyzed by one-way ANOVA and Tukey-Kramer pairwise analyses. Treatments were considered significantly different $p \leq .05$.

Results

Density and Food Stress Fitness Experiment

The winglengths of CM females were significantly greater than those of DFS females ($p < .0001$). When analyzed by mating pair over two gonotrophic cycles, egg size and egg number of mating pairs containing CM females were significantly greater than egg size ($p < .0001$) and egg number (p

<.0001) of mating pairs containing DFS females (Table 1). There was no significant difference in the winglengths (p=0.0775), egg size (p=0.2355) or egg number (p=0.5062) between mating pairs of CM females with either CM or DFS males. Similarly, there was no significant difference for the winglengths (p=0.1439), egg size (p=0.9271) or egg number (p=0.9962) between mating pairs of DFS females with either CM or DFS males. Female size correlated well with egg size (p <.0001) and egg number (p <.0001) with larger females laying larger and more eggs. Male size did not correlate well with either egg size (p=0.7870) or egg number (p=0.5597) indicating that male size has no effect. We expected that male size, and thus treatment, would not have an effect on egg size or egg number as egg development depends almost entirely upon female resources. All CM and DFS females laid eggs.

P. elegans Parasitism Fitness Experiment

The winglengths of CM females were significantly greater (p=0.0005) than those of PEL females. Winglength positively correlated with egg size (p=0.2779); eggs laid by CM females were significantly larger than those of PEL females (p=0.0008) (Table 1). Parasite load was negatively correlated with winglength (p=0.0011) and egg size (p=0.0012) with more heavily parasitized females having shorter winglengths and smaller eggs. The proportion of CM females that laid eggs (76.5%) was significantly greater than the proportion of PEL females that laid eggs (58.6%) (prob>Chisq 0.0068), but the number of eggs laid by those females that did oviposit were not significantly different (prob>Chisq 0.4541). Parasite load had a significant effect on proportions of PEL females laying eggs

(prob>Chisq 0.0127): 76.5% of non-parasitized females laid eggs (parasite load class 0), 50.0% of females with one *P. elegans* metacercariae laid eggs (parasite load class 1) and 64.8% of females with two or more *P. elegans* metacercariae laid eggs (parasite load class 2) (Table 2). There was no significant difference in number of eggs laid between parasite load classes: 0, 1 or 2 (p=0.7164).

Egg hatching experiment

There were no significant differences in the proportion of eggs laid by CM and PEL females that hatched, however, these proportions were significantly greater than those laid by DFS females (Tukey-Kramer q=2.38498; ANOVA p=<.0001) (Table 3).

Gonotrophic cycle analysis

We found no significant differences in any measurement between the numbers or size of eggs produced by the same females over subsequent gonotrophic cycles. [CM: egg number (p=0.2298), egg size (p=0.9530), DFS: egg number (p=0.1865), egg size (p=0.3740), PEL: egg number (p=0.5337), egg size (p=0.2682)].

Quantification of reserves in pupae and eggs

Protein

There were no significant differences in the amounts of total protein between CM and PEL pupae but both groups contained significantly greater amounts of total protein than DFS pupae (q=2.41849; p <.0001) (Figure 3 a)).

Total protein amounts for CM, PEL and DFS eggs were not significantly different (p=0.2420) (Figure 3 d)).

Carbohydrates

There were no significant differences in the total amounts of carbohydrates between CM and PEL pupae but both groups contained significantly greater amounts of total carbohydrates than DFS pupae (q=2.42012; p <.0001) (Figure 3 b)). Total carbohydrates for CM, PEL and DFS eggs were not significantly different (p=0.5320) (Figure 3 e).

Lipids

There were no significant differences in total lipid content of CM and PEL pupae but both groups contained significantly greater amounts of total lipids of DFS pupae (q=2.47942; p <.0001) (Figure 3 c)). Total lipids for CM, PEL and DFS eggs were not significantly different (p=0.2970) (Figure 3 f)).

Discussion

Behavioural patterns contributing to survival and reproductive success are part of an inherited repertoire of predispositions acquired through natural selection (Hart 1990). Fitness is influenced by an individual's genotype as well as phenotype; genetics as well as foraging behaviour, sexual selection, predator avoidance and oviposition behaviour all influence an organism's ability to survive and reproduce. Behavioural avoidance of parasites, as an aspect of parasite resistance (Hart 1994, Bryan-Walker et al. 2007), may conserve energy for other

functions that otherwise would be expended in launching an anti-parasitic immune response. Individuals increase their survival and reproductive potential by using these strategies to protect themselves and their offspring from parasites (Hart 1990).

Winglength, egg number and egg size measurements for CM versus DFS mosquitoes suggest that larval environment can have a significant impact on adult fitness. Adult mosquitoes that emerged from crowded and starvation larval conditions (DFS) were smaller and laid smaller and fewer eggs that were less viable than the larger control (CM) mosquitoes. Body size and bloodmeal volume both can affect egg number and egg size. Smaller mosquitoes ingest less blood with each bloodmeal, have access to a smaller quantity of nutrients and have fewer ovarioles per ovary than larger mosquitoes (Steinwascher 1984) restricting their egg production compared with larger females. The rejection of larval habitats with high larval densities and high competition as suitable oviposition sites by gravid *Ae. aegypti* may reflect an ability to evaluate site quality, which ultimately affects fitness. Females use environmental cues to select optimal larval habitats in which their progeny will develop and can discriminate between good and poor habitats. Although they are dramatic, the detrimental fitness effects caused by poor larval habitat are not genetically imprinted. A small egg laid by a small female in good larval habitat will produce a large, healthy adult, emphasizing the importance of larval habitat selection and the perception of environmental cues.

There was much higher larval and adult mortality in DFS individuals compared with CM individuals. The females that survived the DFS larval treatment and were included in this study were likely more robust than the individuals that died during larval development, shortly after eclosion or before their first oviposition cycle was complete. We expect the fitness effects found here underestimate the effects that would be found in the field because of the strong selection pressure that the larval environment and our experimental design imposed on DFS individuals. Actual fitness consequences of ovipositing in these poor larval habitats would only increase with the consideration of increased immature and imaginal mortality.

Winglength, egg size and proportions of females laying eggs for CM females were significantly greater than those of PEL females but there was no significant difference in numbers of eggs laid by ovipositing females or proportion of eggs hatched between these two treatments. The correlation between increased parasite load and decreased winglength and egg size suggests there is an effect of each parasite on the mosquito host. Each parasite that enters the host haemocoel activates an immune response which can be energetically demanding (Tien et al. 2001). Increased parasite loads require a larger and more costly immune response, possibly taking energy and specific resources from other physiological processes such as growth, development and oogenesis (Ahmed et al. 2002). Parasitized *Ae. aegypti* experience longer development

times, developmental abnormalities and high levels of larval and pupal mortality (Dempster et al. 1986, Schwab et al. 2003) indicating that the reserves required for normal development and survival may be sequestered. Our experimental design did not distinguish between parasite and host provisions.

The difference in proportions of females laying eggs when comparing CM and PEL treatments and for each parasite load class (unparasitized, one parasite, two or more parasites) may represent a behavioural fitness cost rather than a physiological one. The majority of females who did not lay eggs had retained eggs in their abdomen (data not shown). Whether the females were not able to oviposit or chose not to cannot be determined by our experimental design. Potential fecundity or the ability to produce eggs may not be affected by parasite burden, but parasite infection did result in reduced realized fecundity for those females who did not oviposit over both observed gonotrophic cycles.

The significant differences between the total pupal protein, carbohydrate and lipid content between the CM, PEL and DFS treatments may be a result of the size difference between the pupae. Based on the adult winglength measurements as a proxy for individual size, the CM and PEL pupae are significantly larger than DFS pupae. When considering the effect of size on fitness, teneral reserves influence both flight potential and fecundity. At eclosion, small females have a much lower flight potential than large females due to a lack of nutrient reserves (Briegel et al. 2001), potentially making them more

vulnerable to predators and less able to seek bloodmeals. Smaller females also have fewer teneral and blood-meal derived nutrients to allocate to oogenesis resulting in fewer eggs per cycle (Briegel et al. 2002). Furthermore, teneral lipid reserves help to regulate the utilization of blood meal proteins for oogenesis in the first gonotrophic cycle of *Ae. aegypti* (Zhou et al. 2004); larger females being able to utilize bloodmeal proteins more effectively for reproduction than smaller females.

Resource allocation costs are significant if egg production is energetically expensive, if resources are limited and if reproductive efforts deprive other physiological processes of nutrients (Williams 2005). The data for total egg protein, carbohydrate and lipid content indicate that there is no significant difference in the resources apportioned to individual eggs by females raised under the CM, DFS and PEL regimes. Similarly, Briegel (1990b) found that the caloric total per egg for large and small *Ae. aegypti* remained constant regardless of larval regime; these regimes included only starvation and control treatments and did not examine parasitized mosquitoes. There may be a minimum provisional requirement to produce a viable egg and smaller females with limited resources may produce fewer eggs in order to meet these provisional requirements. If these resources can be allocated to smaller eggs such as those produced by DFS females it is unclear why all mosquitoes would not produce more eggs of this size. However, due to the reduced viability of these smaller

eggs there may be other reasons why the larger eggs are produced or specific provisioning or space requirements that were not obvious in this study.

Survivorship of individuals through larval stages is also linked to size. With the DFS larval treatment, crowded and starved individuals exhibit prolonged development times, smaller larval, pupal and adult size and increased larval mortality. Extended development times also can contribute to increased larval mortality by increasing potential contact between the larvae and their parasites and predators (Nguyen et al. 2002). These are direct and detrimental fitness effects as a result of mortality in the poor-quality larval environment; ones that preclude reproductive potential.

Larvae parasitized by *P. elegans* also experience fitness costs regarding larval survival. Survival at all pre-imago stages decreased with exposure to *P. elegans* with the fourth instar and pupal stages being affected the most (Rau et al. 1985, Nguyen et al. 2002). Developmental periods also increased with this treatment while eclosion decreased (Nguyen et al. 2002). Although the size differences are not as pronounced between PEL and CM individuals compared with DFS and CM individuals, pre-imagines the parasitized larval environment also incur injurious fitness consequences before eclosion.

The similarity between CM and PEL pupae in protein, carbohydrate and lipid content may be misleading when considering resources available for oogenesis. Parasitized individuals compete with endoparasites for nutrients, which may deplete specific nutrients required for growth, development, metabolism, oogenesis and other functions. The extraction protocols used in this study did not allow us to discriminate between specific nutrients available to the mosquito and the type and proportions of nutrients incorporated within the *P. elegans* metacercariae during their development. Metacercariae in the haemocoel of the insect are metabolically active and do take up carbohydrate and protein reserves from their hosts (Lowenberger and Rau 1993, Lowenberger et al. 1994) which may not be reflected in our estimate of pupal reserves available for host development.

Larval nutrition determines teneral reserves that ultimately affect fecundity in *Ae. aegypti* (Briegel 1990b, a, Telang and Wells 2004) as egg production depends on both teneral reserves and those accumulated through adult feeding. Anautogenous mosquitoes must ingest a blood meal during each gonotrophic cycle to obtain enough protein to produce a batch of eggs (Klowden 1990) and to synthesize lipids for oogenesis (Ziegler and Ibrahim 2001, Briegel et al. 2002). Mosquitoes with low teneral reserves may allocate nutrients obtained through a first bloodmeal and sugar feeding to maternal protein and lipid reserves rather than to oogenesis (Briegel 1990a). This results in lower egg production for the first gonotrophic cycle and higher egg production for subsequent gonotrophic

cycles (Briegel 1990a). We found no significant difference in egg numbers over two gonotrophic cycles for DFS and PEL females implying that either maternal teneral reserves were not sufficiently depleted during larval development or that further gonotrophic cycles may be required to see this trend.

We initially predicted that DFS females would produce fewer eggs than CM or PEL females, but that eggs from all three regimes would be the same size and have similar provisioning. The decrease in hatching of DFS compared with CM eggs may be related solely to the size of the egg or may be related to differing composition of protein, lipids or carbohydrates allocated to each egg. While total amounts of protein, lipid or carbohydrate content did not differ significantly we did not further break down these categories.

Detrimental fitness effects resulting from poor larval habitat choice may be driving the evolution of behavioural adaptations by gravid female *Ae. aegypti* to avoid ovipositing in undesirable habitats. This choice may be made visually or through chemosensory mechanisms that detect concentrations of waste products and oxygen in areas of high larval densities and potential interspecific or intraspecific competition. How gravid females recognize sites containing low numbers of parasitized larvae is unclear but there is evidence that compounds produced by parasitized larvae are recognized by gravid females and serve to deter oviposition in these sites. The chemical identification of these molecules and investigation of their ecological effects are currently underway.

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Table 1 - A comparison of fitness parameters for control mosquito (CM) vs. density food stress (DFS) and control mosquito (CM) vs. *Plagiorchis elegans* parasitized (PEL) fitness experiments. Winglength, number of eggs laid and egg size are expressed as the mean of three replicates ± standard deviation. Mating pairs with matching symbols (*†) are not significantly different.

Experiment	Mating Groups	n	Mean Winglength (cm)	Mean Number of Eggs Laid	Mean Egg Size (cm)
CM vs. DFS	*CM♀/CM♂	32	0.216 ± 0.01	82 ± 27	0.0453 ± 0.001
	*CM♀/DFS♂	29	0.222 ± 0.01	73 ± 27	0.0456 ± 0.001
	†DFS♀/CM♂	30	0.129 ± 0.02	30 ± 16	0.0311 ± 0.003
	†DFS♀/DFS♂	25	0.136 ± 0.02	30 ± 16	0.0312 ± 0.002
CM vs. PEL	CM♀/CM♂	49	0.324 ± 0.01	58 ± 43	0.0579 ± 0.004
	PEL♀/PEL♂	52	0.320 ± 0.01	41 ± 46	0.0556 ± 0.003

Table 2 - The effects of parasite load on the proportion of ovipositing females for Control Mosquito (CM) vs. *Plagiorchis elegans* parasitized (PEL) fitness experiments. Females who did not lay any eggs are excluded from this table. Parasite load categories are as follows: 0 = unparasitized, 1 = one visible metacercariae in full body smear slide preparation of females following their second gonotrophic cycle, 2 = two or more visible metacercariae in full body smear slide preparation of females following their second gonotrophic cycle.

Parasite load	n	Proportion of females who laid eggs
0	102	0.765
1	32	0.500
2	54	0.648

Table 3 - Proportion of eggs hatched from control mosquito (CM), *P. elegans* parasitized (PEL) and density/food stress (DFS) treated females in egg viability experiment. The proportion of eggs hatched represents the mean of two replicates ± standard deviation. Mating pairs with matching symbols (†) are not significantly different.

Female Treatment	n	Mean Proportion of Eggs Hatched
†CM	30	0.52 ± 0.31
†PEL	30	0.39 ± 0.29
DFS	30	0.16 ± 0.21

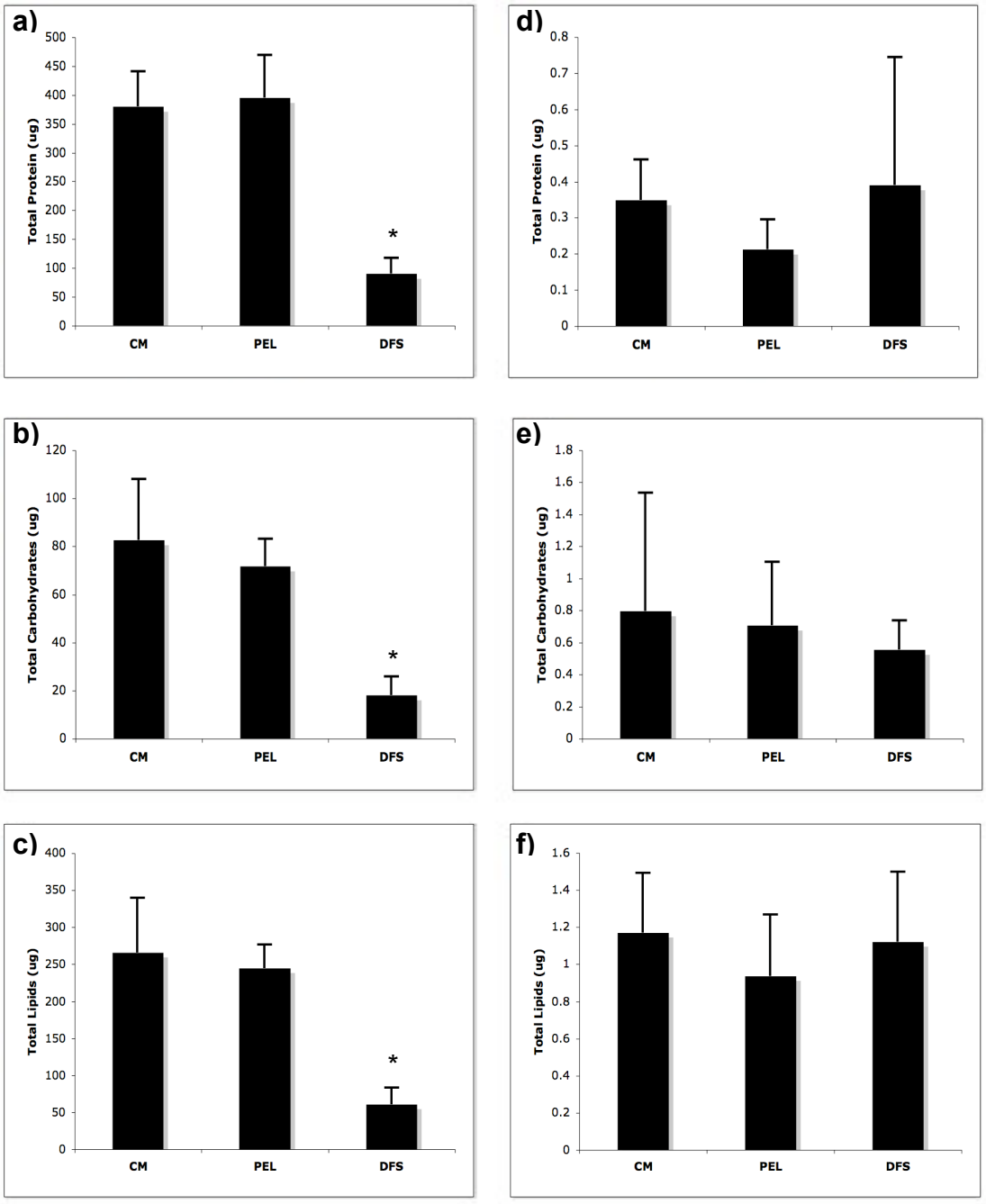

Figure 3 - Protein, carbohydrate and lipid content of pupae (left panel) and eggs (right panel) for each larval regime: Control Mosquito (CM), Density Food Stress (DFS) and Parasitized (PEL). Pupae were taken from each larval environment and total proteins (a), carbohydrates (b) and lipids (c) were extracted from separate whole body samples. Eggs laid by females that had eclosed from each larval environment were analyzed for total protein (d), carbohydrate (e) and lipid (f). The bars represent mean total nutrient content plus bars indicating the standard deviation.

CONNECTING STATEMENT

In Chapter 1 we discussed the concept that female mosquitoes should discriminate between potential oviposition sites to select those sites that would best support the development and growth of her offspring. In Chapter 2 we demonstrated the negative consequences of ovipositing where conditions were not optimal (high density, competition, food stress, and parasites) for larval development and the emergence of large, well nourished adults. In the next chapter we investigate whether females do discriminate and are deterred from ovipositing in specific sites and we take the first steps in evaluating the composition of larval holding waters that are attractant or deterrent to gravid females. Through a series of bioassays and chemical extractions we hope to identify the major compounds that females use to determine that a site is not "suitable" for her offspring.

CHAPTER 3: OVIPOSITION BEHAVIOUR OF *AE. AEGYPTI* (DIPTERA: CULICIDAE) IN RESPONSE TO CONSPECIFIC LARVAL STRESS, INJURY AND PARASITISM: AN INVESTIGATION OF PUTATIVE CHEMICAL CUES

Introduction

Oviposition site selection in insects is a major decision made by females that may affect their overall fitness by determining the survival and performance of their progeny (Bentley and Day 1989, Nguyen et al. 2002). Gravid females respond to environmental cues when evaluating the quality and suitability of an oviposition site and subsequent larval habitat for their offspring. Females that respond to positive environmental cues and select good larval habitats should have increased fitness (Petranka and Fakhoury 1991). These decisions are based on both chemical and physical environmental factors (Bentley and Day 1989). Physical attributes of a site are used in long-range discrimination, whereas chemical cues generally are used over shorter ranges in the evaluation process. The acceptability of an oviposition site may be determined by chemical cues of biotic or abiotic origin (Reeves 2004) and may be affected by the availability of alternate sites, the physiological state of the female and the concentrations of semiochemicals within each site. Semiochemical cues of environmental, conspecific and congeneric origins have been identified as oviposition attractants or repellents to gravid females (Starratt and Osgood 1972,

Klocke et al. 1987, McCall and Cameron 1995, McCall and Eaton 2001, Geetha et al. 2003). As defined for this thesis, gravid females actively move toward attractant cues, but actively move away from repellent cues. In the presence of oviposition deterrent cues, females may orient toward, land upon, and inspect the substrate, but will not oviposit there (Christophers 1960, Bentley and Day 1989, Clements 1999, Hoffmeister and Roitberg 2002). The presence of healthy conspecific larvae in low densities or specific bacteria can act as oviposition attractants for gravid females and may indicate good food availability and low competition (Kalpage and Brust 1973, Kramer and Mulla 1979, Lowenberger and Rau 1994, Allan and Kline 1995, Dhileepan 1997, Edgerly et al. 1998). *Aedes triseriatus*, a treehole mosquito, preferentially oviposits in waters that contain p-cresol or 4-methylcyclohexanol, semiochemicals first identified in a decaying wood infusion (Bentley et al. 1982). *Culex* sp. oviposits in highly eutrophic environments and *Cx. quinquefasciatus* preferentially oviposits in ovitraps infused with blends of phenol, 4-methylphenol, 4-ethylphenol, indole and 3-methylindole; all molecules associated with fermented bermuda grass infusions (Millar et al. 1992). Recently, heneicosane has been identified as a larva-produced pheromone that attracts gravid *Aedes aegypti* (Mendki et al. 2000).

Oviposition repellents and deterrents are associated with unfavourable conditions for larvae within a habitat, such as the presence of predators, parasites, overcrowding, starvation or toxins. *Culex* sp. are deterred from ovipositing in waters containing chemicals associated with the presence of the

predatory mosquitofish, *Gambusia affinis* (Angelon and Petranka 2002), and both *Culex* sp. and *Culiseta* sp. avoid ovipositing in pools containing predatory *Notonecta* sp. (Eitam and Blaustein 2004). Gravid *Ae. atropalpus* and *Ae. aegypti* avoid ovipositing in waters that contain, or had previously contained, conspecific larvae parasitized by the trematode *Plagiorchis elegans* (Lowenberger and Rau 1994, Zahiri et al. 1997a, Tilak et al. 2005). *Aedes aegypti* also is deterred from ovipositing in waters that previously had contained starved and overcrowded conspecific larvae (Bentley and Day 1989, Chadee et al. 1990, Lowenberger and Rau 1994, Zahiri et al. 1997b, Zahiri and Rau 1998), large numbers of conspecific eggs, or high concentrations of chemicals isolated from conspecific eggs (Chadee et al. 1990, Ganesan et al. 2006). A variety of toxins, phytochemicals and larvicides act as repellents and deterrents (Bentley and Day 1989) including eucalyptol for *Ae. aegypti* (Klocke et al. 1987) and *Bacillus thuringiensis israelensis* toxin larvicide for *Cx. quinquefasciatus* (Zahiri and Mulla 2005). The identity of the specific compounds responsible for these attractant, deterrent, and repellent activities are largely unknown.

Repellent and deterrent oviposition behaviour have been associated with parasitized mosquito larvae (Lowenberger and Rau 1994). *Plagiorchis elegans* is a trematode parasite that uses aquatic invertebrates, including mosquito larvae and pupae, as second intermediate hosts. Xiphidiocercariae emerge from the snail first intermediate host, swim freely through aquatic environments until they come in contact with an invertebrate, penetrate the cuticle and enter the host.

The cercariae encyst as metacercariae, absorb nutrients from the host during their development (Lowenberger and Rau 1993, Lowenberger et al. 1994), and await predation on their invertebrate host by a suitable vertebrate definitive host.

Aedes aegypti females preferentially oviposit in waters containing healthy conspecific larvae and avoid sites containing starved or overcrowded larvae, or larvae parasitized by the trematode *P. elegans* (Lowenberger and Rau 1993, Lowenberger et al. 1994). It was assumed that a repellent compound was produced by *P. elegans*-parasitized larvae (Lowenberger and Rau 1994), but the identity of the molecule(s) is not known. Reeves (2004) speculated that the tissue damage caused by *P. elegans* xiphidiocercariae penetrating and migrating through host tissues was the source of these semiochemical cues, but other stressors associated with parasitism should be considered: immune responses to invading pathogens, reduced feeding activity, internal organ damage, other endoparasites, or a specific reaction to parasitism by *P. elegans*.

Aedes aegypti larvae with a heavy burden of *P. elegans* metacercariae display altered behaviour, such as decreased movement and feeding (Webber and Rau 1986), and have increased mortality and morbidity compared with larvae harbouring few or no parasites (Dempster et al. 1986, Zahiri et al. 1997c). The oviposition avoidance by gravid *Ae. aegypti* of larval waters increases as the mean intensity of parasites increases (Zahiri et al. 1997c). Similarly, high larval density and low food availability increase larval mortality and oviposition

deterrent/repellent behaviours by gravid females (Zahiri et al. 1997b). Thus, infected, dead and dying larvae may produce compounds that when encountered by females often leads to them refraining from oviposition. In cases where significant numbers of larvae are killed, it is possible that necromones, including hexadecanoic or palmictic (16:0) and octadecanoic or stearic (18:0) acids, (Rollo et al. 1994, Rollo et al. 1995, Peterson and Coats 2001), mediate oviposition decisions.

Normal larval metabolism may be altered due to starvation, parasitism or stress (Candy et al. 1997). The main haemolymph carbohydrate in mosquitoes is trehalose, a non-reducing disaccharide (Wyatt and Kalf 1957, Wallage et al. 2001) that is catabolized from glycogen reserves in the fat body. Trehalose is cleaved into two glucose molecules by the enzyme trehalase during normal carbohydrate metabolism (Elbein et al. 2003). Alternatively, trehalose may be formed from dietary monosaccharides or by gluconeogenesis (Candy et al. 1997). *Aedes aegypti* larvae infected with *P. elegans* metacercariae have lower levels of serum glucose and higher levels of serum trehalose than uninfected larvae (Zahiri et al. 1998), suggesting that the presence of the parasite disrupts trehalose conversion, possibly by inhibiting the enzyme trehalase (Zahiri et al. 1998, Wallage et al. 2001). Under these conditions, parasitized insects may use lipid-based metabolism to fulfil their energy requirements; high-density lipoproteins can be converted to fatty acids that can be used to generate Acetyl Coenzyme A for use in the Krebs cycle. This process also generates ketone

bodies which can be oxidized to meet energy needs (Candy et al. 1997, Wallage et al. 2001), but excess fatty acids or ketone bodies may be excreted into their aquatic environment. Unsaturated and saturated hexadecanoic and octadecanoic acids (16:0,16:1,18:0, 18:1, 18:2 and 18:3) have been identified as major fatty acids in *Ae. aegypti* haemolymph (Wallage et al. 2001) and these fatty acids also have been identified as oviposition repellents for several mosquito species (Hwang et al. 1980, Hwang et al. 1982, Bentley and Day 1989). Oleic acid [(Z)-9-octadecanoic acid (18:0)] is an oviposition repellent for *Cx. quinquefasciatus* (Hwang et al. 1984) and *Ae. aegypti* (Ganesan et al. 2006). Hexadecanoic or palmictic acid (16:0) is repellent in higher concentrations for *Ae. aegypti* (Ganesan et al. 2006). Methyl esters of fatty acids with 10, 12, 16 and 18 carbon chains also deterred *Ae. aegypti* from ovipositing (Ganesan et al. 2006). Therefore, stresses caused by parasites or starvation may disrupt larval physiology, increasing the excretion of free fatty acids or ketone bodies which may be perceived by gravid females and deter oviposition.

We investigated the oviposition behaviour of gravid *Ae. aegypti* in response to nursery waters (1) containing larvae which had been stressed by cuticular damage; (2) general immune activation via inoculation of bacteria into the haemocoel; (3) exposure to *Bacillus thuringiensis israelensis* toxin larvicide; (4) exposure to the nematode larval parasite, *Romanomermis culicivorax* (Ross & Smith); (5) parasitism with *P. elegans*; and (6) starvation and crowding. I used chemical extractions of oviposition deterrent solutions to isolate compounds that

may contribute to the oviposition deterrence of *P. elegans*-parasitized larval waters. Although *P.elegans*-parasitized larvae had higher larval mortality than control larvae, no significant difference was found in concentrations of known necromones in control or *P. elegans*-parasitized larval waters.

Materials and Methods

Organisms

A laboratory colony of *Ae. aegypti* (LVP strain) was maintained at 27°C and 80% relative humidity with a 14:10 (L:D) photoperiod. Adult mosquitoes were fed 10% sucrose solution *ad libitum* and bloodfed once weekly. Larvae were raised at a density of ~ 100 larvae/L of distilled water and were fed a slurry of ground Nutrafin™ (Hagen Inc., Montreal, Canada) fish food and water ad libitum.

We maintained the pulmonate snails *Stagnicola elodes* (Say) and *Lymnaea stagnalis* (L.) infected with *P. elegans* in aquaria containing decholrinated water conditioned with chalk ($CaCO_3$) under a 16:8 light: dark cycle. Snails were fed Nutrafin™ (Hagen Inc., Montreal, Canada) fish food and lettuce *ad libitum*. To obtain the *P. elegans* xiphidiocercariae that are shed at dusk, infected snails were isolated prior to the scotophase in plastic cups containing 25 ml water. Six to 8h-old cercariae were used to infect mosquito larvae.

Laboratory cultures of *Escherichia coli* ATCC11303 and *Micrococcus luteus* ATTC4698 were maintained on Luria-Bertani (LB) plates and stored at 4 °C. The *E. coli* colonies were cultured in LB broth at 37 °C and *M. luteus* at 25 °C and re-plated weekly.

Romanomermis culicivorax eggs were obtained from a laboratory colony at University of California Riverside (Dr. E. Platzer) and stored at room temperature.

Larval Waters

Control larval waters (CLW) were prepared by incubating 100 3-day-old *Ae. aegypti* larvae in 1L double distilled water (ddH_2O) under normal laboratory conditions for 72 h at 27 °C with 0.15 g of ground Nutrafin™ (Hagen Inc., Montreal, Canada) fish food. Larvae were rinsed with ddH_2O before incubation. The waters were 0.2 μm filter sterilized to remove larvae and any microflora, then stored at 4 °C until use. This has been used to provide an attractive larval environment to gravid *Ae. aegypti* (Lowenberger and Rau 1994, Zahiri et al. 1997a, Zahiri and Rau 1998).

We prepared similar larval holding waters using 100 larvae that had undergone experimental treatments prior to incubation in 1L of ddH_2O. These waters then were used to compare the oviposition response between different treatment groups and CLW. These included: (1) cuticular thoracic puncture

(CTP), (2) inoculated thoracic puncture (ITP), (3) exposure of larvae to 5% *Bacillus thuringiensis israelensis* larvicide (BTI), (4) *R. culicivorax* infection (RCX), (5) *P. elegans* infection (PEL) and (6) high larval density with low food availability stressed (DFS).

Cuticular thoracic puncture (CTP) was used as a stress treatment to mimic the cuticular damage inflicted by the xiphidiocercariae as they penetrate the host. Larvae were punctured once in the thoracic region with a sterile 0.15 minuten insect pin before incubation in larval waters.

Inoculated thoracic puncture (ITP) was used to stimulate a general immune response in the larvae. Larvae were treated as in CTP except the minuten pin was dipped in a mixed pellet of *Escherichia coli* ATTC 11303 and *Micrococcus luteus* ATTC4698 bacterial cultures before puncturing the thoracic cuticle. The bacterial pellet was made as described (Lowenberger et al. 1995).

Bacillus thuringiensis (BTI) was used to mimic severe midgut pathology. One grain of the larvicide (AquaBac®; AFA Environnement Inc., Montréal, Canada) was dissolved in 300 ml of ddH_2O; this stock BTI solution was diluted to 5% of its original concentration. Larvae were incubated in the 5% solution for 3 hours, washed twice with ddH_2O, and then incubated as described above to generate the holding waters.

Romanomermis culicivorax infection (RCX) was used as an alternate endoparasite to *P. elegans*. Gravel containing *R. culicivorax* eggs was flooded with ddH_2O to induce the emergence of pre-parasitic juvenile worms. As they emerged from the gravel they were transferred to a Petri dish in 10 ml of ddH_2O. Two-day-old *Ae. aegypti* larvae were exposed to nematodes in a 4:1 worm: mosquito larva ratio for 3 h. Larvae were washed twice with ddH_2O before being incubated. After holding waters were produced and filtered, RCX larvae were examined microscopically to confirm parasite infection.

Density and food stress (DFS) conditions were created by rearing larvae at densities of ~ 600 larvae/L of distilled water and by limiting food availability (1 ml of fish food slurry per week). At late second or early third instar (similar to the larval stage at 3 days in normal CLW larval development), larvae were incubated as CLW with no food.

P. elegans cercariae (PEL) treatment waters were made by exposing larvae were to *P. elegans* cercariae to recreate the repellency described in Lowenberger and Rau (1994). We exposed 100 third-instar larvae in ~100 ml ddH_2O in a ratio of approximately 8:1 cercariae to larvae, for 3 h. The larvae were rinsed twice with ddH_2O before incubation to generate the holding waters.

After filtration, PEL larvae were examined microscopically to confirm parasite infection.

Water controls were made as CLW without the addition of larvae before incubation at 27°C. Two water controls were used, (1) ddH_2O that was held in a plastic Nalgene® (Nalge Nunc International Corporation, Rochester, NY) carboy before incubation and (2) ddH_2O that was held only in glass before incubation.

General Bioassay Design

After filtration, 250 ml of the larval waters for each treatment were reserved for bioassay purposes. Treatment and control larval waters were always made simultaneously with the same cohort of mosquitoes.

Groups of 10 adult *Ae. aegypti* females and five males were isolated in 12 oz. paper cups with a screen top and fed 10% sucrose solution *ad libitum*. A bloodsource was provided for 10 minutes to allow the females to engorge. Forty-eight hours after bloodfeeding, mosquitoes were transferred to experimental cages (20-40 L aquaria sealed with nylon stocking) and provided with oviposition sites (60 x 15mm round dishes) containing 10 ml of either control or treatment larval waters. Eggs laid in each dish were counted and removed from the dishes daily. Dishes were rinsed with distilled water, refilled with fresh larval waters and replaced. The orientation of the control and treatment larval waters was

randomized daily in an attempt to control for any spatial bias. All experiments were performed under our standard rearing conditions.

Two bioassays that counted eggs from 10 groups of gravid females over five days were done for CTP and ITP vs. NLS and one for BTI vs. NLS comparisons. One bioassay that counted eggs from 5 groups of gravid females over three days was done for RCX vs. NLS comparisons. Twelve bioassays that counted eggs from three to ten groups of gravid females over three to five days were done for DFS vs. NLS comparisons and 31 were done for PEL vs. NLS comparisons (Table 4).

Chemical extraction

Filtered larval waters from each treatment were frozen at -80°C in 250 ml aliquots in plastic bottles and lyophilized to dryness. Dried larval waters were rehydrated to 10% of their original volume with ddH_2O and extracted with one of the following: ether, dichloromethane, hexane or chloroform (Table 6); or were rehydrated to 2% of their original volume and extracted with a 3:1 chloroform: methanol mix. Each 15- or 25-ml aliquot of reconstituted larval water was extracted 5 times with 4 ml of solvent. Solvent fractions for each larval water type were combined. Ether, dichloromethane, pentane and chloroform were used to extract different molecules in the treatment versus control larval waters. The chloroform/methanol mix was used to isolate fatty acids from larval waters.

Half of the ether, hexane, dichloromethane and chloroform extracts were evaporated using a Rotavap™ (BÜCHI Labortechnik AG, Flawil, Switzerland), then under cold nitrogen and immediately rehydrated with ddH_2O. Both the water and the solvent fractions of the extracted larval waters were restored to their original 250 ml volume (to restore original concentrations of molecules) with ddH_2O and bioassayed as previously described to determine the fraction that retained the oviposition deterrent activity. Half of the ether, pentane, dichloromethane and chloroform extracts and all of the chloroform: methanol extracts were used for compound isolation and identification.

Compound isolation and identification

Of the ether, hexane, dichloromethane and chloroform extractions, only the ether extracts retained any deterrent activity. Two batches of PEL waters were extracted with each hexane, dichloromethane and chloroform and four batches of PEL and one of DFS waters were extracted with ether. For each larval water batch, 40 ml of extracted ether fractions were washed with clean ether and dried of absorbed water with anhydrous magnesium sulfate ($MgSO_4$). Extracts were filtered through #1 Whatman™ (Whatman plc, Kent, UK) filter paper and washed 3 times with 5 ml clean ether. Dehydrated ether extracts were rotary-evaporated to a concentrated volume of 4 ml. Two μl of the concentrated ether extracts were injected into a Varian 3800 gas chromatograph (GC) (Varian Inc., Palo Alto, CA, USA) with the temperature of the injector and flame ionization detector at 250°C and a temperature program as follows: 50°C (held 2 min), increase 10°C per minute to 280°C (held 5 min). Gas chromatograph/mass

spectrometer (GC/MS) profiles of the CLW and treatment waters extracts were compared. Ether extracts of samples were analyzed by coupled gas chromatographic-electroantennographic detection (GC-EAD) (Arn et al. 1975, Blackwell and Johnson 2000, Derksen et al. 2007) to determine candidate semiochemicals that elicit a response from *Ae. aegypti* antennae.

Fractions with antennal stimulatory compounds were injected into a Varian 3800 GC/Varian Saturn 2000 Ion Trap (in electron impact mode) gas chromatograph/mass spectrometer (GC/MS) (Varian Inc., Palo Alto, CA, USA) running the same GC program as above. We tentatively identified candidate semiochemicals, confirmed these structural assignments by comparative analyses of authentic standards and tested the authentic standards for differential oviposition responses with oviposition bioassays. Compounds that elicited a response were extracted with ether from PEL, DFS, CLW and water controls and were quantified.

Synthetic tributyl citrate bioassay

Based on initial results, we carried out an oviposition bioassay using synthetic tributyl citrate (TBC). Tributyl citrate (99% pure)(Chem Service Inc., West Chester, PA, USA) was added to ether to make a 2.5 μg/μl stock solution. We added 1.6 μl of stock solution to 200 ml of ddH_2O to make TBC test waters of 0.02 ng/μl, and added 1.6 μl of ether to ddH_2O to make control waters for the bioassay.

Fatty acid investigation

Four batches of CLW, eight of PEL and two of DFS were extracted with a 3:1 chloroform: methanol mix to capture fatty acids in the solvent mixture. Water fractions were discarded and solvent fractions were trans-esterified (Morrison and Smith 1964) so that retention times and mass spectra of the resulting esters could be compared by GC/MS with those of authentic standards. Methyl esters were quantified using SatView® (Varian Inc., Palo Alto, CA, USA) software.

Statistical analysis

All statistical analyses were performed using JMP statistical software (SAS Statistical Institute, Cary, NC). The proportions of eggs laid in treatment larval waters versus CLW were arcsine-square root transformed and analyzed by repeated measures. Concentrations of fatty acid methyl esters were analyzed by larval water treatment using one-way ANOVA.

Results

Larval water bioassays

There was no differential oviposition in treatment waters CTP ($p=0.9081$) or RCX ($p=0.5647$)(Table 5). Gravid *Ae. aegypti* laid significantly more eggs in ITP waters over CLW ($p=0.0416$) (Table 5). Females laid more eggs in CLW than in BTI ($p=0.0002$), DFS ($p=0.0111$) and PEL ($p<.0001$) (Table 5).

Chemical isolation and identification

Solvent fractions of pentane, dichloromethane and chloroform extracts of repellent PEL waters did not retain the oviposition deterrent activity.

Gravid females laid more eggs in the rehydrated solvent fractions of chloroform extracts of PEL than in those of CLW ($p=0.0055$). There was no differential oviposition in rehydrated solvent fractions of PEL and CLW for pentane ($p=0.5105$) or dichloromethane ($p=0.3983$). The differential oviposition response was retained in the water fraction of PEL when extracted with pentane ($p=0.0499$), but not with dichloromethane ($p=0.1155$) or chloroform ($p=0.9322$) (Table 6).

The rehydrated solvent fractions of ether extractions of PEL retained oviposition deterrent activity when compared with that of CLW ($p=0.0408$). There was no differential oviposition in water fractions of ether extracted PEL and CLW ($p=0.7881$). Neither the solvent fraction ($p=0.2918$) nor the water fraction ($p=0.1662$) of ether extracts of DFS retained activity. GC-EAD and GC/MS analyses of ether extracts of PEL and CLW revealed a candidate semiochemical in PEL but not CLW extracts (Fig 4). This compound was present at 1.03 ng/μl and 0.07 ng/μl in two deterrent PEL waters, at 0.0019 ng/μl in non-deterrent PEL waters and was identified as tributyl citrate (TBC) (Table 7). TBC was present at 0.014 ng/μl in DFS waters, at 0.016 ng/ul in CM waters, at 0.798 ng/μl in carboy-held control waters and was not present in glass-held control waters (Table 7).

Synthetic tributyl citrate bioassay

Gravid females oviposited fewer eggs in the 0.02 ng/μl tributyl citrate experimental waters than in the ddH_2O/ether control waters ($p=0.0042$) (Table 5).

Fatty acid investigation

Following the transesterifications, both 16:0 and 18:0 methyl esters were identified in CLW, PEL and DFS solvent extracts. There was no significant difference in either 16:0 ($p=0.3786$; $q=2.70081$) or 18:0 ($p=0.6515$; $q=2.70081$) concentrations between CLW, PEL and DFS extracts (Table 8).

Discussion

Mosquito larvae are restricted to a finite, relatively small larval habitat. They cannot disperse to another site to acquire food or avoid parasites or predators (Christophers 1960, Clements 1999). Selection of larval habitat by a gravid female affects the survival and development of her offspring, directly affecting her overall fitness. Gravid females should assess the suitability of larval habitat in terms of food availability and potential mortality due to parasites, predation and competition before laying eggs (Bentley and Day 1989, Hilker and Meiners 2002). Gravid *Ae. aegypti* avoid ovipositing in larval habitats with high levels of morbidity, mortality, predation, parasitism, starvation and crowding (Bentley and Day 1989, Lowenberger and Rau 1994, Zahiri et al. 1997b, a, Zahiri et al. 1997c, Clements 1999, Hilker and Meiners 2002). Identifying the semiochemical cues may provide insight into mosquito chemical ecology and oviposition strategies. The identified compounds might be used in integrated pest management strategies, improving the success of mosquito monitoring and control efforts.

The cuticular damage (CTP) and *R. culicivorax* (RCX) larval stress treatments did not elicit selective oviposition from gravid *Ae. aegypti* (Table 2). Contrary to assumptions by Reeves (2004), that the violation of cuticular integrity was the cause of the repellence, cuticular injury did not induce the deterrent activity seen in *P. elegans*-infected larvae, despite the fact that the wounds we created were larger than those of penetrating xiphidiocercariae.

Because there was no differential oviposition is seen in CTP experimental trials, the *P. elegans*-induced repellent quality of larval waters must be due to some other aspect of the host-parasite interaction. There was no oviposition deterrence in response to the RCX waters (Table 2). While both *R. culicivorax* and *P. elegans* are endoparasites and develop within host tissues, the endpoint of the host-parasite relationships is different: mosquito larvae hosts are always killed by *R. culicivorax* as it completes its life cycle (Platzer 2007) whereas *P. elegans* normally does not kill its host. Both parasites absorb nutrients from their hosts but may take up different nutrients or impose different stressors on the host. This may cause the production of oviposition deterrent compounds in response to one parasite but not the other. One might predict that the compound should be produced in the larvae destined to die (RCX) rather than in those that are only mildly affected (PEL). It appears that the production of this deterrent compound is not a general response to endoparasitism but may be a specific host response to trematode or to *P. elegans* infection.

We anticipated that inducing a general immune response by inoculating bacteria into the haemocoel would weaken larvae and induce them to produce a deterrent compound. The observed attractant quality of ITP waters (Table 2) was unexpected and might be due to the presence and growth of bacteria or metabolites. Bacteria left behind on the larval cuticle after the inoculated wound was washed or their metabolites may have passed through the 0.2 μm filter, although neither of the bacteria used normally multiply in water. High concentrations of bacteria and their metabolites can act as oviposition attractants (Kramer and Mulla 1979, Millar et al. 1992). Any repellent cues in ITP waters resulting from the larvae's general immune response could have been masked by these bacterial metabolites.

Gravid *Ae. aegypti* did not lay as many eggs in BTI waters as in CLW (Table 2). BTI treated larvae were rinsed repeatedly before incubation to remove any potential direct effect of the BTI on oviposition, Most larvae appeared moribund by the end of the 72-hour incubation period and exhibited reduced movement and feeding. A reduction in feeding could cause starvation and the production of an oviposition deterrent, as seen in other starvation studies (Zahiri et al. 1997b). BTI also destroys midgut epithelia and compromises the integrity of midgut membranes (Gill et al. 1992, Zahiri and Mulla 2005). Severe tissue damage may invoke a repair response in the larvae, diverting resources from other functions which may lead to using alternate metabolic pathways, and the possible production of deterrent ketone bodies.

As expected, PEL and DFS larval waters received fewer eggs than did CLW waters (Table 2). We suspect that compounds produced by PEL and DFS larvae, as a result of their parasite loads and poor larval habitat, are the cause of this differential oviposition. We have established that gravid females are not deterred/repelled from ovipositing in waters of larvae that have been wounded, parasitized by a different endoparasite or immune-activated by a pathogen, supporting the hypothesis that not all types of larval stress induce the production of oviposition repellent or deterrent compounds. Metabolic stress may be the common factor in the cases of BTI, DFS and PEL deterrence. In all three larval treatments, normal metabolism is affected or disrupted; starvation, midgut damage and trehalase inhibition all may cause larvae to switch from normal carbohydrate metabolism to lipid metabolism to satisfy energy requirements.

Tributyl citrate (TBC) was identified initially as the single differential peak found in PEL and not CLW ether extracts. It was, however, also found in both CLW and carboy-held ddH_2O controls (Table 4). We are unsure whether larvae produce this compound because TBC is used as an industrial plasticizer (Labrecque et al. 1997, Ljungberg and Wesslen 2002, Ljungberg et al. 2003). Nonetheless, tributyl citrate is a citrate ester (Labrecque et al. 1997) and citric acid is required for aerobic respiration within the citric acid cycle (Randall et al. 2001). It is conceivable that the precursors of tributyl citrate are present within the larvae, however, the role of this molecule in larvae is unknown. The ddH_2O

used in preparing all larval waters was held in large plastic Nalgene® carboys. The presence of TBC in the carboy-held ddH_2O control and the absence in the non-carboy ddH_2O control suggests that these carboys are a possible source of TBC. The carboy-held ddH_2O control had a higher concentration of TBC than all treatment and control larval waters extracted, with the exception of one batch of PEL deterrent waters (Table 4). The variation of TBC concentration in the extracted waters may be due to the differing lengths of time that the ddH_2O used to make the larval waters was held in the plastic carboys before use. Logically, waters held in carboys for longer periods of time could contain more TBC. Larval waters were also frozen, lyophilized and rehydrated in small plastic bottles; these bottles are another potential, but less likely, source of tributyl citrate. Although its origins in this instance are ambiguous, tributyl citrate is still an effective oviposition repellent in very low concentrations. Further research should evaluate the environmental impact, persistence and long-term effectiveness of this promising compound as an oviposition deterrent.

Our original hypothesis was that the deterrent quality of PEL waters is due to a compound or compounds produced by parasitized larvae. Thus, our experimental protocol targeted compounds that arose in PEL and not CLW waters. There were, however, many other peaks in CLW waters that were not in PEL waters that were not examined. It is possible that the deterrence is not caused by a single compound produced by parasitized larvae. Rather, it may be due to compounds absent in PEL but not CLW waters. A mixture of compounds

with specific ratios and concentrations may be required to elicit the deterrent response by gravid females. It is possible that this chemical mixture was not detected by our extraction and isolation protocols as some compounds may have been soluble in one solvent but not another. We only used one solvent at a time or used solvent mixes that specifically targeted a class of molecules (fatty acids) to extract concentrated larval waters. This approach may have been too narrow in scope to detect compound mixtures. If the deterrence is caused by a lack of compounds in deterrent waters, or by a mixture of compounds, a different approach to chemical isolation and identification may be needed.

The repellent quality of larval waters was lost in the extraction processes with the sole exception of the water fraction of PEL pentane extractions and the repellent activity of tributyl citrate (Table 3). The repellent compound of larval origin may be obliterated at any step within the extraction protocol. Freezing at -80°C or lyophilizing over several days could destroy the active compound(s); this would explain why virtually none of the solvent or water fractions retained repellent activity of suspected larval origin post-extraction. Alternatively, if the deterrent activity is due to the presence of two or more compounds, the elimination of a single component of a repellent blend might render the solution no longer repellent.

Both palmictic (16:0) and stearic acid (18:0) methyl esters were found in transesterified PEL and CLW extracts. We expected to find higher

concentrations of both esters in PEL and DFS extracts than in CLW extracts as they are necromones, and PEL and DFS larval waters should have higher larval mortality. There was no significant difference in 16:0 or 18:0 methyl ester concentrations between any of the larval treatments, CLW, PEL or DFS (Table 5). Although not statistically different, these data show trends. There was a 58% increase in mean16:0 methyl ester concentrations in PEL over CLW extracts, but relatively no difference in mean 18:0 concentrations (Table 5). Thus, palmictic acid may contribute to the deterrence of PEL waters, whereas stearic acid likely does not. The concentrations of both 16:0 and 18:0 methyl esters in DFS extracts were only half those of CLW extracts (Table 5). It is not likely that either palmictic or stearic acid contributes to the oviposition deterrent quality of DFS waters. Future research in this area should focus on investigating palmictic acid and its methyl ester as deterrent compounds of possible larval origin.

Necromones are not released from living organisms (Rollo et al. 1994, Rollo et al. 1995), including moribund larvae. A larva with a high parasite load may have altered behaviour, reduced feeding and mobility (Zahiri et al. 1997c), but if the larva does not die, necromones will not be released. Lowenberger and Rau (1994) reported an oviposition repellent of larval origin, attributing it to a host-parasite interaction in live larvae. Sub-lethal effects of *P. elegans* parasitism in *Ae. aegypti* are most likely responsible for the production of this repellent.

Further research into the metabolic stresses that immune responses, parasite burdens and specific host-parasite interactions place on *Ae. aegypti* larvae is needed to identify molecules used for site quality determination by gravid mosquitoes. Physiological experiments measuring secreted metabolic products or by-products from stressed or starved larvae coupled with electroantennogram analyses might reveal the types of molecules that are the best candidates to pursue. Specifically, investigations into the ability of stressed larvae to produce and secrete long-chain fatty acids and citrate esters would support the role that molecules such as tributyl citrate and saturated and unsaturated hexadecanoic and octadecanoic acids might play in the chemical ecology of oviposition by *Ae aegypti*.

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Table 4 - Bioassays performed to assess deterrent qualities of treatment larval waters when compared to control larval waters (CLW). Larval treatment waters tested were: clean thoracic puncture (CTP), inoculated thoracic puncture (ITP), exposure to *B. thuringiensis israelensis* larvicide (BTI), *R. culicivorax* infection (RCX), high-density low-food stress (DFS), *P. elegans* parasitized (PEL).

Larval waters assayed	Replicates in trial	Duration of trial (d)	Number of trials
CTP	10	5	2
ITP	10	5	2
BTI	10	5	1
RCX	5	3	1
DFS	3 to 10	3 to 5	12
PEL	3 to 10	3 to 5	31

Table 5 - Mean proportions of eggs laid in oviposition response bioassays of gravid *Ae. aegypti*. In each bioassay, the total number and proportions of eggs laid on control or treatment larval waters were compared in a binary choice design. The treatment groups included: clean thoracic puncture (CTP), inoculated thoracic puncture (ITP), *R. culicvorax* infection (RCX), BTI larvicide exposure (BTI), density/food stressed (DFS) and *P. elegans* infected (PEL). The differential oviposition on distilled water or distilled water containing synthetic tributyl citrate (TBC) also are presented. The data were generated to determine which treatments rendered larval waters deterrent to gravid females. Deterrent waters then were used in subsequent studies to isolate oviposition deterrent compounds. Proportions of eggs laid in treatment waters are expressed as the mean of daily proportions taken over all replicates and all days for individual bioassays. = indicates no differential oviposition, + indicates an attraction to treatment waters, – indicates a deterrennce to treatment waters. The data were analyzed in pairwise comparisons using repeated measures analysis. The proportional data were arcsin transformed prior to analysis. Differential oviposition was considered significant if the P≤0.05.

Larval Water Treatment	n	# eggs laid in control waters	# eggs laid in treatment waters	Mean Proportion of Eggs Laid in Treatment Waters (± SD)	Oviposition Response	p value
CTP	10	2366	2315	0.509 ± 0.26	=	0.9081
ITP	10	3914	5084	0.570 ± 0.29	+	0.0416
RCX	5	1100	1157	0.433 ± 0.30	=	0.5647
BTI	10	2807	737	0.298 ± 0.30	−	0.0002
DFS	4	2228	840	0.293 ± 0.23	−	0.0111
PEL	10	3614	927	0.242 ± 0.29	−	<0.0001
TBC	4	1051	292	0.225 ± 0.24	–	0.0042

Table 6 - Larval water fractions retaining oviposition deterrent(s) after extraction with solvents. Pentane, ether, chloroform and dichloromethane were used to extract PEL and DFS larval waters to isolate deterrent compound(s). Fractions marked with + retained deterrence after the extraction process, whereas those marked with – did not.

	PEL deterrent waters		DFS deterrent waters	
Solvent used	H_2O fraction	Solvent fraction	H_2O fraction	Solvent fraction
Pentane	**+**	**–**	**–**	**–**
Ether	**–**	**+**	**–**	**–**
Chloroform	**–**	**–**	**–**	**–**
Dichloromethane	**–**	**–**	**–**	**–**

Table 7 - Concentrations of tributyl citrate (TBC) in larval waters and water controls. Larval waters of *P. elegans*-infected larvae (PEL), high density low food stressed larvae (DSF) and control mosquito larvae (CM) as well as carboy water control (H_2O) and water that had no contact with plastic carboys (H_2O plastic control) were extracted with ether and TBC was quantified using GC/MS analysis and standards. Larval waters sharing the same symbols (§) were bioassayed against one another. An n/a indication for deterrence indicates waters not bioassayed for deterrence.

Waters Extracted	volume extracted (ml)	[TBC] (ng/ul)	deterrence
PEL 1	400	1.0278	y
PEL 2	1000	0.0714	y
PEL 3§	1000	0.0019	n
DFS	2000	0.0142	n/a
CM§	1000	0.0159	n
H_2O in carboy	1000	0.7928	n/a
H_2O in glass	1000	0	n/a

Table 8 - Concentrations of palmictic (16:0) and stearic (18:0) acid methyl esters transesterified from chloroform/methanol extracts of control larval waters (CLW), *Plagiorchis elegans* infected (PEL) larval waters and density/food stressed (DFS) larval waters. Methyl ester concentrations are expressed in ng/μl as a mean taken over multiple replicates plus or minus standard deviation.

Larval Water Treatment	n	Mean concentration 16:0 methyl ester (± SD) (ng/μl)	Mean concentration 18:0 methyl ester (± SD) (ng/μl)
control larval waters (CLW)	4	1.96 ± 2.33	1.38 ± 1.31
P. elegans infected (PEL)	8	3.09 ± 2.32	1.30 ± 0.83
density/food stressed (DFS)	2	0.698 ± 0.029	0.652 ± 0.014

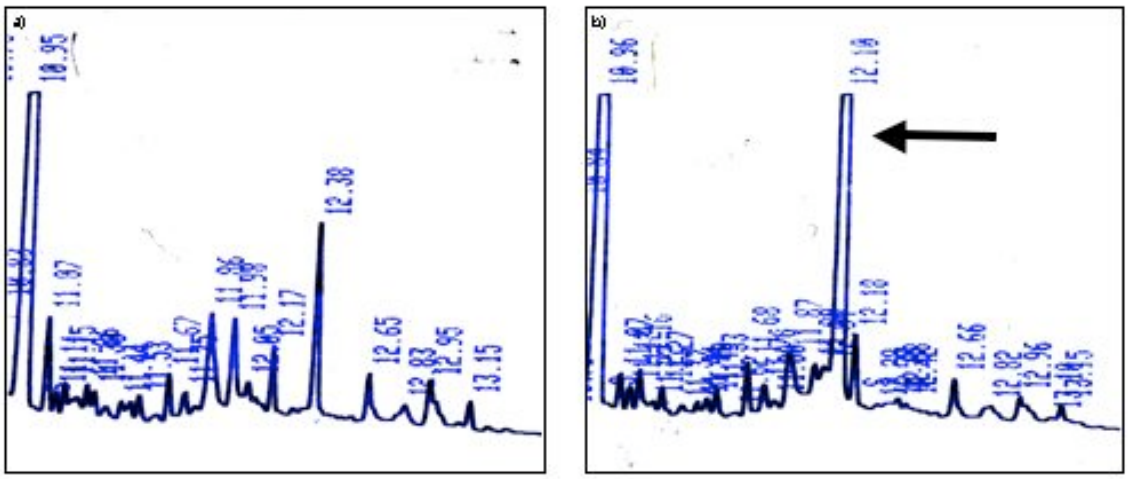

Figure 4 - GC/Electroantennogram output for ether extracts of a) control larval water (CLW) and b) *Plagiorchis elegans* infected larval water (PEL). The biologically active differential peak in PEL ether extracts at 12.1 minutes (indicated by the arrow) was later identified as tributyl citrate by GC/MS analysis.

CHAPTER 4: THESIS CONCLUSIONS

Mosquito-borne diseases such as Malaria and Dengue have become major human health problems that place an inordinate strain on health care systems in developing countries (WHO 2006). Because there is no vaccine for many of these diseases, the main strategy used to reduce transmission continues to be mosquito control (Rigau-Pérez et al. 1998, Solomon and Mallewa 2001, Spiegel et al. 2005). Much of this is concentrated on larviciding to reduce populations, or adulticiding during outbreaks. Investigating the oviposition ecology of *Ae. aegypti*, the main vector of Dengue viruses, the effects of good and poor larval habitat on the fitness of emerging adults, and semiochemicals that females use to determine the quality of site selection can provide information useful to the improvement of existing vector control and monitoring programs or implementation of new approaches.

Effects of oviposition behaviour on fitness

Because *Ae. aegypti* invests no parental care in its offspring (Christophers 1960, Clements 1999) and the larvae are restricted to the habitat in which they hatch, oviposition site selection is their main contribution to the survival and fitness of their offspring. We investigated the fitness parameters, F1 adult size, fecundity, egg size, egg provisioning and egg viability, and compared these

parameters under the stresses of high-density low-food environment (DFS), good environments, and good environments plus an endoparasite (PEL).

Adults emerging from the DFS regime were smaller, had smaller pupal reserves of protein, carbohydrates and lipid, and laid fewer and smaller eggs that were less viable than CM mosquitoes. Therefore, DFS females have a distinct fitness disadvantage over CM mosquitoes in the laboratory environment, and these data support the overall conclusions of Briegel (1990b) on the importance of nutrient reserves.

PEL females were only slightly smaller and laid slightly smaller eggs than CM females, however, winglengths of PEL females decreased as parasite load increased. The total protein, carbohydrate and lipid content of both pupae and eggs were not different between these two larval treatments, nor were egg viability and numbers of eggs laid. But a smaller proportion of PEL females oviposited than CM females.

Ovipositing in either the DFS or PEL larval environment adversely affects the overall fitness of the ovipositing female by reducing the fitness of her offspring. Smaller body size is correlated with lower flight potential and lower fecundity in *Ae. aegypti* (Briegel 1990b, Briegel et al. 2001). Lower fecundity may be the result of smaller reproductive organs, smaller teneral reserve stocks,

smaller maximum bloodmeal volume or a combination of these effects (Steinwascher 1984, Briegel 1990b, Briegel et al. 2001). Both DFS and PEL larval habitats also increase pre-imaginal mortality, adult viability and adult longevity (Hawley 1985, Dempster et al. 1986b, Dempster and Rau 1991, Agnew et al. 2000).

Current control programs aim at reducing mosquito populations. This may increase the size and fitness of those that survive and emerge. Theoretically we could increase larval density in oviposition sites in the field by seeding them with large quantities of early stage mosquito larvae should reduce the quality of adult mosquitoes emerging from treated sites. Those adults that do emerge would be less fit, would have shorter lifespans, and would be smaller and lay fewer and less viable eggs. This theoretical situation would simply not be practical, feasible or palatable to health policy makers or the general public and could create a public health disaster. Bloodfeeding frequency is negatively correlated with adult size in the field for *Ae. aegypti*; smaller mosquitoes have a higher bloodfeeding frequency than do larger mosquitoes (Scott et al. 2000). Smaller adults emerging from high-larval-density treated sites may have an increased capacity to transmit arboviruses because: (1) they feed on more hosts per unit time than larger mosquitoes do, so for a given time frame in an area endemic for mosquito-borne disease, they would have (2) increased probability of picking up pathogens from infected hosts and (3) may have an increased probability of passing on pathogens to uninfected hosts.

Parasites of mosquito larvae, including entomopathogenic xiphidiocercariae, have been effectively used to reduce mosquito populations in the field (Chapman 1974, Rao et al. 1985, Mijares et al. 1999). *Plagiorchis elegans* could be integrated as part of mosquito control programs. Aside from increasing mosquito pre-imago mortality, parasitism with *P. elegans* can prolong larval development and reduce the proportion of females who oviposit in adult mosquito populations. Longer larval development times provide a larger window of opportunity for immature mosquito populations to be targeted by predators or larvicides, possibly increasing the efficacy of control programs. However, *P. elegans* would not be effective against populations of *Ae. aegypti* that uses small, man-made containers as larval habitat that is not be suitable for the pulmonate snail hosts of *P. elegans.* Given that *P. elegans* does not appear to exhibit host-specificity for its second intermediate host (Zakikhani and Rau 1999), this system could be used to control populations of mosquito species that oviposit in larger, permanent eutrophic water sources (*Culex sp.*) that would support the growth of pulmonate snail populations. Periodic sowing of infective *P. elegans* eggs would be required to maintain parasitic infection in a sufficient proportion of the snail population to ensure effective densities of xiphidiocercariae (Zakikhani and Rau 1999).

Oviposition deterrent investigation: Tributyl citrate and palmictic acid

We have established that there are serious fitness consequences to ovipositing in poor quality larval habitat or habitat containing parasites. How can a gravid female avoid these detrimental fitness effects for her progeny?

Gravid *Ae. aegypti* are deterred from ovipositing in waters that had previously contained starved conspecific larvae and conspecific larvae parasitized by *P. elegans* (Lowenberger and Rau 1994c, Zahiri et al. 1997b, a, Hilker and Meiners 2002). It has been suggested that the chemical cues responsible for the oviposition deterrence are produced by the larvae as the result of environmental or physiological stress. We demonstrated that this was nod due to the penetrating mechanism, nor due to a general parasitism of larvae. We extracted CM, PEL and DFS larval waters and distilled water controls with and used GC-EAD and GC/MS analysis of the extracts to isolate and identify putative semiochemicals present in treatment waters and absent from controls.

Tributyl citrate (TBC) was initially found only in PEL waters and not CM, but later was found in PEL, DFS, CM and water controls. This molecule is an oviposition deterrent for *Ae. aegypti* in low concentrations but our data suggests it may not be of larval origin. Nevertheless, it may prove useful in mosquito monitoring and control strategies. Ovitraps are useful tools in mosquito monitoring programs (Zeichner and Perich 1999) but only prove effective if females lay eggs in them rather than surrounding larval habitats. Many control

programs use oviposition attractants in their ovitraps to increase their success (Santos et al. 2003). To the same effect, oviposition deterrents could make oviposition sites surrounding the ovitraps undesirable to gravid females. TBC could be used to many water sources that cannot be eliminated, or otherwise controlled, undesireable as oviposition sites to gravid *Ae. aegypti,* forcing them to use sites such as ovitraps, which will increase the effectiveness of these as population monitors. TBC may be an effective oviposition deterrent to mosquito species other than *Ae. aegypti*, further experimentation investigating other taxa susceptible to this deterrent is needed. Before it can be used in mosquito control efforts it is imperative to determine toxicity and persistence of TBC in the environment, minimum effective concentrations and non-target effects.

Due to interrupting carbohydrate metabolism, *P. elegans* may cause larvae to use fatty acid or lipid metabolism to generate glucose for energetic needs (Candy et al. 1997). Stearic (18:0) and palmictic (16:0) acids are common in mosquitoes and are also necromones. We extracted and transesterified 18:0 and 16:0 fatty acids from PEL, DFS and CM waters. There was no significant difference in the concentrations of either 18:0 or 16:0 methyl esters for any of the larval waters, however, trends in the data suggest that 16:0 fatty acids may be of some importance in the deterrence of PEL waters. PEL had a higher mean concentration of 16:0 methyl esters than did CM waters suggesting palmictic acid as a molecule of interest for future studies. Further experiments should be done to quantify the methyl esters in larval waters prior to the transesterification of free

fatty acids as the presence of methyl esters in the water may be confounding our results.

Concluding Points

We have shown in this thesis that:

1) There are fitness consequences to ovipositing in poor larval habitats.
2) Our fitness data agree with Briegel (1990) that parasitized mosquitoes or mosquitoes eclosed from poor larval habitat are less fit than healthy mosquitoes from good larval habitat.
3) Our data disagree with Reeves' (2004) hypothesis that suggested the deterrent effects of PEL larval waters are due to cuticular damage in larvae caused by penetrating xiphidiocercariae.
4) We have compared the larval holding waters and assessed potential compounds for their contribution to the oviposition deterrence effect.
5) We identified a potentially important deterrent molecule, tributyl citrate (TBC), that might be used in increasing monitoring success of existing ovitraps by deterring oviposition in surrounding sites containing TBC.
6) We have laid a foundation for subsequent studies on other deterrent molecules produced by *Ae. aegypti* larvae, including ketone bodies and necromones.

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