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AEDES AEGYPTI (DIPTERA: CULICIDAE)

OVIPOSITION ATTRACTION/REPELLENCY

By

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A thesis submitted to the
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Doctor of Philosophy

Dedicated to my Dad, my husband, and my son.

ABSTRACT

Waters from normal larvae of *Aedes aegypti* (L.) are highly attractive to ovipositing conspecific females, whereas waters from larvae infected with the entomopathogenic digenean parasite, *Plagiorchis elegans* Rudolphi, are rendered strongly repellent. The production of the repellent appears to be mediated by the degree of environmentally induced stress experienced by the larvae. Whereas waters from fully fed larvae were highly attractive as an oviposition site, these were rendered progressively less attractive, and eventually strongly repellent as the larvae were deprived of food over a period of 7 days. Crowding of the larvae elicited similar repellent effects as did close contact between larvae and the walls of the container in which they were reared. The site of infection in the tissues of the mosquito larvae also influenced the intensity of repellency. Thus, infections of the head and thorax induced the highest degree of repellency, and infections of the abdomen the lowest. The repellent effect overrode attraction and remained stable for more than one week at 27° C, and even longer at lower temperatures. Stressors which induced repellency all precipitated similar physical and physiological changes in mosquito larvae. They reduced wet and dry weights and the concentration of serum carbohydrates, amino acids, proteins and lipids. Both infection and crowding rendered larvae anorexic. As well, infected larvae appeared to be unable to convert trehalose to glucose, thereby exacerbating the energy deficit. Incubating infected larvae in a dilute glucose solution significantly reduced the repellent effect of their waters. Addition of glucose to already repellent waters had little effect. Larvae of another species, *Aedes atropalpus* Coquillett, were equally capable of

producing repellent effects when infected with *P. elegans*, and gravid females of *Ae. aegypti* were equally sensitive to these as to conspecific waters. This sensitivity, however, was not reciprocal. *Aedes. atropalpus* females were not repelled by waters from infected *Ae. aegypti* larvae. This work provided new insight into the biology of mosquitoes and offers an unconventional approach to their biological control.

RÉSUMÉ

Les eaux de larves normales d'*Aedes aegypti* (L.) attirent fortement les femelles de la même espèce qui ovipositent, alors que les eaux de larves infectées par le parasite digénien entomopathogène, *Plagiorchis elegans* Rudolphi deviennent fortement repoussantes. La production de l'effet répulsif semble être modifiée par le degré de stress imposé à la larve par le milieu. Les eaux de larves nourries à satiété étaient hautement attirantes comme site de ponte alors que les eaux de larves privées de nourriture devenaient progressivement moins attirantes et éventuellement fortement repoussantes au cours des 7 jours de famine.

Le surpeuplement des larves ainsi que leur contact avec les parois du contenant dans lequel se faisait l'élevage ont engendré le même effet répulsif. Le site de l'infection dans la larve du moustique a aussi influencé l'intensité de l'effet répulsif. Notamment les infections à la tête et au thorax ont produit un degré de répulsion supérieur à celui causé par les infections de l'abdomen. L'effet répulsif a surmonté l'attraction et est resté stable pendant plus d'une semaine à 27°C et même plus longtemps à des températures inférieures. Les différents facteurs stressants, cause de répulsion, ont tous amené chez la larve de moustique des changements physiques et physiologiques semblables. Ainsi ils ont abaissé les poids secs et humides ainsi que la concentration d'hydrate de carbone, d'acides aminés, de protéines et de lipides du sérum. L'infection et le surpeuplement ont rendu les larves anorexiques. De même, les larves infectées semblaient incapables de convertir le tréhalose en glucose. L'incubation de larves infectées dans une solution diluée de glucose a réduit l'effet répulsif de l'eau de façon significative. L'addition de glucose à des eaux ayant déjà un effet répulsif ne produisit aucun changement. Les larves d'*Aedes atropalpus* produisent également le même effet répulsif lorsque infectées par *P. elegans* et les femelles d'*Ae. aegypti* sont aussi sensibles à ces effets qu'aux produits de leur espèce. Toutefois, cette sensibilité n'était pas réciproque: les femelles d'*Ae. atropalpus* n'étaient pas repoussées par les eaux ayant contenues préalablement des larves infectées d'*Ae. aegypti*. Cette recherche nous permet une nouvelle compréhension de la biologie des moustiques et offre une approche originale pour leur contrôle.

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PREFACE

Chapter 1 is an introduction to the theoretical and practical context in which the present studies were carried out. In this chapter, I review the literature dealing with *Ae. aegypti* as an experimental host of *P. elegans,* the phenomenon of oviposition, and aspects of the biology of various other species of mosquitoes. I also outline the concepts of intra- and inter-specific competition among insects, the effects of the parasite, starvation and crowding on the functioning of the insect, and an outline of the function and composition of insect hemolymph.

In **Chapter 2,** I determine whether factors, such as laval instar and the intensity and site of infection with *P. elegans,* influence the expression of oviposition repellency.

In **Chapter 3,** I examine the responses of various mosquito instars to another common stressor, starvation, in the context of induction of oviposition repellency.

In **Chapter 4,** I assess the oviposition responses of gravid female mosquitoes to larval waters in order to determine if oviposition site selection is influenced by various other stressors such as the population density of the larvae, and physical interference with their normal behaviour and posture. The activities and persistence of the repellent and attractant effects on ovipositing, conspecific females are examined in this chapter, and the manner in which oviposition attraction and repellency interact at various dilutions is determined.

In **Chapter 5,** I examine some of the basic biochemical changes in the composition of the hemolymph of larvae which have been exposed to stressors that induce oviposition repellency.

In **Chapter 6,** I evaluate the capacity of the larvae of another mosquito species, *Ae. atropalpus*, to induce oviposition repellency when infected with *P. elegans* metacercariae, and whether such responses of the adult females are in the conspecific and /or heterospecific domain.

Chapter 7 is a general summary and discussion of the findings of this study.

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Additional material (procedural and design data, as well as descriptions of equipment used) must be provided where appropriate and in sufficient detail (e.g. in appendices) to allow a clear precise judgment to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, the candidate is required to make a explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers.

In this context, Professor Rau has provided supervision in all aspects of my research and appears as a co-author in all publications resulting from this work.

Chapter 2 is drawn from a paper co-authored with Professor D.J. Lewis and Dr. Sh. Khanizadeh. Professor Lewis contributed to my understanding of insect biology and Dr. Khanizadeh provided insight and advice regarding the statistical analysis of this data set.

Chapters 3 and 6 are drawn from papers co-authored with Professor Lewis who provided expertise on the biology of biting flies and who reviewed the manuscript in its preparation.

Chapter 5 is drawn from a paper co-authored with Professor G. Dunphy who lent his expertise in insect physiology and biochemistry to this project, particularly in regards to the composition and functions of hemolymph.

I designed, executed and interpreted all of the above experiments.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Oviposition site selection by mosquitoes is a critical factor which influences the survival and population dynamics of these insects and therefore, has important implications with regards to mosquito control. Virtually the whole range of available aquatic niches, including artificial containers, treeholes, ponds, and marshes serve as oviposition sites in nature (Gillett 1972). Mosquitoes are quite discriminating in their selection of sites for egg deposition (Macan 1961), and there is considerable evidence that such site discrimination by ovipositing females is a key factor in determining larval distribution (Rudolfs and Lackey 1929, Beattie 1932, Kennedy 1942, Wallis 1954).

Most entomologists agree that gravid females of many mosquito species have a marked oviposition site preference for aquatic habitats which already harbour their immature stages (Maire 1982, 1983). Nevertheless, the study of factors and mechanisms inducing such oviposition behavior is still a relatively new area of research. For both

oviposition attractants and repellents, almost all laboratory experiments have been conducted with mosquito species that have a tropical-temperate distribution, particularly *Aedes aegypti* (L.). This species has become prominent as a target of research primarily because of its importance as a vector of disease and also because laboratory colonies of this insect are easy to maintain.

Wilton (1968) investigated the influence of water contamination with bacteria, surface area, temperature, and light intensity on oviposition. *Aedes triseriatus* (Say) showed a preference for rough-textured containers with horizontal openings and dark colors. *Aedes vexans* (Meigen) prefers to oviposit on waters more than 60 cm deep (Curtis 1985). Similarly, Fay and Perry (1965) reported that surfaces with rough texture and dark color are attractive to *Ae. aegypti* females, and Perry and Fay (1967) found that olfactory and other stimuli, such as light, are involved in the selection of oviposition sites. Given a choice, females strongly prefer to oviposit in the shade (Fay and Perry 1965). Above all, *Ae. aegypti* females show a distinct preference for waters containing immature stages of their own species (Maire 1982). Ovipositing females are thought to respond to attractant pheromones produced by conspecific larvae and pupae (Soman and Reuben 1970, Chadee 1990). This attraction appears to be somewhat species specific, as *Aedes atropalpus* (Coquillett) larvae fail to attract ovipositing *Aedes communis* (DeGeer) females (Maire 1985), but *Ae. communis* females do not distinguish between *Ae. atropalpus* and *Ae. communis* larval holding waters (Maire and Langis 1985).

In most oviposition studies, the focus has been on the attractiveness of specific sites with the aim of determining which factor(s) contribute to site selection. There are avoidance reactions as well. Females of *Ae. aegypti* avoid laying eggs in ovipots in which they themselves have already laid (Chadee *et al.* 1990). Very little is known about the repellent effects of predators, parasites, pathogens and other stressors on the ability of mosquito larvae to produce semiochemicals and to use them to communicate with adults. However, Chesson (1984) showed that *Culex pipiens* L. oviposits less frequently in sites containing predaceous notonectids. Kuthiala *et al.* (1992) reported that the mosquito repellent 'DEET' greatly reduced the behavioral oviposition response when presented together with ethyl propionate.

Species that avoid ovipositing in sites deleterious to larval development should be favored by natural selection. Lowenberger and Rau (1994) demonstrated that water containing larvae parasitized by the digenean *Plagiorchis elegans* (Rudolphi) received significantly fewer eggs than waters containing unparasitized larvae. Waters that had previously held unparasitized larvae received significantly more eggs than waters that had previously contained no larvae or parasitized larvae. Mosquitoes landed with equal frequency on both waters, and laid similar numbers of eggs / minute spent on the surface. However, mosquitoes remained for significantly shorter periods of time on waters from parasitized larvae and consequently laid significantly fewer eggs there.

Parasitized *Ae. aegypti* larvae produce a compound that repels adults and deters oviposition in sites unsuitable for larval development (Lowenberger and Rau 1994).

Although natural selection should favor female mosquitoes that do not oviposit in environments unsuitable for larvae development, it would be surprising to find that this response is so finely tuned as to be specific to *P. elegans* infections alone. It is, more probably, a general response mediated by the degree of environmentally induced stress in the larvae.

LITERATURE REVIEW

MOSQUITOES

Over 3000 species of mosquitoes belonging to 34 genera have been described (Harwood and James 1979). They are two-winged flies belonging to the order Diptera and family Culicidae. The adults are free living, have scales on the veins and posterior margin of the wing, and possess an elongated proboscis for feeding. The eggs are laid in or near water. The larval stages are aquatic, but different species prefer different environments, and different genera may occupy widely different strata within the same environment. The larvae are largely filter feeders, feeding on algae, rotifers, protozoa, pollen, and yeasts. The larvae collect these by means of brush-like structures on either side of the mouth which, in a rhythmic motion of 180-240 beats / second, draw a current of water towards the mouth (Clements 1963).

The larvae pass through four instars: their rate of growth as well as their eventual size is a function of temperature and nutrition. The pupae are comma-shaped with two respiratory trumpets and paddle-like flaps on the abdomen. Pupae do not feed. Following 2-3 days of tissue restructuring and reorganization, the pupae give rise to adults. The adults feed largely on nectar. However, the females of anautogenous species require a blood meal to supply the protein required for egg development. The adults of autogenous species produce eggs in the absence of blood feeding (Roubaud 1933). Mosquitoes have a holometabolous life cycle which consists of the egg, 4 larval instars, the pupa, and the adult stage (Christophers 1960, Harwood and James 1979).

The most important genera are *Anopheles, Culex* and *Aedes* (Chapman 1969). The genus *Aedes* has been separated into species on the basis of adult morphology (Service 1980). Harwood and James (1979) have conveniently separated the genus *Aedes* according to larval habitat as salt marsh, flood water, boreal, snow pool and tree hole species.

***Aedes aegypti* (L.)**

Aedes aegypti breeds in tree-holes and artificial containers (Welch and Long 1984) and is best known as the vector of yellow fever and dengue (Smith 1956, Gould *et al.* 1968). The process of hatching is a reflex behavior triggered by a variety of stimuli, but primarily by low or decreasing levels of dissolved oxygen in the water (Judson 1960). Hatching may also be influenced by factors such as genotype (Gillett 1955), humidity (Judson 1960), sex (Elzinga 1961) and age (Burgess 1959). First instar larvae undergo metamorphosis in which certain structures are replaced and others differentiate as they grow towards the fourth instar. The larvae are phytophagous, using bacteria and microorganisms as their main food source. However, waters overly contaminated with bacteria can kill the larvae (Lewis 1933) and pupation ceases in waters with inadequate food. Excessive larval competition for food, or overcrowding in the presence of an adequate food supply (>1000 larvae/L) can prevent moulting or may produce smaller larvae, pupae, and adults (Christophers 1960, Clements 1963, Wada 1965). The optimum temperature range for growth and development of larvae is 27-30°C. Larvae can

recuperate from brief episodes of low temperatures but will not mature if exposed continuously to temperatures <12 or >39°C (Christophers 1960).

Following emergence, the activities of feeding, mating, host selection, and oviposition site selection follow temporal and hormonally-regulated behavioral patterns. Many of these stereotypic behaviors are genetically controlled and are triggered by various endogenous or exogenous factors (Klowden 1990). Nectar provides nutrition, but a blood meal is required by females to obtain proteins for oogenesis (Harwood and James 1979, Briegel 1985). Males are sexually mature and begin searching for females within 15-24 h following emergence, whereas females have a post-emergent refractive period (Christophers 1960). *Aedes aegypti* females are monogamous: all offspring result from the first copulation (Craig 1967).

Host-seeking behavior is dependent on parous status (Klowden *et al.* 1987): females which have fed are either developing eggs or are in oviposition mode and are not receptive to blood-feeding cues. Inhibition of host seeking is lost following oviposition (Davis 1984). A single blood meal is usually sufficient for a complete gonotrophic cycle but multiple host feeding may be affected by their nutritional status during the larval stage (MacDonald 1956), water supply (Khan and Maibach 1970), or by the interruption of blood feeding by the host (Edman *et al.* 1975).

Gonotrophic cycles are initiated by the blood meal, and the number of eggs produced per cycle depends largely on the volume of the blood ingested, initial body weight of the mosquitoes (Colless and Chellapah 1960) and genetics (Briegel 1985). *Aedes aegypti*

shows an endogenously regulated diel oviposition pattern (Haddow and Gillett 1957, Klowden 1990) that may be influenced by the length of the photophase (Chadee and Corbet 1987) and by means of a light-activated hormone (Haddow and Gillett 1957).

Oviposition site selection in *Ae. aegypti* is governed by visual cues, substrate texture, reflectivity of water, odors (Wood 1961, Russo 1978, Adham 1979, McIver 1982) and by the presence of aquatic microbes and their metabolites (Gjullin and Johnson 1965, Ikeshoji *et al.* 1979, Benzon and Apperson 1988). While gravid females are attracted to waters containing conspecific larvae (Soman and Reuben 1970), they oviposit preferentially in sites containing eggs of other conspecifics over sites containing their own eggs from previous gonotrophic cycles (Chadee *et al.* 1990). Since the flight range of females may be as short as 25-30m from their site of emergence (Soper 1935), the probability of genetically related females using the same oviposition sites is high. Following oviposition, females resume host-seeking for another blood meal to develop subsequent batches of eggs.

Two forms of *Ae. aegypti* have been described: a widely distributed domestic species which is found only in or near houses, and a darker, feral species which is confined to Africa south of the Sahara (Paterson *et al.* 1976). Adults are chacteristically marked with transverse bands of silvery white or yellowish white on the abdomen. Vertical, thin stripes occur on the dorsal surface of the thorax, and the legs are banded. The tarsi of the last pair of legs are white (Cheng 1986). Harwood and James (1979) noted that, world

wide, the species occurs within the limits of 40°N and 40°S latitude, but it is highly susceptible to temperature extremes and does not thrive in dry, hot climates.

***Aedes atropalpus* (Coquillett)**

Aedes atropalpus is known as a rock-pool breeder, and larvae are found in rock depressions along the shores of lakes, mountain streams, or along rivers where natural or man-made impoundments occur (O'Meara and Craig 1970). Hedeen (1953) has collected this species from tree-holes on two occasions, and from artificial containers once. The female is facultatively autogenous, which means that the genetically autogenous female will engorge readily during the first ovarian cycle if it meets a host, but can mature one batch of eggs without a blood meal. It is well known that *Ae. atropalpus* will attack humans, and can transmit the eastern variety of equine encephalitis (Carpenter *et al.* 1946, Wood 1961). It is also a vector of *Plasmodium gallinaceum* Brumpt, a variety of malaria found in chickens and other birds (Tremblay 1946). This mosquito is found in southern Canada, the United States, Mexico and Central America (Carpenter *et al.* 1946). Developing larvae and pupae are known to release a soluble substance into the aquatic environment which acts as an oviposition attractant (Kalpage and Brust 1973, Bentley *et al.* 1976, Maire 1984, 1985).

OVIPOSITION

Selection of a site for oviposition involves a choice of the type of site, and a choice of the position where the eggs are to be laid. The former choice deals with the nature of the

breeding places used by the species; the latter choice is concerned with exactly where in the chosen water receptacle the female places her eggs (Christophers 1960). There are similarities between mosquito host-seeking and oviposition behaviors in that they require complex integration of physical and chemical cues. Long-range cues, probably involving vision, allow mosquitoes to identify different habitats, specific hosts and oviposition site characteristics. Short-range cues include temperature and chemical signals received by contact chemoreceptors (Bentley and Day 1989). Mosquitoes may respond to oviposition attractants or stimulants and repellents or deterrents. Foster and Harris (1997) proposed that after arriving at a resource, an insect is likely to encounter additional stimuli. These either stimulate a behavior, keeping the insect at the resource, or inhibit that behavior, resulting in rejection of, and possibly movement away from the resource. Most known stimulants are involved in either feeding or oviposition (Renwick 1990). A deterrent is a chemical that inhibits specific behaviors such as feeding or oviposition (Bernays and Chapman 1994).

The location of the oviposition site is governed by visual cues such as color, reflectivity of the water, humidity, surface texture, light intensity, and odors (Wood 1961, Snow 1971, McIver 1982). Christophers (1960) reported that air-borne chemical stimuli may also influence location and selection. Davis (1976) noted that oviposition attractants may be sufficiently volatile to be detected by the olfactory receptors of the mosquito over a short distance. Surface texture and moisture stimulate the actual process of egg-laying (Christophers 1960). The presence of microbes or their metabolites influence site

selection (Gjullin and Johnson 1965). As well, abiotic factors or chemical substances may elicit either preferential oviposition (Osgood 1971) or avoidance by conspecifics (Chadee *et al.* 1990). Gravid females of *Ae. aegypti* are also attracted to waters containing conspecific larvae, which may involve the action of species specific factors (Soman and Reuben 1970, Roberts and Hsi 1977). However, *Ae. atropalpus* females are attracted to waters containing conspecific or *Ae. communis* larvae (Kalpage and Brust 1973, Maire 1984). Bentley *et al.* (1976), however, demonstrated that females of *Ae. triseriatus* are also attracted by *Ae. atropalpus* larval holding waters.

Mosquitoes should select oviposition sites that maximize their fitness. Factors affecting the choice of oviposition site may include the availability of food for developing offspring and the presence of competitors and predators (Blaustein and Kotler 1993). Larval population density also affects site selection. Rearing water of high larval density is less attractive to ovipositing females *Ae. atropalpus* reared under axenic conditions (Maire 1985). *Culex tritaeniorhynchus* Giles prefers water that has held uncrowded rather than crowded conspecific larvae (Reisen and Siddiqui 1978). This crowding factor, whether an inhibitor produced by the larvae or a result of excess excretory products, may have evolved as a mechanism to avoid oviposition in such less favorable sites.

A recently discovered phenomenon may represent a refinement of the response to such selective pressures influencing the choice of an oviposition site. *Ae. aegypti* larvae infected with the entomopathogenic larvae of *P. elegans* elicit a potent oviposition repellent effect (Lowenberger and Rau 1994).

PARASITE

Plagiorchis elegans (Rudolphi)

The genus *Plagiorchis* (Trematoda) constitutes a large, heterogenous group of ecto- and endoparasites that are variable in size, shape and habitat (Olsen 1967, Kassai *et al.* 1988). All species within the genus use aquatic snails as their first intermediate host and characteristically use aquatic insects as the second intermediate host (Schmidt and Roberts 1981). At least 140 species of *Plagiorchis* have been described (Blankespoor 1974). Since many proved to be indistinguishable in their adult stages, classification was based, to a great extent, on the host in which they were found.

Plagiorchis elegans is a cosmopolitan and ubiquitous intestinal parasite of birds and small mammals. Monoecious adults are found in the upper regions of the small intestine where they adhere by means of the ventral sucker and browse on the mucosal tissue and food material undigested by the host. The size of adults is dependent on their age and on the definitive host (Styczynska-Jurewicz 1962, Blankespoor 1974, Genov and Samnaliev 1984).

Eggs (30 - 40 X 20 - 30 μm) are produced as early as 6 days post-infection (Genov and Samnaliev 1984) and are released into the external environment with the feces of the definitive host. When an egg is ingested by the lymnaeid snail, *Stagnicola elodes* (Say), the miracidium hatches from the egg, penetrates the gut lining, and forms an elongated mother sporocyst on the external surface of the intestine. Asexually produced daughter sporocysts migrate and establish in large numbers in the tissues of the hepatopancreas

(Styczynska-Jurewicz 1962). The snails start to shed cercariae on approximately the 40th day post-infection. Cercariae have a well-developed stylet, a large pharynx, intestinal caecae which reach the end of body and a Y-shaped excretory system. Cercarial emergence is largely nocturnal (Styczynska-Jurewicz 1962). Patterns of cercarial emergence are photocycle dependent (Macy 1960), but other factors such as host-parasite interactions, temperature, and the time course of the infection can all affect this pattern (Pfluger 1980). Cercarial infectivity is inversely related to temperature but usually does not exeed 24 h (Lowenberger and Rau 1993).

Plagiorchis elegans cercariae penetrate members of several orders of aquatic insects as well as some other aquatic invertebrates (Williams 1963, Blankespoor 1974). Once a suitable host is found, the cercariae attach by means of suckers and actively penetrate the host using the stylet and histolytic enzymes (Bock 1986). Taft (1990) followed the penetration sequence of *Plagiorchis* sp. cercariae using cinephotomicrography and reported leech-like crawling over the host's cuticle, random probing with the stylet, loss of the tail, penetration, and subsequent encystment.

Encystment begins with the cercaria rotating around itself and secreting an elastic cyst wall. The rate of rotation decreases over the first 10 min., during which time, two cyst walls of parasite origin are deposited. The first cyst wall comprises mucosubstances and a low proportion of proteins, whereas the inner cyst wall comprises a layer of carbohydrates and proteins (Bock 1988). A third layer consisting of disintegrating haemocytes and non-cellular haemolymph components is laid down later by the host

(Taft 1990). Metacercariae measure 90 - 130 X 80 - 110 μm (Styczynska-Jurewicz 1962, Genov and Samnaliev 1984) and are generally immobile within the insect host, showing little growth over time. The Y-shaped excretory vesicle progressively fills with dark, globular excretory bodies, increasing in both size and conspicuousness over time (Styczynska-Jurewicz 1962). Metacercariae require 2-7 d to become infective to the definitive host (Blankespoor 1974, Genov and Samnaliev 1984) and the life cycle is completed when the infected intermediate host is ingested by a suitable definitive host. The excystment of *Plagiorchis* metacercariae depends on temperature, the rates of development increasing significantly with temperature (Lowenberger and Rau 1993).

STARVATION

The amount of food available is an important factor affecting the population of species in a given community. It is not uncommon for a species to utilize its entire available food supply, with a resulting sharp reduction in population because of starvation (Ross *et al.* 1982). Although there may be considerable variation in size between different individuals of the same species, a definite minimum quantity and quality of food is required for normal development. Larvae of *Ae. aegypti* reared in absence of sufficient food experience an increase in the time required for development, and the size of the resulting adults is reduced (Brust 1968, Gilpin and McClelland 1979). If starvation is nearly or quite complete, as when larvae are placed in clean water, they may remain for long periods, up to a week or more, with little or no development and may not reach the adult

stage. Their success depends on stored energy, mainly in the form of lipids, acquired during their larval stages (Wigglesworth 1942, Gilpin and McClelland 1979). However, the longevity of adult female *Ae. aegypti* under condition of starvation is shorter than most other species (Klowden and Chambers 1992). If they do complete their development, they give rise to small pupae and adults characteristic of starvation forms (Brust 1968). Such forms have been reported by a number of authors. Thus, Weidling (1928) described the result of deficient food during the larval stage as leading to prolonged development, deficient size of adults, fewer ovarioles tubes in the females, smaller eggs and fewer eggs laid. Normal, well-fed larvae increase in length to slightly over 7 mm over a period of 8 days. The starved forms grow at a lower rate, achieving a maximum size of 4 mm after 10 days. Kuno and Moore (1975) have shown that starved, axenically-reared *Ae. aegypti* larvae elaborate a growth retardant factor (GRF) that delays pupation. Exposure of first and fourth instars to growth retardant factor leads to significantly higher mortality.

Larvae exposed to severe starvation remain motionless at the surface of the water column and exhibit significantly reduced respiratory rates (Barrera 1996). When such larvae are given food, fourth instars were unable to pupate, but third instar were able to molt to the fourth instar (Brust 1968). In very advanced conditions of starvation, larvae come to lie flat at the surface of the water; with time they lose all traces of fat and become virtually transparent.

CROWDING

Overcrowding of organisms produces adverse effects on their survival, rate of development and population growth (Andrewartha and Birch 1954, Odum 1969, Ikeshoji and Mulla 1970a, Kuno and Moore 1975). Mosquito larvae reared under crowded conditions experience higher mortality, and develop more slowly into smaller adults than those reared under normal conditions (Weidling 1928, Terzian and Stahler 1949, Ikeshoji 1965, Moore and Fisher 1969, Peters *et al.* 1969). Production of growth retarding factors (GRF) by *Ae. aegypti* larvae reared under stress conditions was independently discovered by Peters *et al.* (1969) and by Moore and Fisher (1969). Intraspecific competition among larvae for food and space has been considered to cause these adverse effects (Ikeshoji and Mulla 1970a, Dye 1984). Ikeshoji (1965) demonstrated that the amount of food available per larva and larval density in the rearing units influence the size of emerging adult mosquitoes and the number of ovarian follicles in the females. To produce mosquitoes of the same size, proportionately more food must be given to larvae reared at the higher densities. At the same larval density, adult mosquito size is directly proportional to food quantity provided. Conversely, with the same quantity of larval food, mosquito size and fecundity are inversely proportional to larval density. Even with unlimited food, larvae of *Ae. aegypti* maintained under overcrowded conditions still manifested a prolongation of the pupal stage and the presence of undigested food in the alimentary tracts in spite of normal food intake (Shannon and Putman 1934). Klomp (1964) interpreted this to be the result of mutual interference, brought on by the intense mutual bodily contact under

crowded conditions. Ikesoji and Mulla (1970b) described chemical factors in the water of *Culex pipiens quinquefasciatus* (Say) larvae maintained under crowded conditions which manifested a high toxicity against conspecific and heterospecific first instars. Similarly, *C. p. quinquefasciatus* larvae die when exposed to the waters from overcrowded *Ae. aegypti* larvae (Peters *et al.* 1969).

Larval density also affects the attractiveness of potential oviposition sites to gravid females. Rearing water of higher larval density (900 larvae / L) was unattractive to conspceific ovipositing *Ae. atropalpus* (Maire 1985). Similarly, *C. tritaeniorhychus* and *Ae. aegypti* prefer waters from uncrowded conspecific larvae to waters from crowded conspecific larvae (Reisen and Siddiqui 1978, Benzon and Apperson 1988, respectively). For *Anopheles stephensi* Liston, no such preference was evident.

COMPETITION

Competition is the interaction between organisms using the same limited resource; the resource may be food, water, light, etc. It may take place among organisms of the same species (intraspecific), or among those of different species (interspecific). The availability of suitable and sufficient food is one of the most important factors influencing the distribution and abundance of insects. Many populations increase until they reach the carrying capacity of the environment. The inevitable result is that the resource in shortest supply becomes the limiting factor.

There are key factors responsible for population changes, and these can act in a regulatory role. They may be exogenous (extrinsic), which include density-dependent factors such as food, space, predation or a combination, and density-independent factors such as weather, or they may be endogenous (intrinsic) such as the pathological effects of crowding, disease, genetics and social interaction (Price 1984). Density-dependent factors often involve intraspecific competition for food so that any adaptation that will reduce this competition will be strongly favored. Individuals with requirements that differ most from those of the majority of the population will suffer least from competition. Thus, a diverse array of qualities within a population will be selected for by intraspecific competition.

There are two types of interspecific competition. Nicholson (1954) assigned the term "contest competition" to those situations where the winner of the competition "obtains as much of the governing requisite as it needs for survival and reproduction" and the loser "relinquishes the requisite to its successful competitor". The second type of intraspecific competition is described as "scramble competition". Here all members of a population have equal access to the limited resource and a free-for-all results. Nicholson (1954) pointed out that one characteristic of scramble competition is that success may often be incomplete, so that some, and at times all of the requisite resources secured by the competing animals are insufficient to sustain the population. The term more commonly used today is "exploitation competition" (Price 1984).

It is widely held that populations of *Ae. aegypti* are regulated by intraspecific competition among larvae (Southwood *et al.* 1972, McDonald *et al.* 1977, Gilpin and McClelland 1979). Mixed conclusions about the relative importance of different competitive mechanisms have been reached through laboratory experiments (Dye 1984). Bar-Zeev (1957) found that food shortage alone could substantially increase larval mortality and greatly lengthen larval developmental times; space can be similarly restrictive. It is not clear whether these effects are attributable to chemical interference among larvae competing for food or space, or physical interference. Ikeshoji and Mulla (1974) identified toxic and growth-retarding compounds isolated from water holding a high density of *C. p. quinquefasciatus* larvae.

HEMOLYMPH AND SERUM

The blood or hemolymph of insects is the only extracellular liquid circulating throughout the insect body bathing the internal organs. The hemolymph is part of an open circulatory system and flows through closed ducts for only a short part of its course. Its chief progress is through the body tissues within a system of cavities, such that the blood is in direct contact with tissues. The body cavity with this open circulatory system is known as the hemocoel. Blood is composed of liquid, plasma, and various types of circulating cells. The plasma contains many substances, including dissolved salts, amino acids, proteins, carbohydrates, uric acid, lipids, fatty acids, organic metabolites and organic phosphates (Ross *et al.* 1982).

The blood of insects has five functions. The first is transportation of nutrients and waste products. Digested food materials are absorbed by the blood from the digestive system and conveyed to the tissues, and waste products are carried from the tissues to the excretory system. In addition, certain hormones are transported from their sites of secretion to their sites of activity. Respiration is the second function. Some insect cells are not provided with tracheoles for direct respiratory exchange. These cells presumably obtain their oxygen from the dissolved store in the blood. The third function of insect blood is protection. Hemocytes dispose of a variety of bacteria and parasites. The healing of wounds is effected by the blood or its hemocytes. The fourth function is metabolism. Blood serves as a medium for ongoing metabolic reactions. Biochemical materials carried in the blood are converted to other substances as the blood circulates in the insect body. For example, the carbohydrate trehalose is converted to glucose. The last function of blood is mechanical. Blood acts as a hydraulic pressure system, which is important in the movement of soft-bodied larvae and in the expansion of the insect body after molting (Ross *et al.* 1982).

Among insects, most free energy is obtained by the oxidation of carbohydrates (Ross *et al.* 1982). The store of carbohydrates is the ultimate source of energy for insects during flight, metamorphosis, and periods of starvation. Trehalose is converted by the enzyme trehalase to glucose as energy is needed by the insect. The adaptive advantage of insect blood carrying trehalose rather than the usual animal sugar, glucose, may be that it is a relatively unreactive molecule and, therefore, can be stored in high concentrations in the

hemolymph without posssible osmotic consequences or side reactions with amino acids, which also are stored in high concentrations in insect blood. Thompson and Lee (1992) have shown that starved or crowded *Manduca sexta* (L.) larvae had lower concentrations of serum trehalose and D-glucose than normal larvae. Similarly, Gordon *et al.* (1978) showed that mermithid parasitism significantly affects the level of trehalose in the hemolymph of black fly larvae. The major neutral lipids in *Ae. aegypti* are triacylglycerol, monoacylglycerols, tripalmitin, phosphatidyl ethanolamine and phosphatidyl inositol (Ford and Van Heusden 1994). Lipids are broken down into fatty acids and glycerol, providing energy reserves during starvation and sustained insect flight (Ross *et al.* 1982). Larval hemolymph proteins have been studied in several insects (Kanost *et al.* 1990). The best-studied storage-protein class is known as the arylphorins (Telfer and Kunkel 1991). The major hemolymph protein of *Ae. aegypti* larvae was characterized and designated P1. It is hexameric and is composed of subunits with molecular weights estimated to be 83kDa (Jarrouge *et al.* 1997). This protein accumulates during the last larval instar and is not detected in adult mosquitoes. Furthermore, from analysis of the amino acid composition of P1 there was a high content of aromatic amino acids. The composition of hemolymph provides a window on the various metabolic processes occurring in the body of the insect and is an indicator of its general nutritional status. The serum composition of aquatic insect larvae has been reported to change in response to infection with digeneans and mermithid nematodes (Chamber *et al.* 1975, Schmidt and Platzer 1980).

BIOLOGICAL CONTROL OF MOSQUITOES

Insects are responsible for transmitting a wide variety of pathogenic organisms to humans. Attempts to control transmission through the large scale application of chemical insecticides have resulted in resistance target species and environmental pollution with concomitant intoxication of human and other non-target species (Pimentel *et al.* 1980). The development of insecticide resistance by vector insects, the cost of developing and registering new insecticidal compounds, and the increase in legislation to combat the detrimental effects of insecticidal residues on the environment, are emphasis that we need to assess alternative strategies for vector control. New approaches to biological control are required in order to suppress vector populations. This is particularly true in tropical countries where insects transmit the organisms which cause diseases such as malaria, filariasis, leishmaniasis, river blindness, yellow fever and various other viral diseases which respresent the most important health care problems.

Classical biological control relies on agents which establish and cycle through the target species and maintain it at acceptable levels. Several methods of biological control of interest in targeting vectors include larvivorous fish (Bay 1967), invertebrate predators (Bennet and Hu 1951), parasitic nematodes (Petersen *et al.* 1969), parasitic protozoans (Jenkins 1964), parasitic fungi (Couch 1972), and pathogenic bacteria (Heimpel 1967, Pimentel *et al.* 1980).

In recent years, we have explored the use of entomopathogenic digeneans as agents in the biological control of mosquitoes. *Plagiorchis elegans* in *Ae. aegypti* has been used as

a convenient model system. Entomopathogenic digeneans have complex life cycles and long generation times when compared with the mosquito host. Such discrepancies are generally considered to be detrimental to the effective functioning of an organism as a biological control agent: the organism cannot respond numerically to the inevitable rebound of the mosquito population which follows its initial crash following effective treatment. However, due to the sustained asexual production of cercariae in the molluscan intermediate host, large numbers of cercariae are produced daily, whether or not the target species is present, and recycling within the target population is not required for extended periods of time.

Cercariae of the genus *Plagiorchis* can kill mosquito larvae (Dempster and Rau 1990), inhibit pupation (Dempster and Rau 1991), and sublethal infections can reduce the fecundity and longevity of surviving adults (Kimoro 1990). They can also inhibit oviposition by conspecific, gravid females (Lowenberger and Rau 1994).

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CONNECTING STATEMENT 1

Chapter 1 is a review of various aspects of mosquito biology that form the conceptual framework of the present study. Central to my dissertation is the twin phenomenon of oviposition attraction and repellency, and how various stressors such as starvation, crowding and parasitic infection may influence their expression. In subsequent chapters, I will deal with these questions, and as permitted by the McGill Faculty of Graduate Studies and Research, each chapter is in the form of a manuscript, either in press or submitted for publication.

Oviposition attraction of gravid females by waters from normal, conspecific pre-imagoes has long been recognized as an important mechanism whereby mosquito females may maximize the return on their reproductive investment. Oviposition repellency, in contrast, was only discovered recently as one of the sequelae of sublethal infections with the entomopathogenic digenean *P. elegans*. It would appear that the semiochemical message varies with the quality of the larval environment. Since the direct lethal effects of this parasite are influenced by the size of the host, and the intensity and site of the infection, it raises the question whether these factors also influence the intensity of the oviposition repellent effect. This question is addressed in Chapter 2.

CHAPTER 2

INTENSITY AND SITE OF *PLAGIORCHIS ELEGANS* (TREMATODA: PLAGIORCHIIDAE) INFECTIONS IN *AEDES AEGYPTI* (DIPTERA: CULICIDAE) LARVAE AFFECT THE ATTRACTIVENESS OF THEIR WATERS TO OVIPOSITING, CONSPECIFIC FEMALES

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ABSTRACT A series of biological assays was conducted in the laboratory to assess the oviposition responses of *Aedes aegypti* (L.) to waters that had harbored conspecific larvae parasitized with the entomopathogenic digenean, *Plagiorchis elegans* (Rudolphi). Infections were of various intensities and locations within the bodies of 2nd, 3rd, and 4th instars. Regardless of instar and location of infection, repellency of the waters to ovipositing females increased with the intensity of infections of the larvae. Similarly, waters derived from larvae with infections of the head and thorax tended to be more repellent than waters from larvae with abdominal infections, regardless of instar and intensity of infection. Oviposition repellency was greatest in response to waters from 2nd and 4th instars; the mean numbers of eggs laid on these waters was 1/2 that on distilled water controls. Sublethal infections of *Ae. aegypti* larvae with *P. elegans* may reduce the recruitment of 1st instars into a potentially hazardous environment.

KEY WORDS *Plagiorchis elegans, Aedes aegypti,* Oviposition

INTRODUCTION

Entomopathogenic digeneans of the genus *Plagiorchis* have been used as a convenient model system to assess the suitability of these parasites for biological control of mosquitoes (Rau *et al.* 1991). Asexual production of the parasite within the molluscan intermediate host provides sustained, daily inundatory releases of the effector organisms, the cercariae (Blankespoor 1974). *Plagiorchis elegans* (Rudolphi) not only kills *Aedes aegypti* (L.) larvae, but sublethal infections render larval waters repellent to conspecific, ovipositing females (Lowenberger and Rau 1994). This repellency of waters from infected larvae is of particular significance because waters from normal larvae act as potent oviposition attractants (Soman and Reuben 1970).

In an earlier study on the effects of *P. elegans* infections on the production of the repellent compound(s) Lowenberger and Rau (1994) examined only the collective response to *Ae. aegypti* larvae consisting primarily of 4th instars. Most, but not all, of these larvae were infected. Furthermore, their infections were of various intensities and located in various parts of their bodies (e.g., head, thorax, and abdomen). All of the above factors: the size of the larvae, the intensity of infection with such digeneans, as well as infection site have been shown to influence the development and survival of *Ae. aegypti* preimagos (Dempster *et al.* 1986). The objective of the current study is to determines whether these factors have a similar effect on oviposition repellency.

MATERIALS AND METHODS

A colony of adult *Ae. aegypti* was maintained under a photoperiod of 14:10 (L:D) h at 27 ± 2 °C. Adults were provided with a 10% sucrose solution ad libitum, and females were blood-fed every 48 h on newly born rat pups. Larvae were fed finely ground Tetramin ® fish food ad libitum. A colony of aquatic snails, *Stagnicola elodes* (Say), infected with *P. elegans* was maintained at 20 ± 4 °C under a photoperiod of 16:8 (L:D) h and were fed fresh lettuce ad libitum. Because *P. elegans* cercariae emerge from the snail host at dusk, infected snails were placed in 20 ml of aerated tap water just before the scotophase and allowed to shed cercariae normally. A pool of cercariae from 10 snails was used at the peak of their infectivity, ≈ 8 h after emergence, to induce experimental infections (Lowenberger and Rau 1993).

Groups of 100 2nd, 3rd, and 4th instar larve of *Ae. aegypti* were exposed to ≈ 1,000 cercariae of *P. elegans* for 30 min in 50 ml of aerated tap water. After exposure, mosquito larvae were removed, washed, and maintained individually for 48 h in tissue culture wells containing 2 ml of distilled water. Larvae were then removed, lightly crushed under a coverslip and examined for metacercariae under a compound microscope (40X). The location of the infection (head, thorax, or abdomen) and the number of metacercariae at each site were recorded for each larva. Individual waters were sterilized by filtration (0.2 μm) and stored at 4 °C until used.

Waters derived from infected larvae of the three instars were tested for repellency or attraction and compared with distilled water controls. Distilled water provided a

convenient point of comparison because all larvae had been incubated previously in this medium. Waters from larvae with intensities of infections ranging from 1 to 5 metacercariae distributed among 7 sites or combination of sites (head, thorax, abdomen, head and thorax, head and abdomen, thorax and abdomen, head and thorax and abdomen) were tested. Waters from each instar were tested separately. Thus, 35 two-ml samples, all from infected larvae, as well as 7 distilled water controls were arranged randomly on tissue culture plates, each well separated from the next by a distance of 2.20 cm. The surface area of water presented to ovipositing females in such wells was 2.52 cm^2. Wells of this size containing distilled water attracted disproportionately large number of eggs when compared with wells >10 times the surface area. Each well array was tested in a mosquito flight cage (30 by 40 by 55 cm) containing 100 ovipositing adult females and an approximately equal number of males at 27 ± 2 °C under a photoperiod of 14:10 (L:D) h. A 10% saturated sucrose solution was provided ad libitum. Three replicate arrays were presented to fresh populations of ovipositing females for a period of 26 h, providing 8 h of light before and 8 h after the 10 h scotophase.

Statistical analysis. The numbers of eggs laid in each well were determined. To allow comparison between larval instars, numbers were expressed as a percentage of the highest egg count within each instar group, and arcsine transformed before statistical analysis. This reduced the impact of possible differences in the numbers of eggs laid among instar groups. The effects of host instar, infection intensity and site of

infection on oviposition by conspecific females were assessed by general linear model and analysis of variance (ANOVA) of SAS Institute (1994). The least significant difference procedure was used to determine significant differences between individual means.

RESULTS

The numbers of eggs laid by gravid *Ae. aegypti* females on waters from conspecific larvae harboring metacercariae of *P. elegans* varied with the intensity of infection, the location of the parasite in the body of the host, and larval instar ($P < 0.01$). Because interaction among treatments was uniformly nonsignificant ($P = 0.999$), comparisons were restricted to main effects.

As the intensity of infection of larvae rose from 1 to 5 metacercariae, their respective waters attracted progressively fewer eggs (Table. 1). Thus, waters from larvae of all 3 instars bearing 1 metacercaria of *P. elegans* attracted 70.63 ± 3.58 of eggs, which is not significantly different from the proportion laid on the distilled water controls (77.08 ± 1.03). The proportion of eggs laid decreased significantly with each increase in the number of metacercariae harbored from 1 to 4 and was indistinguishable thereafter. The lowest proportion of eggs was laid on waters from larvae harboring 5 metacercariae of *P. elegans* (38.76 ± 1.96). Waters from larvae infected with >1 metacercaria invariably attracted a significantly lower proportion of

eggs than the distilled water controls. The relationship between infection intensity and number of eggs laid was linear: $Y = 17.248\text{-}0.657x$; $R^2 = 0.797$.

The site of infection within the body of the larvae significantly influenced the mean proportion of eggs laid on their waters ($P < 0.05$) (Table. 2). Waters from larvae with purely abdominal infections attracted significantly more eggs from conspecific females (63.08 ± 2.58) than did waters from larvae with only head infections (56.85 ± 1.16). Waters from larvae with thoracic infections were intermediate in terms of the percentage of eggs attracted. Infections involving >1 body region of the hosts rendered their waters progressively less attractive to ovipositing conspecifics. Thus, waters from larvae with mixed thoracic, and abdominal infections attracted a significantly larger proportion of eggs than mixed head, thoracic and abdominal infections (56.23 ± 2.18 and 45.63 ± 6.59, respectively). Waters from larvae with mixed infections that included a head component attracted few eggs. Regardless of the site of infection, waters from larvae harboring metacercariae invariably yielded a significantly lower proportion of eggs than did the distilled water controls (77.08 ± 1.03).

The instar of infected *Ae. aegypti* larvae significantly influenced the proportion of eggs laid by females on their waters ($P < 0.05$) (Table. 3). Females deposited a higher proportion of eggs on waters from 3rd instars (59.41 ± 8.05) than on waters from 2nd and 4th instars (51.94 ± 8.22 and 52.06 ± 6.30, respectively). Regardless of instar, waters from infected larvae received fewer eggs than the distilled water controls (77.08 ± 1.03).

DISCUSSION

Sublethal infection of *Ae. aegypti* larvae with *P. elegans* fundamentally alters their chemical message to conspecific females. Whereas waters from uninfected larvae are attractive to ovipositing females (Soman and Reuben 1970, Roberts and Hsi 1977), infection renders waters strongly repellent (Lowenberger and Rau 1994). This effect is governed by the intensity of infection, the location of metacercariae within the body of the larvae, and the instar of the host. Waters from normal, uninfected mosquito larvae are invariably more attractive to ovipositing females than are distilled water controls (Lowenberger and Rau 1994). Infections of larvae with only 1 metacercaria completely nullified this attraction, rendering the response of ovipositing females to such waters indistinguishable from that to distilled water controls. Infections of larvae with >1 metacercaria rendered waters significantly more repellent. Degree of repellency increased linearly as the intensity of infection increased. Each metacercaria contributed equally to the combined effect (Table. 1).

Penetrating cercariae and encysted metacercariae of *Plagiorchis* damage the host. The cercariae broach the integrity of the insect cuticle by means of histolytic enzymes and a spear-like boring apparatus (Bock 1989), whereas the metacercariae are lodged in host tissue where they absorb nutrients, metabolize and stimulate host defense mechanisms (Lowenberger *et al.* 1994). The manifestations of pathology in this host/parasite association are varied. Massive infections rapidly kill larvae (Dempster and Rau 1990), whereas lesser infections delay development (Dempster *et al.* 1986),

modify the behavior of the host (Webber *et al.* 1987a), and increase its susceptibility to predation (Webber *et al.* 1987b). Infections of the head, particularly in combination with infection of other body regions, induced the most pronounced repellent effects. This may reflect the relatively small size of this site and the concentration of neurological tissues. Cercarial migration and eventual encystment within such sensitive tissues may interfere with the normal functions of the mosquito larvae more severely than infection of the thorax or abdomen.

Host instar also appeared to influence the impact of *P. elegans* infection on the attractiveness / repellency of waters to ovipositing females. However, because the 3 instars were tested separately, the results of the statistical analysis must be interpreted with some caution. Nevertheless, infections of 2nd instar affected the number of eggs laid in their waters more severely than infection of 3rd instars. As larval size and development increased, the impact of infection declined. Conceivably, increased body mass may be of advantage in coping with the pathogenic consequences of infection. Although significantly larger than 3rd instars, 4th instars were more sensitive to the effects of infection. This may conceivably be an artifact of their experimental environment in that all infected larvae, regardless of size, were maintained in only 2 ml of distilled water. This volume may have been adequate for 2nd and 3rd instars, but inadequate for the much larger 4th instars. Maire (1985) and Benzon and Apperson (1988) reported that the crowding of larvae reduces the inherent attractiveness of their waters to ovipositing females. It is conceivable that the relatively small volume of

water used in our studies may have had effects akin to crowding, because the large 4th instars may have been stressed by the frequent contact with the walls of their relatively small container, by a buildup of waste products, or by both. These stresses may have added to the effects of *P. elegans* infection and therefore enhanced repellency.

The effects of infection intensity, site of infection, and host instar on the induction of oviposition repellency find a close parallel in the ability of these same factors to induce mortality. Thus, Dempster and Rau (1990) found that infected 1st and 2nd instars suffer the greatest mortality. Third and 4th instars are somewhat robust and can tolerate higher intensities of infection. Furthermore, infection of the head and thorax tend to cause greater mortality than abdominal infection. As the pathological effects of infection mount and approach the physiological limits of the larvae, there is a concomitant increase in the repellency of their waters to ovipositing conspecifics.

The presence of sublethal infections with the entomopathogenic digenean *P. elegans* in populations of *Ae. aegypti* larvae may induce gravid conspecific females to oviposit elsewhere. If alternate, suitable larval habitats are not readily available, eggs may be deposited into habitats suboptimal for larval development. This may reduce the recruitment of 1st instars into preimago populations of the insect and reduce adult emergence.

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Table 1. Mean proportion of eggs laid by *Aedes aegypti* on waters from conspecific larvae infected with 1-5 metacercariae of *Plagiorchis elegans*.

No. metacercariae	Mean % eggs laid ± SEM and range
1	70.63 ± 3.58 a (27)
	66.34-75.02
2	59.17 ± 2.95 b (54)
	56.34-63.18
3	46.26 ± 2.49 c (60)
	43.87-49.63
4	42.13 ± 2.25 d (48)
	39.85-44.94
5	38.78 ± 1.96 d (30)
	36.25-41.51
Distilled water control	77.08 ± 1.03 a (63)
	76.21-78.52

Means (arcsine transformed) followed by the same letter are not significantly different at the 0.05 level (LSD test). Numbers in parentheses represent number of waters tested.

Table 2. Mean proportion of eggs laid by *Aedes aegypti* in waters from conspecific larvae infected with metacercariae (1-5 per 2nd, 3rd, and 4th instar larvae) of *Plagiorchis elegans* in various parts of their bodies.

Site of infection	Mean % eggs laid ± SEM and range
Abdomen (A)	63.08 ± 2.58 b (48) 60.50-66.01
Thorax (T)	59.87 ± 2.65 bc (39) 57.78-63.40
Head (H)	56.85 ± 1.16 cd (33) 55.50-58.23
T and A	56.23 ± 2.18 de (42) 54.14-58.39
H and A	49.89 ± 4.81 e (39) 43.86-55.21
H and T	49.60 ± 4.64 ef (45) 44.02-55.23
H and T and A	45.63 ± 6.59 f (36) 39.21-55.26
Distilled water control	77.08 ± 1.03 a (63) 76.21-78.52

Means (arcsine transformed) followed by the same letter are not significantly different at the 0.05 level (LSD test). Numbers in parentheses represent the number of waters tested.

Table 3. Mean number of eggs laid by *Aedes aegypti* on waters from conspecific second, third, and fourth instars infected with 1-5 metacercariae of *Plagiorchis elegans*.

Larval instar	Mean % eggs laid ± SEM and range
Second	51.94 ± 8.22 c (90)
	42.38-63.01
Third	59.41 ± 8.05 b (96)
	49.16-68.90
Fourth	52.06 ± 6.30 c (96)
	44.43-59.82
Distilled water control	77.08 ± 1.03 a (63)
	76.21-78.52

Means (arcsine transformed) followed by the same letter are not significantly different at the 0.05 level (LSD test). Numbers in parentheses represent the number of waters tested.

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CONNECTING STATEMENT 2

In Chapter 2 evidence was presented that the severity of the infection with *Plagiorchis elegans* in terms of intensity and location in the body of the host is reflected in the level of oviposition attraction / repellency. As the pathological effects of infection mount and approach the physiological limits of each instar, there is a concomitant decrease in oviposition attraction and an increase in repellency. The chemical message changes with the status of health the larvae and may provide a general index of the suitability of the environment for larval growth and development. Thus, ovipositing females would avoid habitats harboring pathogens, such as *P. elegans*, which reduce the probability of survival their offspring. This raises the question whether other environmental factors crucial for larval of growth and development, such as the availability of food, are reflected in the oviposition attraction / repellency response as well. In Chapter 3, I address this question by assessing the effects of progressive starvation of *Aedes aegypti* larvae on the oviposition responses of gravid conspecific females to their waters.

CHAPTER 3

STARVED LARVAE OF *AEDES AEGYPTI* (DIPTERA: CULICIDAE) RENDER WATERS UNATTRACTIVE TO OVIPOSITING CONSPECIFIC FEMALES

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ABSTRACT Gravid, laboratory-reared females of *Aedes aegypti* (L.) were allowed free choice of oviposition sites among a full array of test waters derived from fed larvae, from larvae starved for varying lengths of time, and distilled water. Females consistently preferred to oviposit on waters from fed larvae rather than on any other medium (407.8 ± 22.0 [mean ± SE] eggs). Waters from larvae starved for 3 or 5 d were progressively less attractive and, in some cases, repellent. After 7 d of starvation, the attractiveness of 3rd instar waters (256.8 ± 3.9 eggs) was indistinguishable from that of distilled water controls (230.5 ± 20.5 eggs). Corresponding 2nd and 4th instars rendered waters repellent to ovipositing females (156.8 ± 8.6 and 159.5 ± 16.5 eggs, respectively). The significance of these findings are discussed in light of oviposition repellence elicited by other means.

KEY WORDS *Aedes aegypti*, Conspecific, Starvation, Oviposition Preference, Attraction / Repellency

INTRODUCTION

The reproductive success of mosquitoes depends, in part, on the ability of gravid females to locate and select oviposition sites that will support the growth, development, and survival of their offspring. Thus, ovipositing females of *Aedes aegypti* (L.) are attracted by the presence of some species of bacteria and bacterial metabolites in the aquatic environment (Hasselschwert and Rockett 1988). Such bacteria may serve as a source of food for their progeny. Perhaps an even more reliable indicator of environmental suitability is the presence of developing, conspecific preimagos. Larvae and pupae of *Ae. aegypti* are reported to aid conspecific females in the selection of suitable oviposition sites by producing an attractant pheromone, thereby enhancing recruitment of eggs into previously colonized aquatic environments (Soman and Reuben 1970). Similar types of chemical communication have been reported from *Culex tarsalis* Coquillett (Hudson and McLintock 1967), *Anopheles stephensi* Liston (Reisen and Siddiqui 1978), *Aedes communis* deGeer (Maire and Langis 1985), and from both normal (Maire 1984, Kalpage and Brust 1973) and axenically reared *Aedes atropalpus* Coquillett larvae (Maire 1985). Similarly, *Aedes togoi* Theobald females preferred bacteria-free waters from kaolin-treated conspecific larvae (Trimble and Wellington 1980).

Aedes aegypti larvae also may be able to communicate adverse environmental conditions to ovipositing conspecifics. Thus, infection of larvae with metacercariae of the entomopathogenic digenean, *Plagiorchis elegans* Rudolphi, profoundly alters the

chemical message. Waters from infected larvae repel gravid females (Lowenberger and Rau 1994). Although gravid females landed on waters from infected larvae and distilled water controls with equal frequency, their stay on the former was severely curtailed (Lowenberger and Rau 1994). The repellent effect is of a type that acts only over short distances as described by Foster and Harris (1997). During the course of their development, mosquito larvae may encounter not only pathogens, but a wide variety of other adverse factors. These may include predators, competitors, and toxins, all of which may influence their chances of survival. It follows that any mechanisms that can transmit information regarding the quality of the environment to ovipositing conspecifics would be of adaptive significance. Thus, larvae encountering a nutritionally suboptimal environment may be able to transmit this information to ovipositing conspecifics and render the site less attractive. In the current study, the influence of varying degrees of food deprivation of 2nd, 3rd and 4th instars of *Ae. aegypti* on the character of the chemical message received by ovipositing, conspecific females is examined.

MATERIALS AND METHODS

Larvae were reared in the laboratory on a diet of finely ground Tetramin ® fish food at a concentration of 0.25g of dry powder per liter of distilled water. Adults (≈ 100 females and 100 males) were kept in mesh cages (80 x 40 x 40 cm) at 27 ± 3 °C, 80% RH, and a photoperiod of 16:8 (L:D) h. Four days after emergence, females were

blood fed daily for 1 h on newly born rat pups. Cotton wool pads saturated with 10% sucrose solution were provided as an alternative food source. Oviposition assays began four day after the first blood feeding.

Larva-holding waters were prepared using 2nd, 3rd and 4th instars. Larvae were starved for a maximum period of 7 d because preliminary experiments 2nd instars starved longer had mortality rates of almost 80%. Starved 3rd and 4th instars manifested only 10% mortality over corresponding time frames. First instars were even less tolerant of starvation than 2nd instars and, therefore, were not used.

Larvae about to moult were removed from their rearing containers by pipette and allowed to moult in the absence of food. Newly moulted larvae were then rinsed 3 times in distilled water and transferred to flat-bottomed tissue culture wells (1.70 cm diameter, 1.60 cm deep; Linbro, Flow Laboratories, Virginia, USA) filled to within 3 mm of the top with 2.2 ml of distilled water. Individual larvae were incubated at 27 ± 2 °C for varying lengths of time. To prepare waters from fed larvae, individuals of each instar were maintained without food for 12 h, transferred to a well with food (Tetramin fish food at concentration of 0.04g/10 ml distilled water) for 1 h, then rinsed as described above and returned to their respective wells for an additional 11 h without further access to food. To obtain waters from starved larvae, individuals were maintained in 2.2 ml of distilled water for 3, 5, or 7 d without access to food. At 24-h intervals, larvae were removed, rinsed, and transferred to wells containing fresh distilled water. Only waters from the last day of each series were collected for

bioassays, and all were collected at the same time to avoid possible effects of differential storage duration. Waters were sterilized by filtration (Millipore, 0.2 μm), stored in sealed containers at 4 °C and bioassayed within 24 h for attractiveness or repellency to ovipositing conspecific females. The bioassays were carried out in cages (75 x 39 x 32 cm) containing 100 male and 100 female mosquitoes at 27 ± 2°C under a photoperiod of 14:10 (L:D) h, with a 10% sucrose solution available ad libitum. Mosquitoes were blood fed daily for 4 consecutive days before they were given the opportunity to oviposit. The oviposition wells (1.70 cm diameter) were filled with 2.2 ml of the samples. Two samples each of waters from individual 2nd, 3rd and 4th instars starved for 0, 3, 5, or 7 d, as well as 6 distilled water controls, were arranged at random among 30 oviposition wells, each separated from the next by a distance of 3.20 cm. Ovipositing *Ae. aegypti* females preferred the 1.7-cm-diameter wells over larger containers (4 cm diameter); densities of eggs in the small wells reached 4 times those in the larger containers. The above 30-sample test arrays were presented to ovipositing females for 26 h, providing 10 h of light before and 8 h after the 8-h scotophase. Four replicate arrays were presented to fresh populations of ovipositing females on 4 consecutive days. The numbers of eggs in each well was counted; the number of eggs in duplicate wells were summed. Total means were calculated for the 4 replicates.

Statistical analysis. The experimental design was factorial with four replicates. The factors were 3 levels of larval development (i.e., 2nd, 3rd or 4th instars) and 4

levels of starvation (i.e., fed, starved 3 d, starved 5 d, and starved 7 d), plus distilled water controls. The data were analyzed using the SAS package GLM procedure (SAS Institute 1994, Steel and Torrie 1980). The number of eggs was square root-transformed before statistical analysis to satisfy the assumptions of analysis of variance (ANOVA). Differences between means were determined using an approximation of the 95% confidence interval (± 2 standard errors).

RESULTS

Larval instar, the duration of starvation, and the interaction of these two factors significantly influenced the distribution of eggs deposited by gravid female *Ae. aegypti* in the test array (Table. 1). Waters from fed larvae tended to attract more eggs than waters from starved larvae or the distilled water control (Fig. 1). Thus, fed 3rd instars attracted 476.8 ± 15.8 (mean ± SE) eggs. Starvation for 3 or 5 d significantly reduced the number of eggs deposited (314.0 ± 17.2 and 300.3 ± 10.8 eggs, respectively), but not to the level of the corresponding distilled water controls (230.5 ± 20.5 eggs). Only after 7 d of starvation did the attractiveness of 3rd-instar waters become statistically indistinguishable from that of the distilled water controls (256.8 ± 3.9 and 230.5 ± 20.5 eggs, respectively).

Although waters from 4th instars starved for 3 or 5 d attracted fewer eggs than waters from corresponding fed individuals (312.3 ± 15.0, 278.0 ± 4.7, and 401.5 ± 7.5 eggs, respectively), they nevertheless attracted more eggs than did their corresponding

distilled water controls (208.5 ± 8.9). After 7 d of starvation, however, 4th instars yielded waters that attracted even fewer eggs than did these same control waters (159.5 ± 16.5 and 208.5 ± 8.9 eggs, respectively).

After 3 d of starvation, waters from 2nd instars attracted as many eggs as did corresponding 3rd and 4th instars (267.8 ± 15.7, 314.0 ± 17.2, and 312.3 ± 15.0 eggs, respectively). Starvation of the larvae for 3 d rendered their waters significantly less attractive than those of fed larvae (267.8 ± 15.7 and 345.3 ± 13.4 eggs, respectively) and no more attractive than waters from the corresponding distilled water controls (230.0 ± 9.0 eggs). Five or 7 d of starvation of 2nd instars yielded waters that were significantly less attractive than the distilled water controls (176.0 ± 5.1, 156.8 ± 8.6, and 230.0 ± 9.0 eggs, respectively). Starvation of 2nd and 4th instars for 7 d yielded waters that were significantly more repellent than waters from corresponding 3rd instars. There were no significant differences among the numbers of eggs laid on the distilled water controls (230.0 ± 9.0, 230.5 ± 20.5, and 208.5 ± 8.9 eggs, respectively).

DISCUSSION

As the duration of starvation increased, the attractiveness of larval waters to ovipositing conspecific females declined (Fig. 1). Waters from fed *Ae. aegypti* larvae were invariably attractive to ovipositing conspecific females when compared with distilled water. This supports the findings of Maire (1984) and Soman and Reuben (1970). Hasselschwert and Rockett (1988) noted that the presence of bacteria,

particularly *Bacillus cereus*, serves as a strong ovipositional attractant for *Ae. aegypti*, as do the feces of larvae. Because both bacteria and feces were removed by filtration (0.2 μm), the attraction may be attributable to substances produced by the larvae themselves, although an effect of soluble components of feces or bacterial metabolites (or both) cannot be ruled out. Third-instar waters were more attractive than waters from 2nd instars (Fig. 1; Table. 1). We suggest that the magnitude of the attractant effect may be a function of larval mass and associated metabolic activity. As the larvae molt and grow, the attractiveness of their waters to ovipositing conspecific females may increase. The fact that waters from 4th instars appeared to be less attractive than those from 3rd instars may be a reflection of our experimental conditions; we maintained larvae in relatively small volumes of water (2.2 ml). Fed larvae grow rapidly (Christophers 1960, Brust 1968), and whereas 2.2 ml of water may be an adequate volume for 2nd and 3rd instars, this may not necessarily be the case for 4th instars. Even when food is abundant, a small volume of water per larva, as exists under conditions of crowding, will reduce attractiveness (Maire 1985, Benzon and Apperson 1988). Relatively small volumes of water in the absence of other larvae may have effects similar to crowding.

Starvation of larvae for as few as 3 d invariably reduced the attractiveness of their waters. The severity of this effect increased with the duration of starvation. Within the 7-d time frame of this study, starvation reduced the attractiveness of waters from 2nd and 4th instars to levels below those of the distilled water controls. Seven days of

starvation approached the physical limits of 2nd instars. In contrast, 7 d of starvation of 3rd instars did not reduce the attractiveness of their waters below that of the distilled water controls. It should be noted that the repellency expressed by 4th instars may be a function of both starvation and crowding.

Whereas it required 7 d of starvation in the current study to render the waters of larvae repellent to ovipositing conspecifics, this was achieved in only 2 d by an infection with the entomopathogenic digenean *P. elegans* (Lowenberger and Rau 1994). It is conceivable that a wide variety of adverse environmental conditions, including the presence of pathogens, will induce repellency, but it is not clear whether the same or different messenger substances are involved. We are currently in the process of identifying these messenger substances with a view to eventual synthesis as oviposition repellents that may reduce recruitment into *Ae. aegypti* populations.

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Table 1. ANOVA results for the number of eggs laid by females of *Aedes aegypti* on waters from conspecific larvae of various instars subjected to periods of starvation of various duration.

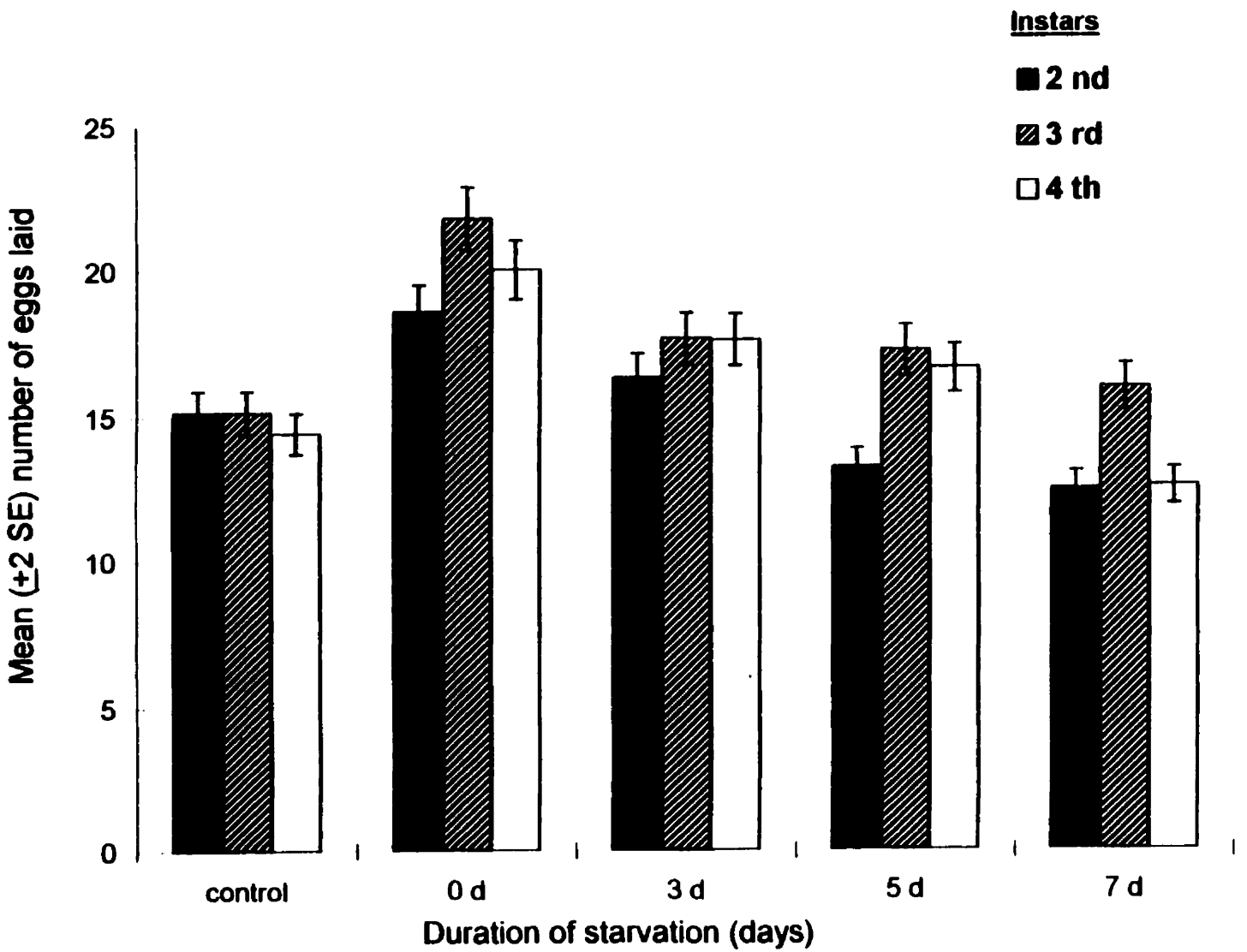
Source of variation [a]	*F*	df	*P*
Instar [b]	22.29	2, 45	0.0001
Starvation time [c]	55.93	4, 45	0.0001
Instar × starvation time	3.62	8, 45	0.0025

[a] The dependent variable was the number of eggs laid by females of *Ae. aegypti*. Data were square root-transformed before analysis.

[b] Second, 3rd, and 4th instars were tested.

[c] Effects tested were full feeding; starvation for 3, 5 or 7 d and distilled water controls.

Fig. 1. Mean number (± 2 SE, or an approximate 95% CI) of eggs laid by females of *Aedes aegypti* on waters from conspecific larvae subjected to periods of starvation of various duration and compared with distilled water controls. Square root-transformed data are graphed.


Instars
2 nd
3 rd
4 th
Mean (±2 SE) number of eggs laid
25
20
15
10
5
0
control
0 d
3 d
5 d
7 d
Duration of starvation (days)

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CONNECTING STATEMENT 3

In Chapter 3, it was shown that starvation of *Aedes aegypti* larvae progressively reduces the attractiveness and increases the repellency of their waters to ovipositing, conspecific females. The twin phenomenon of oviposition attraction / repellency is a response to a wider range of environmental stressors. Chapter 4 deals with the question whether crowding and close confinement of larvae elicit similar oviposition responses as pathogens and starvation, and if so, how the magnitude of the responses compare. The work also provides some insight into the sensitivity and persistence of the oviposition response and the interaction between oviposition repellency and attraction.

CHAPTER 4

OVIPOSITION ATTRACTION AND REPELLENCY OF *AEDES AEGYPTI* (DIPTERA: CULICIDAE) TO WATERS FROM CONSPECIFIC LARVAE SUBJECTED TO CROWDING, CONFINEMENT, STARVATION OR INFECTION

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ABSTRACT Waters from *Aedes aegypti* larvae which had been starved, crowded, confined or infected with the entomopathogenic digenean, *Plagiorchis elegans,* were tested for oviposition attraction and repellency. Oviposition attraction declined and repellency increased as the experimental population density of the larvae rose. The larger the larvae, the greater was their sensitivity to crowding. Similar effects were elicited by confining larvae in small volumes of water. As incubation volumes increased, oviposition repellency declined and attraction rose to a broad peak. Further increases in volume diluted the attractant effect. Again, the larger the larvae the more sensitive they were to confinement. Not only the incubation volume but also the surface diameter and water depth of the container in relation to the size of the larvae influenced the degree of repellency and attractiveness. If these dimensions interfered with the normal behavior or posture of the larvae, there was a decrease in oviposition attractiveness and an increase in repellency. When the repellent effect was titrated against distilled water, infections of the larvae with *P. elegans* generated a significantly more powerful effect than starvation or crowding. Extensive serial double dilution of the repellent did not restore attractiveness. When the repellent was titrated against attractant waters, repellency overrode attraction. Whereas repellency to ovipositing females was affected only to a slight extent by the presence of the attractant in the larval test waters. the attractant was effectively neutralized in the presence of even minute amounts of the repellent. The findings are discussed in the context of possible biological control effects on mosquito populations and their control.

KEY WORDS *Aedes aegypti*, Oviposition, Attraction/Repellency, Crowding/ Confinement, Starvation, Infection

INTRODUCTION

Overcrowding of mosquito larvae generally results in retarded growth and high mortality of the larvae, small and non-uniform size of adults, and decreased fecundity (Terzian and Stahler 1949, Shannon and Putman 1934, Ikeshoji 1965, Moore and Fisher 1969). Larval density also affects the attractiveness of potential oviposition sites to gravid females. Whereas waters from uncrowded *Aedes atropalpus* (Coquillett) larvae attract ovipositing, conspecific females, waters from crowded larvae are unattractive. Maire (1985) suggested that this may be due to excessively high concentrations of the otherwise attractant compound. Similarly, *Culex tritaeniorhynchus* and *Ae. aegypti* (L.) adults prefer waters from uncrowded over waters from crowded conspecific larvae (Reisen and Siddiqui 1978, and Benzon and Apperson 1988 respectively). Zahiri *et al.* (1997) suggested that confinement of *Ae. aegypti* larvae in small volumes of water elicited effects similar to crowding. It was proposed that confinement of this nature may render fourth instars more susceptible to the stress of starvation and therefore enhance the induction of oviposition repellency.

The repellent effect is powerful. A ten-fold dilution of waters rendered repellent to ovipositing conspecific females by infecting larvae with the entomopathogenic digenean, *Plagiorchis elegans* (Rudolphi), does not elicit a return to attractiveness (Lowenberger and Rau 1994). The waters tested by these authors were derived from a population of third and fourth instars exposed to infection. Most, but not all, of these larvae were infected: as a result, the properties of their waters may

represent the combined effects of attraction elicited by a small number of normal larvae and the repellency of a majority of infected individuals.

The objective of this study was to explore the effects of larval population density, confinement and infection on the twin phenomena of oviposition attraction and repellency. Some insight into the biological character of repellencies elicited by means as varied as infection, starvation and crowding is provided.

MATERIALS AND METHODS

A colony of *Ae. aegypti* adults was maintained under a 14 h light and 10 h dark regime at 27 ± 2°C, provided with a 10% saturated sucrose solution ad libitum, and blood-fed twice weekly. Larvae were reared under the above temperature and light conditions in clear plastic containers (13 cm diam.x 13.5 cm), filled to a depth of 7.6 cm with 1 L of distilled water. Larvae were fed finely ground Tetramin (0.25g / 1L) fish food ad libitum.

Crowding. In order to assess the impact of crowding of larvae on the attractiveness and repellency of their waters to ovipositing conspecifics, 0, 1, 2, 3, 4, 5 or 6 second, third or fourth instars were rinsed with distilled water and transferred to tissue culture wells (diameter 1.4 cm, depth 1.7 cm) filled with 2 ml of distilled water and provided with a scant excess of food. Larvae were incubated at 27 ± 2°C and a photoperiod of 14 h light and 10h darkness for 48 h. Larvae were then removed and the waters were filter sterilized (Millipore, 0.2 μm), stored in sterile serum vials at 4°C

and bioassayed for oviposition attraction/repellency within 24h. Four replicate arrays were presented to fresh sets of ovipositing females (Zahiri *et al.* 1997).

In order to assess the influence of larval incubation volumes on the attractiveness and repellency of waters to ovipositing, conspecific females, individual second, third, and fourth instars were rinsed with distilled water and transferred to tissue culture wells (diameter 1.4 cm, depth 1.7 cm) containing 0.5, 1.0, 2.0, or 4.0 ml of distilled water and a scant excess of food. Larvae were incubated for 48 h and waters were pooled according to incubation volume and instar, filter sterilized and stored as above. Four replicate were tested.

Confinement. The influence of confinement of larvae on the attraction and repellency of their waters was assessed by incubating individual fourth instars for 48h in 2 ml of distilled water but varying water depth and surface area (Moderate: diameter 1.40 cm, water depth 1.37 cm; Narrow/deep: diameter 0.58 cm, water depth 7.8 cm; Wide/shallow: diameter 3.0 cm, water depth 0.30 cm). A scant excess of food was provided. Waters were treated as above.

Dilution. Waters from larvae starved for 7 d according to method of Zahiri *et al.* (1997), waters from larvae infected with *P. elegans* according to the method of Lowenberger and Rau (1994), and crowded larvae maintained at a density of three larvae per ml for two days were double diluted serially with distilled water to a final concentration of 1:1024. All three sources of water were repellent. The first two classes of waters were first shown to be repellent to ovipositing conspecifics by Zahiri *et al.* (1997) and Lowenberger and Rau (1994) respectively. The third water proved to

be repellent as determined in the current study. Attractant waters derived from normal larvae were diluted in the same manner and treated as above. In order to assess the interaction between oviposition repellency and attraction, waters from infected, crowded or starved larvae were titrated as above, but against waters from normal larvae rather than distilled water.

Stability. As well, the persistence of oviposition attraction and repellency over a range of environmental temperatures was assessed. Waters from fourth instars were stored in sealed serum vials at 27, 4, and -20°C and tested weekly over a period of 4 weeks for oviposition activity.

Oviposition assays. Various waters or their dilutions were assessed for attraction or repellency to ovipositing conspecific females in mosquito flight cages (80 x 40 x 40 cm) at 27 ± 2°C with a photoperiod of 14 light and 10 dark. Dilute sucrose solution was provided ad libitum. Newly emerged mosquitoes (100 males and 100 females) were introduced into each cage and blood fed daily. Oviposition assays began on day four after the first blood feeding, and eggs were collected after 26 h. Within experiments, each test solution was presented in triplicate to the ovipositing mosquito population in tissue culture wells (diameter 1.9 cm, volume 4.0 ml) filled to a depth of 1.5 cm and placed 4.8 cm apart. The number of eggs laid in triplicate sets of test wells and distilled water standards was summed for each replicate.

Statistical analysis. The relative attractiveness or repellency of test samples was expressed as an oviposition activity index (OAI) calculated according to Kramer and Mulla (1979).

$$OAI = \frac{NT - NS}{NT + NS}$$

NT denotes the number of eggs laid in test wells, and NS the number laid in distilled water control wells. Index values lie within the range from +1 to -1. For positive values, more eggs were deposited in the test wells than in the controls, and therefore test waters were attractive. Conversely, more eggs in the controls than in the test samples yielded a negative OAI, and the test waters were repellent. The data were arcsine transformed prior to statistical analysis by Proc GLM, SAS Institute, (1994). Alpha was set at 0.05.

RESULTS

At a density of one larva per 2 ml of water, the OAI was marginally above neutrality. As density was increased by one larva, the index increased by a factor of 4, only to decline again at larval densities of and 4 per 2 ml of water. At a density of 5 larvae, the OAI was negative, as it was at 6 larvae per 2 ml. Third and fourth instars, at a density of one larva per 2 ml manifested significantly greater OAI's than corresponding second instars. However, increases in larval densities progressively reduced the index until, at a density of 4 larvae per 2 ml the OAI of waters from third instars was negative. The OAI of waters from fourth instars was negative at a density of only 2 larvae per 2 ml of water, and declined progressively as the density increased to 4 and 5 larvae (Fig 1).

A similar pattern was generated when the number of larvae was kept constant and the volume of water was increased progressively from 0.5 ml to 4.0 ml. Thus, second instars manifested a positive OAI at a volume of 0.5 ml. This increased significantly as the volume doubled, but declined as the volume increased to 2.0 ml. A further increase in volume to 4.0 ml neither reduced nor enhanced the OAI. Third instars in 0.5 ml yielded a negative OAI which converted to positive as volume increased to 1.0 ml. Third instars reached a peak OAI in a volume of 2.0 ml and declined as volumes increased further. Fourth instars in 0.5 ml manifested an OAI lower than corresponding third instars which remained negative until a volume of 2.0 ml was reached: thereafter the OAI continued to increase with the volume of incubation (Fig 2).

Oviposition indices were significantly higher in response to waters from larvae incubated in containers of moderate dimensions than to waters from wide/shallow and narrow/deep containers when presented to females in containers of uniform dimensions. Waters from the latter two incubation containers were statistically indistinguishable in terms of their OAI values (Table 1).

When waters from normal larvae were titrated, their positive OAI values were indistinguishable from distilled water standards at a dilution of 1 in 32 parts. Negative OAI waters from starved larvae reach this point at a concentration of 1 part in 32, waters from crowded larvae at 1 part in 64, and waters from parasitized larvae at a concentration of 1 part in 512. Negative OAI values did not convert to positive values as dilution proceeded (Table 2).

Negative OAI waters from parasitized larvae titrated against positive waters from normal larvae manifested a progressive decline in repellency, and converted to positive values at a dilution of 1 part in 512. At this concentration, the OAI was significantly lower than that of undiluted waters from normal larvae. Waters from crowded larvae reached the point of conversion at concentrations of 1 part in 32, whereas waters from starved larvae did so at 1 part in 16. The OAI values increased as the concentration of water from normal larvae rose (Table 3).

Storage, regardless of temperature, brought both positive and negative OAI values progressively closer to neutrality, but the process was retarded at low temperatures. Negative OAI values became neutral more quickly than positive values. Thus, at above freezing temperatures, OAI values remained largely unchanged for approximately two weeks, whereas negative values changed significantly within that period (Figs 3 and 4).

DISCUSSION

The small second instars were close to optimally spaced at densities as high as 2 per 2 ml of water, generating a rise in oviposition attraction as densities climbed to these levels. However, further density increases engendered crowding, which manifested itself in reduced oviposition attraction and eventually in repellency. The somewhat larger third instars induced a significantly higher level of attraction at a density of one larva per 2ml than did second instars at a corresponding density, but this declined as soon as concentrations of larvae rose to 2. It seems that crowding was in

effect, and conditions were no longer adequate for the normal functioning of the insects (Benzon and Apperson 1988; Maire 1985, Reisen and Siddiqui 1978). Repellency was expressed at concentrations of as few as 4 second instars in 2 ml of water and increased subsequently with larval population density. This effect was significantly more pronounced among the larger fourth instars, so that even densities lower than one larva per 2 ml were suboptimal.

Effects akin to those of crowding were also elicited by maintaining a constant number of larvae in containers of graded volumes. Thus, second instars incubated individually in tissue culture wells (diameter 10 mm) and filled with 0.5 ml of distilled water were mildly attractive to ovipositing conspecific females. These larvae did not function normally in such small volumes since incubation in 1 ml enhances oviposition attractant. Further increases in the incubation volume, however, led to a dilution of the chemical message to near neutrality. The somewhat larger third instars in the same volume of distilled water clearly rendered the medium repellent, but as the incubation volume was increased, so was oviposition attraction. This reached its highest level at a volume of approximately 2 ml. Thereafter, as the incubation volume increased, attraction decreased, again probably due to dilution. The largest larvae, fourth instars, appeared to be the most sensitive to confinement in a small volume. One larva in 0.5 ml of water rendered the medium highly repellent. Repellency decreased as the incubation volume increased. Repellency changed to attraction at a volume of approximately 2 ml and reached its highest levels at volumes of 4 ml or above. These findings confirm earlier suggestions that crowding and starvation can act

in concert to reduce the oviposition attractiveness of waters from fourth instars (Zahiri *et al* 1997). Volumes required for eliciting optimal attractive responses increase with larval size. As incubation volumes increased beyond these levels, dilution diminished the effect.

The effects of volume may be direct and may be due to the build-up of metabolic waste products or semiochemicals (Ikeshoji *et al.* 1979, Ikeshoji and Mulla 1970). However, it may also be mediated through enforced changes in the posture and/or behavior of the larvae. Thus, the relative dimensions of the rearing container for larvae (diameter and water depth) influenced the attractiveness/repellency of their waters when presented in containers of uniform dimensions to ovipositing conspecific females. Containers of moderate dimensions allowed larvae to hang suspended from the water surface by their siphons without any other surface contact and to swim in the horizontal and vertical plane. The wide / shallow dimensions forced larvae into a semi-prone posture so that their bodies made partial contact with the bottom of the containers when the larvae were attached by their siphons to the water surface. Swimming movements were restricted to the horizontal plane, in close contact with the bottom. The narrow / deep dimensions forced resting larvae into contact with the sides of the container imposing a more vertical posture and restricting swimming to the vertical plane. Larvae confined vertically or horizontally rendered their waters repellent, whereas their relatively free-swimming conspecific counterparts rendered them attractive to ovipositing females. Enforced contact of larvae with the surfaces of the container appeared to reduce attractiveness and enhance repellency when compared

with unrestricted counterparts. The larger the larvae, the more sensitive they were to relative volumetric changes in their environment, whether this was due to an increase in the larval population or a diminution of volume. Thus, in the field, both recruitment of larvae into the population by means of oviposition or a decline in the volume or dimensions of the habitat due to evaporation or drainage may elicit oviposition repellency. Conversely, population decline or a replenishing of water may trigger the return of oviposition attraction.

When repellent and attractive waters were titrated against distilled water, infection with *P. elegans* generated the most powerful repellent effect, whereas crowding or starvation induced significantly weaker responses. Waters from infected larvae (intensity of infection >3 metacercariae) was significantly less attractive at a dilution as low as 1 in 512 of distilled water, whereas waters from starved and crowded larvae were repellent at a maximum dilution of 1 in 32 and 64 parts of water, respectively. At no time did dilution of the repellent waters restore attractive properties. This refutes the suggestion by Maire (1985) that the observed decline in oviposition attractiveness as larval densities rise is generated by an excess of attractant substance.

Serial double dilution of repellent waters with attractive waters from normal larvae, as would occur if only a subset of the population was affected by factors inducing oviposition repellency, suggests that the effects of dilution followed the same pattern as when distilled water was used. Dilutions as low as 1 in 512 parts of attractant water still manifested significant repellency, whereas waters from starved

and crowded larvae were less potent (1 in 16 and 32 respectively). Even beyond this level of dilution (1 in 1024), the expression of oviposition attraction was diminished. Repellency seemed to override attraction. Whereas repellency was affected only to a slightly extent by the presence of the attractant, the latter was effectively neutralized by even minute amounts of the repellent. In a population of mosquito larvae consisting primarily of fourth instars exposed to cercariae of *P. elegans*, a prevalence of sublethal infections as low as 3% may render the environment strongly repellent to conspecific, ovipositing females, and a 0.2% prevalence may render the waters neutral. Even if not renewed continuously, the repellent effects may persist for more than one week between 4 and 27°C.

The presence of starving or crowded larvae may have similar effects. Although not as strongly repellent individually, most larvae within a particular environment would be similarly affected by such stressors, and the combined effect may be as great as that of limited infection. Waters from starved or crowded larvae are toxic to early instars (Kuno and Moore 1975). It is not known if waters from infected larvae have similar effects. Regardless, avoidance of habitats detrimental to the survival of larvae, whether due to parasitic infection or toxins, would be of selective significance. The deflection of ovipositing females from such sites by introduced infections with entomopathogenic digeneans may augment the direct lethal effects of such control agents and reduce the subsequent production of adults even further (Rau *et al.* 1991). This may lead to new, and perhaps more effective methods to control mosquitoes and the diseases they transmit.

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Table 1. Oviposition activity indices (OAI) of *Aedes aegypti* females on waters from individual 4th instars incubated in containers of different dimensions, all filled with 2 ml of distilled water (distilled water as a standard control).

	Dimensions (cm)			
	Surface diameter	water depth	OAI [1]	
Moderate	1.40	1.37	+ 0.33 ± 0.03 * [2]	a (4) [3]
Narrow/deep	0.58	7.80	- 0.20 ± 0.07 *	b (4)
Wide/shallow	3.0	0.30	- 0.14 ± 0.02 *	b (4)

[1] Positive values = oviposition attraction, negative values = repellency.

[2] Significantly different from distilled water standard at the 0.05 level.

[3] Means followed by the same letter are not significantly different at the 0.05 level of probability based on the LSD test, (number of replicates in parentheses).

Table 2. Oviposition activity indices (OAI) of gravid *Aedes aegypti* in response to repellent waters from crowded or starved larvae and larvae parasitized with *P. elegans*, as well as to attractant waters from normal larvae at various dilutions. [1]

	OAI ± SE [2]			
Dilution titer	parasitized larvae (4) [3]	crowded larvae(4)	starved larvae(3)	normal larvae (3)
1:1	-0.51 ± 0.09 e [4]	-0.41 ± 0.07 d	-0.42 ± 0.06 c	+0.41 ± 0.02 a
1:2	-0.43 ± 0.06 de	-0.44 ± 0.03 d	-0.40 ± 0.10 c	+0.40 ± 0.09 a
1:4	-0.46 ± 0.05 e	-0.32 ± 0.08 c	-0.37 ± 0.09 c	+0.31 ± 0.15 a
1:8	-0.35 ± 0.07 cd	-0.18 ± 0.05 b	-0.33 ± 0.08 c	+0.29 ± 0.07 a
1:16	-0.33 ± 0.07 c	-0.21 ± 0.02 b	-0.20 ± 0.06 b	+0.24 ± 0.10 ab
1:32	-0.30 ± 0.01 c	-0.18 ± 0.01 b	-0.32 ± 0.06 bc	+0.03 ± 0.11 c
1:64	-0.17 ± 0.12 b	-0.20 ± 0.04 b	-0.03 ± 0.04 a	+0.07 ± 0.07 c
1:128	-0.16 ± 0.01 b	-0.02 ± 0.01 a	-0.04 ± 0.12 a	+0.05 ± 0.07 c
1:256	-0.17 ± 0.06 b	-0.07 ± 0.05 a	-0.02 ± 0.05 a	+0.07 ± 0.05 c
1:512	-0.12 ± 0.01 b	-0.07 ± 0.05 a	+0.03 ± 0.05 a	+0.03 ± 0.01 c
1:1024	-0.01 ± 0.01 a	-0.01 ± 0.02 a	-0.04 ± 0.04 a	+0.09 ± 0.11 bc
0	0 a	0 a	0 a	0 c

[1] Larvae were incubated for 2 days following infection with cercariae of *P.* elegans, crowded for 2 d at a density of 3 larvae/ml, or starved for 7 d. Normal larvae were incubated for 2 d. All waters were compared to distilled waters standards.

[2] Positive values = attraction to test solution; negative values = repellency (± one standard error).

[3] Number of replicates in parentheses.

[4] Values in columns followed by the same letter are not significantly different at the 0.05 level of probability based on Duncan's multiple-range test.

Table 3. Oviposition activity indices (OAI) of gravid *Aedes aegypti* in response to repellent waters from crowded or starved larvae and larvae parasitized with *P. elegans* at various dilutions with attractant waters from normal larvae .[1]

	OAI ± SE [2]		
Dilution titer	parasitized larvae (3) [3]	crowded larvae(3)	starved larvae(3)
1:1	-0.54 ± 0.02 h [4]	-0.44 ± 0.02 i	-0.42 ± 0.02 g
1:2	-0.49 ± 0.03 h	-0.43 ± 0.03 i	-0.40 ± 0.04 g
1:4	-0.42 ± 0.02 g	-0.34 ± 0.01 h	-0.35 ± 0.03 f
1:8	-0.31 ± 0.04 g	-0.32 ± 0.03 h	-0.35 ± 0.04 f
1:16	-0.38 ± 0.07 fg	-0.23 ± 0.02 g	-0.20 ± 0.06 e
1:32	-0.33 ± 0.01 f	-0.15 ± 0.02 f	-0.08 ± 0.05 de
1:64	-0.24 ± 0.03 e	-0.04 ± 0.04 ed	+0.01 ± 0.04 d
1:128	-0.20 ± 0.02 e	+0.21 ± 0.05 c	+0.13 ± 0.02 c
1:256	-0.07 ± 0.01 d	+0.27 ± 0.02 b	+0.29 ± 0.05 b
1:512	-0.08 ± 0.03 d	+0.28 ± 0.05 ab	+0.33 ± 0.06 ab
1:1024	+0.13 ± 0.03 b	+0.29 ± 0.04 ab	+0.35 ± 0.03 a
0	+0.35 ± 0.07 a	+0.33 ± 0.05 a	+0.37 ± 0.02 a
DW	0.00 ± 0.00 c	0.00 ± 0.00 d	0.00 ± 0.00 d

[1] Larvae were incubated for 2 days following infection with cercariae of *P. elegans*, crowded for 2 d at a density of 3 larvae / ml, or starved for 7 d. All waters were compared to distilled water standards (DW).

[2] Positive values = attraction to the test solution; negative values = repellency (± one standard error).

[3] Number of replicates in parentheses.

[4] Values in columns followed by the same letter are not significantly different at the 0.05 level of probability based on Duncan's multiple-range test.

Fig. 1. Oviposition activity indices (OAI) of gravid *Aedes aegypti* in response to waters from second, third and fourth instars maintained at various population densities. All waters compared to distilled water standards. Points shown are mean values of four replicates and vertical lines represent standard errors of the means.

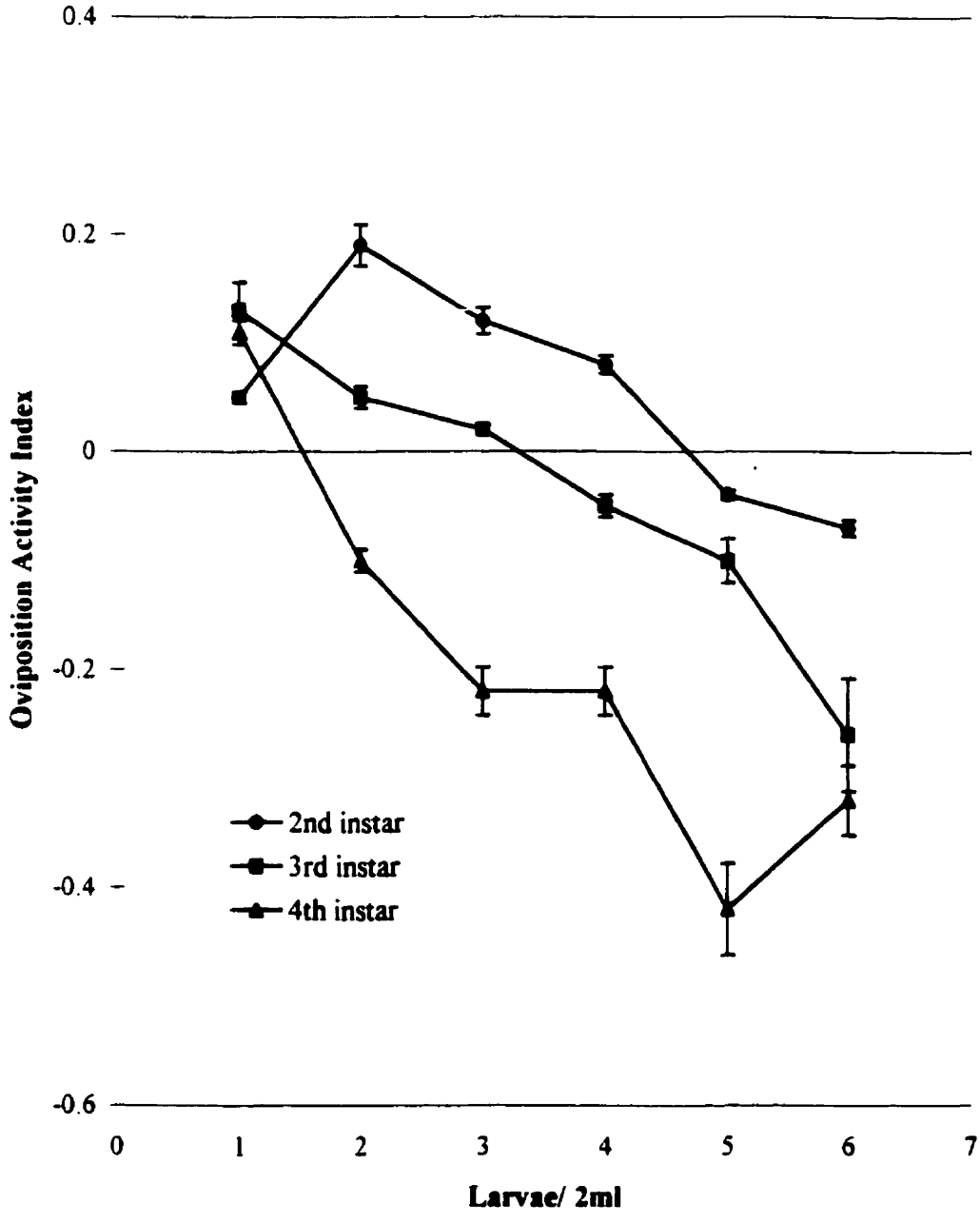

0.4
0.2
0
-0.2
-0.4
-0.6
Oviposition Activity Index
2nd instar
3rd instar
4th instar
0
1
2
3
4
5
6
7
Larvae/ 2ml

Fig. 2. Oviposition activity indices (OAI) of gravid *Aedes aegypti* in response to waters from second, third and fourth instars incubated in graded volumes of water. All waters compared to distilled water standards. Points shown are mean values of four replicates and vertical lines represent standard of the means.

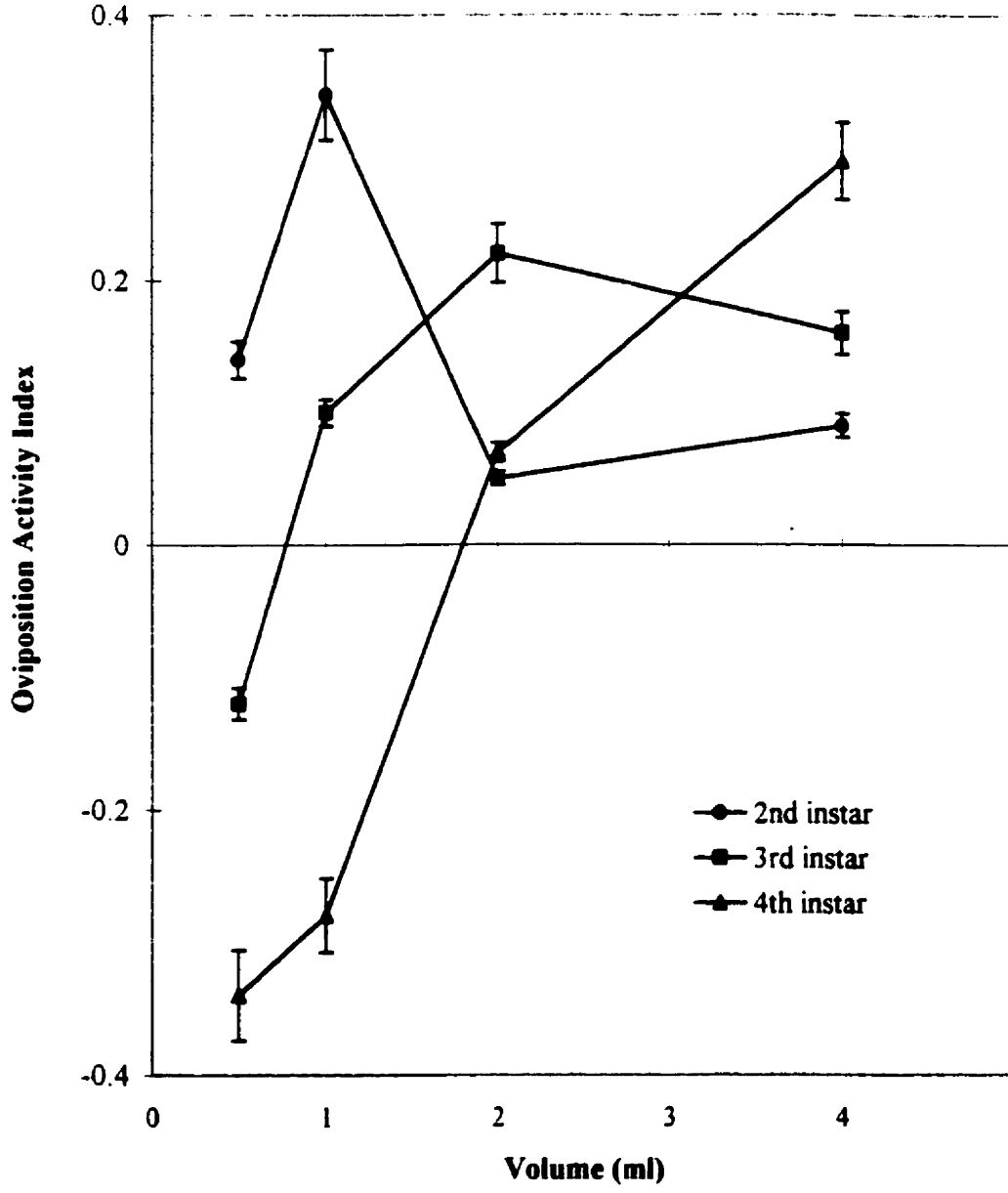

0.4
0.2
0
-0.2
-0.4
0
1
2
3
4
5
Oviposition Activity Index
Volume (ml)
2nd instar
3rd instar
4th instar

Fig. 3. Oviposition activity indices (OAI) of gravid *Aedes aegypti* in response to waters from normal larvae stored for various period of time. All waters compared to distilled water standards. Points shown are mean values of three replicates and vertical lines represent standard errors of the means.

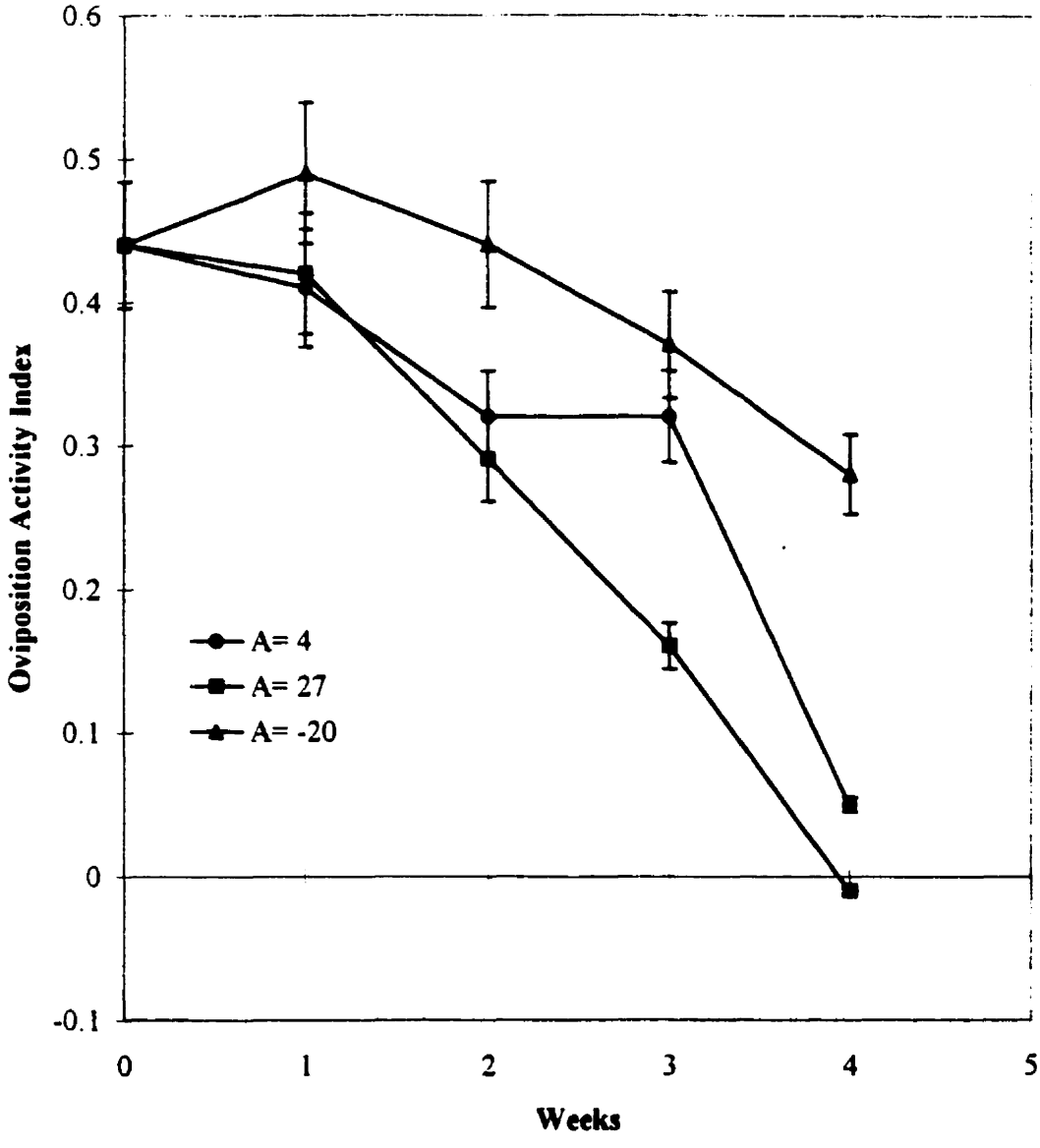
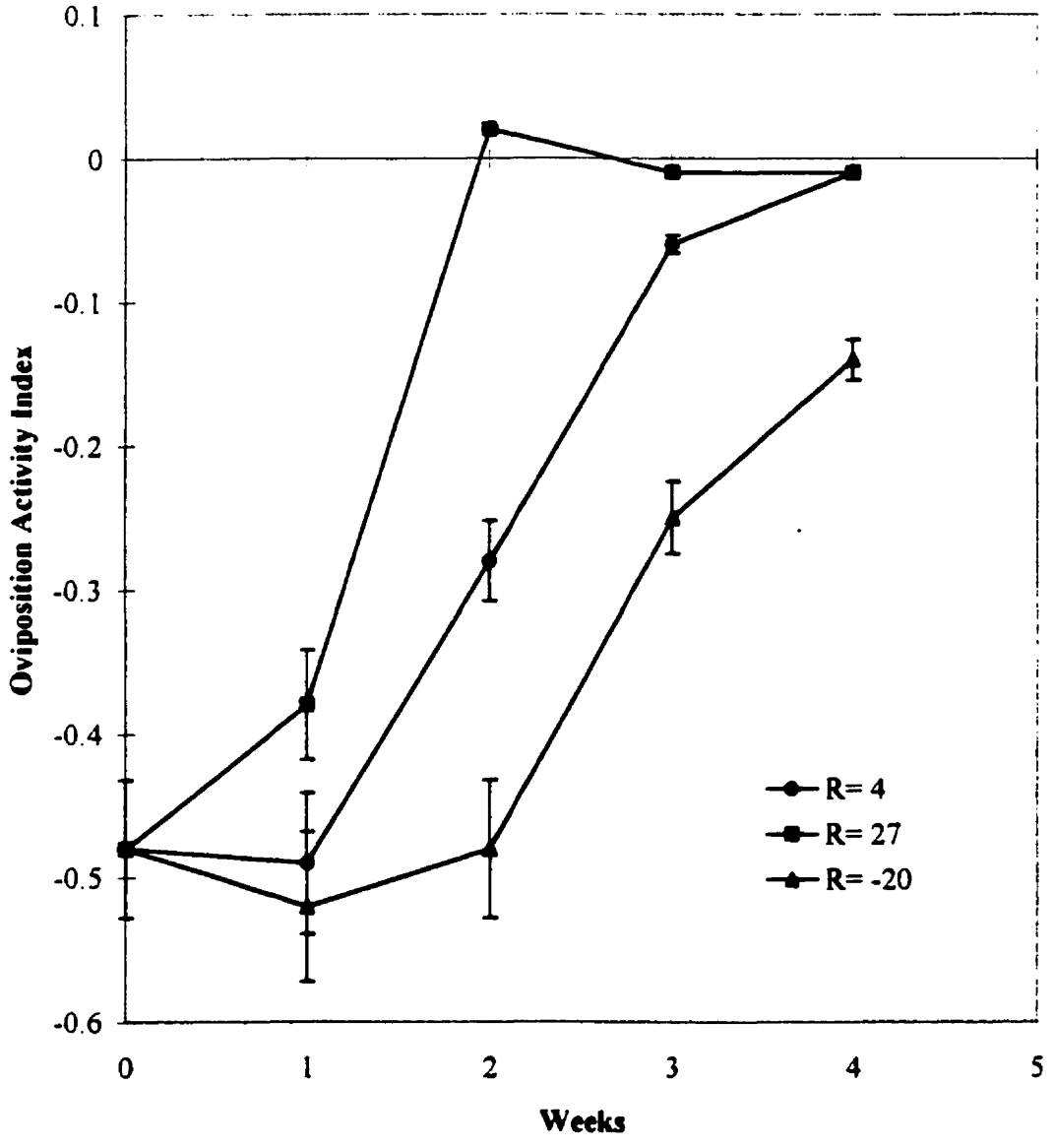

0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
Oviposition Activity Index
A= 4
A= 27
A= -20
0
1
2
3
4
5
Weeks

Fig. 4. Oviposition activity indices (OAI) of gravid *Aedes aegypti* in response to waters from *Plagiorchis elegans* infected larvae stored for various period of time. All waters compared to distilled water standards. Points shown are mean values of three replicates and vertical lines represent standard errors of the means.


0.1
0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
Oviposition Activity Index
0
1
2
3
4
5
Weeks
R= 4
R= 27
R= -20

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CONNECTING STATEMENT 4

In Chapter 4 it was demonstrated that oviposition attraction of waters declined and repellency increased as the population density of *Aedes aegypti* larvae rose. The larger the larvae, the more sensitive they were to crowding. Close confinement of individual larvae elicited oviposition effects similar to crowding and interference with the normal posture and behavior of the larvae may induce oviposition repellency. Within the specific time frame, the effects of parasitic infection induced a more potent repellent effect than starvation and crowding. Titration of the repellent against attractive waters revealed that oviposition repellency overrode attraction. Dilutions of as low as 1 part of repellent in 512 parts of attractant effect may persist for more than one week at ambient temperatures. Both infection with *Plagiorchis elegans* and crowding cause a decrease in food consumption in mosquito larvae, and may mimic the effects of starvation. In Chapter 5 are documented some of the physical and physiological changes that accompany infection with *P. elegans* , crowding and starvation in order to determine if the production of a repellent is due to the non-specific effect of nutritional stress, and to detect similarities and differences among the 3 treatments that may shed some light on some of the major factors that may precipitate the induction of oviposition repellency.

CHAPTER 5

THE SERUM COMPOSITION OF AEDES AEGYPTI (DIPTERA: CULICIDAE) LARVAE AND THE PRODUCTION OF AN OVIPOSITION REPELLENT ARE INFLUENCED BY INFECTION WITH THE ENTOMOPATHOGENIC DIGENEAN *PLAGIORCHIS ELEGANS* (TREMATODA: PLAGIORCHIIDAE), STARVATION AND CROWDING

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ABSTRACT Subjecting *Aedes aegypti* (L.) larvae to conditions that induced the production of oviposition repellency reduced their wet and dry weights and the concentration of total serum carbohydrates, amino acids and proteins. Thus, infection with metacercariae of the entomopathogenic digenean, *Plagiorchis elegans* (Rudolphi), starvation for seven days or two days of crowding reduced larval dry weights by as much as 32, 20, 23% respectively, and wet weights by 20, 14, 11%, respectively. Total serum carbohydrates declined by as much as 36, 21, 29% for infected, starved and crowded larvae, respectively, amino acids by 39, 48, 44% and protein concentrations by 72, 63, 62%, respectively. Repellency dilution titers were negatively correlated with the movement of mouth parts and gut. Incubation of infected, starved and crowded larvae in 0.01 g/L glucose greatly reduced the level of repellency of their waters, whereas adding glucose to repellent waters had only minor effects. The induction of repellency is strongly associated with the depletion effects of starvation.

KEY WORDS Repellency, Parasitism, Starvation, Crowding, Serum Composition

INTRODUCTION

The reproductive success of mosquitoes is governed, in part, by the ability of females to locate and select oviposition sites suitable for the growth, development and survival of their offspring. Larvae and pupae of *Aedes aegypti* (L.) assist conspecific females in this task by producing an attractant pheromone, thereby enhancing recruitment of eggs into previously colonized aquatic environments (Soman and Reuben 1970, Roberts and Hsi 1977).

The entomopathogenic cercariae of some digeneans show promise as biological control agents, impairing the development and survival of mosquito larvae, including *Ae. aegypti* (Rao *et al.* 1985, Dempster *et al.* 1986, Rau *et al.* 1991). *Plagiorchis elegans* Rudolphi shows particular potential because larvae bearing sublethal infections release a non-volatile repellent that renders their waters less acceptable as oviposition sites than waters containing non-parasitized larvae: females spend less time on repellent water than attractant water (Lowenberger and Rau 1994). The repellent effect is of a type that acts only over short distances as discussed by Foster and Harris (1997). As well, physical crowding and food deprivation of *Ae. aegypti* larvae render their waters less attractive to conspecific, ovipositing females (Moore 1977, Benzon and Apperson 1988, Zahiri *et al.* 1997). Infection, crowding and starvation affect the behavior of larvae. The penetration of cercariae induces violent escape responses in mosquito larvae. This is followed by the movement of the larvae to the water surface where they remain motionless and do not feed (Webber *et al.*

1987). Crowding increases physical activity of larvae and interferes with digestion (Shannon and Putman 1934), whereas prolonged food deprivation severely reduces activity (Christophers 1960).

Changes in serum composition induced by digenean and mermithid nematode infection in aquatic insect larvae have been used to characterize the physiological status of the host insects (Chambers *et al.* 1975, Schmidt and Platzer 1980). Both infection and crowding cause a decrease in food consumption of larvae and may have effects similar to starvation. The present study was undertaken to determine whether physical and physiological changes that accompany infection, crowding and starvation of larvae can be linked to nutritional deprivation and may underlie the induction of oviposition repellency in gravid females.

MATERIALS AND METHODS

Propagation of the digenean and mosquitoes. *Plagiorchis elegans* was reared according to the method of Lowenberger and Rau (1993). A colony of *Ae. aegypti* was maintained at 27 ± 2°C, 14:10 h (L:D) photoperiod, and 75% RH (Lowenberger and Rau 1994). Adult mosquitoes had free access to 10% saturated sucrose and water from cotton wicks. Larvae were reared under the same temperature and light conditions and were fed finely ground Tetramin fish food (Tetra Werke, Melle, Germany) ad libitum.

Exposure of larvae to infection, starvation, and crowding. One hundred fourth instar *Ae. aegypti* larvae were exposed for 20 min to 1,000 cercariae of *P. elegans* in 1 liter of distilled water. Cercariae were 8 h old and near their peak of infectivity after emergence from their snail host, *Stagnicola elodes* (Say) (Lowenberger and Rau 1993). Exposed larvae subsequently were maintained for 48 h in fresh, distilled water prior to hemolymph collection.

To induce starvation, individual larvae were maintained in 2 ml of distilled water without food. Larvae were rinsed and placed in fresh distilled water each day. Repellency due to starvation is first seen after 5 d of starvation (Zahiri *et al.* 1997), and hemolymph was collected after 7 d.

To create crowded conditions, 150 larvae were maintained for 72 h in 50 ml of distilled water with 0.012g of Tetramin fish food. Repellency due to crowding is first detected after 48 h (unpublished data), and hemolymph was collected at 72 h.

Control larvae were maintained in distilled water (1 larva per 5 ml) with sufficient food (0.25g/1L) for 48 h. Attraction is first detected after 24 h (Lowenberger and Rau 1994), and hemolymph was collected at 48 h.

Repellency titers. Larval waters were serially double diluted with distilled water and each dilution was tested for oviposition attraction or repellency to ovipositing conspecifics by exposure to populations of approximately 100 gravid females in flight cages (80 x 40 x 40 cm) maintained at 27 ± 2°C and a photoperiod of 14:10 h (L:D). Oviposition assays began on day 4 after the first blood feeding, and

eggs were collected after 26 h (Zahiri *et al.* 1997). The dilution at which the oviposition response of gravid females to repellent water first becomes indistinguishable from the response to distilled water was used to characterize the relative strength of the repellent effect.

Effects on gut activity and content, and weight. Larvae were examined under the microscope (88 X) for gross departures from normalcy attributable to treatment. Gut activity (number of contractions / min) and the presence or absence of gut contents were recorded. Movement of the mouth parts / min) were also ascertained.

To determine the relationship between the wet and dry weights of control and treated 4th instars, wet weights were obtained from groups of 5 surface-dried parasitized, crowded, starved and control larvae. To obtain corresponding dry weights, larvae were placed in a vacuum dessicator for 5 days and subsequently reweighed.

Serum collection and analyses. Hemolymph was collected by puncturing the 4th abdominal segment of surface-dried larvae and aspirating the fluid into 25 µl micropipettes coated with phenythiourea (to inhibit melanization). Pooled serum from 50 to 60 larvae was centrifuged (8,700 g, 1 min, 25°C), and the supernatant (serum) stored at -20°C until needed.

Proteins. Total protein content of aliquots of sera from each group of larvae diluted 1:10 (v/v) with distilled water was determined using the Bradford procedure with bovine serum albumin as a standard (Bradford 1976). To determine which sizes

of serum proteins were influenced, 10 µl of sample containing 23 µg total protein from parasitized, non-parasitized, crowded, and starved larvae were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis using a 4% (w/v) acrylamide stacking gel and a 12% (w/v) acrylamide separating gel under constant voltage (200 V) (Laemmli 1970). The gel was stained with Coomassie blue and destained with a solution of 40% (v/v) methanol and 10% (v/v) acetic acid to reveal protein bands.

Amino acids. Estimates of the total amino compounds (which include amino acids, amino alcohols, primary amides and urea) and imino compounds were collectively obtained by adapting the ninhydrin method of Rosen (1957) for small volumes of serum by increasing the concentration of NaCN and sodium acetate buffer (pH 5.3) 10 fold. This adaptation allowed the analysis of µl volumes of sample at the same sensitivity as the 1 ml volumes recommended by Rosen (1957). Serum (10 µl) from crowded, starved, parasitized and control insects were deproteinized with 500 µl of 95% ethanol followed by centrifugation (13,000 g, 2 min, 5°C) and 10 µl of the supernatant was used. The volume of reagents and standards were 1/50 that given by Rosen (1957). The total imino acids and amino compounds of the supernatants were determined spectrophotometerically at 570 nm with leucine as a standard.

Lipids. To tentatively determine the types of lipids in the serum, 10µl of serum from each group of larvae was deproteinized by exhaustive vortexing with 500µl of chloroform (Maniatis et al. 1982) followed by centrifugation (8,700 g 2 min., 2°C). The chloroform phase was used to identify the classes of neutral lipids and

phospholipids by analyzing the equivalent of 5µl of serum by two-step, one-dimensional thin layer chromatography on silica gel G (with $CaSO_4$ binder) plates. Phospholipids were resolved using chloroform: methanol: water (80: 35: 5, v/v/v) and subsequently neutral lipids were separated with a hexane:ether (4:1, v/v) solution. Plates were stained with 2' 7'- dichorofluorescein reagent (Sigma) and lipids tentatively identified with authentic standards (Jewell and Dunphy 1995).

Carbohydrates. The aqueous phase of the extracted serum was used to measure total carbohydrates spectrophotometerically using the anthrone method (Roe 1954), measuring absorbance at 625 nm and using D-glucose as the standard.

Thin-layer-chromatography was used to tentatively identify the types of sugars in the serum of each test group of *Ae. aegypti* (Boos *et al.* 1990). Samples of serum (5 µl) and the standards (trehalose, D-glucose and D-fructose) were applied to commercially prepared silica gel plates (250 µm x 20 cm x 20 cm) impregnated with 0.02M sodium acetate (Fisher Scientific). The plates were developed in butanol:ethanol:water (5:3:2 v/v/v/) which selectively extracts carbohydrate via TLC, and the carbohydrates were rendered visible by spraying the plates with 50% H_2SO_4 and charring at 115°C for 5 min. Concentrations were ranked visually according to stain intensity, after which each sugar was scraped from the TLC plates and extracted from the silica gel by dissolving the sugar in 100 µl of distilled water and removing the silica by centrifugation (8,700 g 1 min, 2°C). The concentrations of sugars in the supernatants were measured using the anthrone method (Roe 1954). There were two determinations.

Effect of glucose incubation on repellency. Because a relationship between serum glucose levels and repellency was detected (see results), groups of 10 infected, crowded, starved and control larvae were incubated in solutions of D-glucose (0.01 g/L), for 2, 2, 7 and 2 d, respectively, to determine if glucose replacement would alleviate the production of the repellent from the stressed insects. The glucose solutions were changed daily. Solutions from the designated day of incubation were sterilized by vacuum filtration through a cellulose nitrate filter (0.22 μm), and stored at -20°C to be tested later for attraction/repellency. To determine if D-glucose would offset the effect of the repellency, waters from infected, crowded, starved and control larvae containing repellency/attractant were incubated in distilled water after which the waters were filter-sterilized and glucose was added (final concentration of 0.01 g/L). The attractant/repellency properties of waters from larvae incubated with glucose and from larvae incubated in water to which glucose had been added subsequently were assessed in separate trials using the oviposition bioassay methods described by Zahiri *et al.* (1997). Briefly, samples (2 ml) and controls (distilled water alone and glucose solution) were arranged randomly in tissue culture wells (depth 2.2 cm, diameter 1.8 cm, separated by a distance ≥ 2.2 cm). Samples and controls were presented in triplicate for a total of 30 wells. Arrays of samples and controls were introduced into mosquito flight cages (80 by 40 by 40 cm) at 27 ± 2°C, and photoperiod of 14:10 [L:D] h, with a 10% sucrose solution available ad libitum. Newly emerged mosquitoes (100 males and 100 females) were introduced into each cage and blood fed daily.

Oviposition assays began on day 4 after blood feeding. The numbers of eggs laid in each well were counted at 26-h intervals: one 26 h period consisted of 8:10:8 h (L:D:L). There were 3 replicates. Numbers from triplicate wells were summed for each plate, expressed as a percentage of the total number of eggs and arcsine transformed prior to analysis of variance.

RESULTS

Repellency titers. The repellency titers were 1:16 (reciprocal dilution 4) for starved larvae, 1:32 (reciprocal dilution 5) for crowded larvae and 1:1024 (reciprocal dilution 10) for *P. elegans*-infected larvae ($F = 11.99, 35.32, 25.93$ respectively, df = 11, $P < 0.05$).

Effects on mandibular movement, gut activity and weight. Microscopical examination of starved, crowded and infected larvae revealed few, if any, food particles in the hind gut. Such larvae also manifested significantly fewer gut contractions and mandibular movements than controls (Table. 1). Repellency titers significantly increased with decreasing frequency of mandibular movement and gut contractions ($r = 0.97, 0.90$, $P < 0.05$, $n = 10$, respectively).

The wet weights of parasitized, crowded and starved larvae were significantly lower than those of controls: infected larvae were the most severely affected (Table. 1). Mean dry weights followed a similar pattern with infected larvae being significantly lighter than starved and crowded larvae. The latter 2 treatments, although not statistically different from each other, resulted in larvae that were significantly

lighter than the controls. Mean dry weights of larvae were correlated with repellency titers ($r = 1.0$, $P < 0.05$). Wet weight:dry weight ratios for parasitized, crowded and starved larvae were greater than for controls (Table. 1). The wet weight:dry weight ratios also were correlated with repellency titers ($r = 0.925$, $P < 0.05$).

Serum analyses. The total serum protein levels of *Ae. aegypti* larvae decreased significantly to similar level as a result of starvation (20.0 ± 0.2 μg/μl) and crowding (20.4 ± 0.2 μg/μl) and to even lower levels due to infection (15.0 ± 0.5 μg/μl), when compared with the controls (53.7 ± 0.5 μg/μl) (Table. 2) (LSD = 0.65, $P < 0.05$, n = 5). Total protein levels were not correlated with repellency titers ($r = 0.85$, $P > 0.05$) (Table. 2).

The same numbers of readily discernible protein bands were present in the serum of normal, starved, crowded, and parasitized larvae (Fig. 1). The protein band with a molecular weight of 200 KDa (a) decreased in intensity in parasitized and crowded insects, the level in the parasitized larvae being less than in the crowded insects. The 180 KDa (b) protein band was faint in control insects and increased in starved, parasitized, and crowded larvae. The intensity of this protein band was the same in the latter two groups and greatest in starved larvae. The 132 KDa (c) protein band was more noticeable in starved and crowded larvae and equally less in normal and parasitized insects. The 96 KDa (d) band in normal and starved larvae had a similar intensity, but decreased to the same level in the crowded and parasitized larvae. The 82 KDa (e) band increased to the same intensity in all treatments.

In general, there was a decrease on the quantity of a number of low molecular weight proteins (62-17 KDa), the effect being least in samples from starved larvae, moderate in crowded insects, and greatest in parasitized larvae (Fig. 1). The 62 KDa (f) protein band was constant in normal and starved larvae, but decreased in crowded and parasitized larvae. Protein with molecular weight of 51 KDa (g) was not detected in parasitized larvae. Protein bands 34 KDa (h) and 30 KDa (i) decreased to equal level in the starved and crowded groups and to a lower level in parasitized insects. The 28 KDa band (j) decreased more in parasitized than crowded or starved larvae. The 26 KDa (k) protein was not detectable in parasitized insects, and decreased in magnitude in crowded and starved larvae. The 17 KDa (l) and 15 KDa (m) bands were not discernible in parasitized and crowded insects, but were present in starved larvae at a diminished concentration compared with the control group.

The concentration of total amino compounds in the serum declined by approximately 50% in larvae infected by *P. elegans* (0.14 ± 0.02 μg/μl) and larvae starved for 7 d (0.12 ± 0.02 μg/μl) or crowding for 48 h (0.13 ± 0.03 μg/μl) as compared with control larvae (0.23 ± 0.02 μg/μl) (Table. 2) (LSD = 0.04, $P < 0.05$, n = 6). There was no correlation between the concentration of amino compounds and repellency titer (r = 0.735, $P > 0.05$) (Table. 2).

The sera of normal mosquitoes contained 9 lipid groups, a triglyceride similar to triolein, phosphtidyl glycerol, phosphatidylcholine, tripalmitin, phosphatidyl ethanolamine, phosphatidyl inositol, and 3 unidentified groups. All stressed larvae

lacked tripalmitin and phosphatidyl ethanolamine, but possessed a lipid tentatively identified as cholesterol. Starved and crowded larvae had less phosphatidyl inositol than the controls, whereas parasitized insects lacked the lipid. The three unidentified bands were absent in all treated groups.

The concentration of serum total carbohydrates decreased significantly from control larvae (10.2 ± 0.7 μg/μl) to infected (6.5 ± 0.8 μg/μl), crowded (7.2 ± 0.7 μg/μl), and starved (8.0 ± 0.5 μg/μl) larvae (LSD = 1.2, $P < 0.05$, n = 5) (Table. 2). This paralleled the general activity of the insect and was correlated with repellency titer (r = 0.935, $P < 0.05$) (Table. 2). Trehalose and D-glucose (the latter at lower concentration than the former), were present in serum of all groups of instars but not D-fructose. Starved and crowded *Ae. aegypti* larvae had lower concentrations of serum trehalose (3.1 ± 0.1μg/μl and 3.1 ± 0.8μg/μl respectively) and D-glucose (2.7 ± 0.2μg/μl and 3.3 ± 0.1μg/μl respectively) than the control groups (5.8 ± 0.5μg/μl and 3.9 ± 0.2μg/μl respectively). The concentration of D-glucose in parasitized *Ae. aegypti* (1.3 ± 0.2μg/μl) was lower than in the starved (2.7 ± 0.2μg/μl) and crowded (3.3 ± 0.1μg/μl) insects. The concentration of serum trehalose in the parasitized larvae (5.1 ± 0.3μg/μl) was comparable to the control groups (5.8 ± 0.5μg/μl) and higher than in the starved (3.1 ± 0.1μg/μl) and crowded (3.1 ± 0.8μg/μl) groups.

Incubation of starved, crowded or parasitized larvae in D-glucose rendered these solutions significantly less repellent than corresponding waters to which glucose was added after incubation (Table. 3).

DISCUSSION

Crowded larvae, starved larvae, and larvae infected with *P. elegans* were stressed nutritionally and the level of oviposition repellency was proportional to the severity of the stress. Microscopical examination of larvae from the 3 treatments revealed few, if any food particles, in the hind gut. These larvae had probably ceased to feed. This is supported by the reduced number of gut and mandibular movements (Table. 1). In the absence of food, larvae quickly become inactive and remain at the surface of the water column (Christophers 1960). This may be an adaptive response to reduce energy expenditure and may serve to delay the onset of stressful levels of nutritional deprivation. In contrast, crowded larvae experience intense mutual body contact which leads to increased physical activity, and interferes with feeding behavior and digestion (Shannon and Putman 1934). Such activity may exacerbate the energy drain and the accompanying stress above the level experienced by food deprived larvae. Similarly, exposure of *Ae. aegypti* larvae to entomopathogenic cercariae and their subsequent penetration into host tissue elicit violent locomotory activity. Eventually, infected larvae withdraw to the surface of the water column where they remain virtually immobile, particularly if infections are heavy (Webber *et al.* 1987). The initial escape responses in concert with the subsequent cessation of feeding represents a considerable energy drain. Declines in wet and dry weights of stressed larvae are the gross manifestations of food deprivation.

Stressed larvae also experienced a reduction of the total protein, especially parasitized larvae. This reduction may be the result of dilution due to the accumulation of water in tissues and/or proteolysis directly or indirectly induced by the parasite. The same number of readily discernible protein bands was present in the serum of control, starved, crowded and parasitized larvae (Fig. 1). Proteins with high molecular weight increased in stressed larvae, particularly in infected individuals. Such increases have not been observed in other aquatic insects infected with digeneans (Chambers *et al.* 1975) or *Romanomermis culicivorax* (Ross and Smith) (Schmidt and Platzer 1980), and its significance is unknown. Many of the changes in the serum of parasitized larvae were not of similar magnitude, so that changes in band intensity represented more than a dilution effect due to increased fluid content. Protein with low molecular weight declined in parasitized and crowded larvae, and was faint in starved larvae when compared with controls. This parallels what has been observed in mermithid infections of 3 species of mosquitoes where infections lowered larval weights and the concentrations of protein in bands below 68 KDa (Schmidt and Platzer 1980, Womersley and Platzer 1982). The decline/loss of these proteins from host's serum during crowding and parasitism may be explained by nutritional depletion related to increased activity. This effect was greater in parasitized larvae; *P. elegans* acts as a "protein sink" similar to *R. culicivorax* in *Culex pipiens* Say (Schmidt and Platzer 1980) and digeneans in *Hexagenia recurvata* (Morgan) and *Sialis* sp. (Chambers *et al.* 1975).

The concentration of total amino compounds in the serum of stressed larvae declined by approximately one half. Parasitism is known to lower the amino compound concentration of insect concomitantly with inducing hypoproteinema (Webster and Dunphy 1987), and *P. elegans* is known to assimilate leucine *in vitro* (Lowenberger *et al.* 1994). However, because there was no correlation between the concentration of amino compounds and repellency titers, these compounds may not be linked to oviposition repellency.

The serum of normal mosquitoes contained 9 lipid groups. The major neutral lipids in *Ae. aegypti* are tricylglycerol, monoacylglycerols, tripalmitin, phosphatidyl ethanolamine and phosphatidyl inositol (Gordon *et al.* 1979, Ford and Van Heusden 1994). Starved and crowded larvae of the present study had less phosphatidyl inositol than controls, whereas parasitized larvae lacked the lipid. Such changes were not found in *Ae. aegypti* parasitized by mermithids (Gordon *et al.* 1979), and may reflect differences in host parasite associations.

The effects of the 3 stressors on carbohydrate levels in the serum of larvae were variable. Starved and crowded larvae had relatively low concentration of serum trehalose and D-glucose. This is similar to what is found among other species of nutritionally stressed insects such as *Manduca sexta* (L.) infected with *Cotesia congregata* (Masson) (Thompson and Lee 1993). In the current study, the concentration of serum trehalose in the parasitized mosquitoes was comparable to that in the control groups. This is similar to the effect of mermithid infections in black flies

(Gordon *et al.* 1978) but unlike their effect on *C. pipiens* (Schmidt and Platzer 1980). D-glucose levels in parasitized larvae were lower in parasitized than in starved and crowded individuals. Conceivably, *P. elegans* inhibited trehalose mobilization resulting in greater D-glucose depletion caused by the needs of both the host and parasite.

Incubation of starved, crowded or parasitized larvae in D-glucose solutions rendered these solutions significantly less repellent than waters from corresponding larvae to which glucose was added after incubation (Table. 2). Provision of glucose ameliorates the effects of stress on the insects, and concomitantly the manifestation of oviposition repellency. These effects could not be attributed to a direct attractant effect of glucose on ovipositing females, because the addition of glucose to repellent waters only marginally enhanced their attractiveness. Incubation of control larvae with glucose did not enhance the attractiveness of their waters, presumably because such larvae are already well nourished.

In summary, the observed changes in the serum composition of starved, crowded and parasitized larvae are characteristic of nutritional depletion effects and there is a strong association with the induction of repellency.

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Table 1. Mean total wet, dry and wet/dry weight ratios, frequency of mandibular movement, and frequency of gut contractions from normal, starved, crowded or *Plagiorchis elegans*-infected *Aedes aegypti* larvae.

Treatment groups	Mandibular movements/ min (10) [a]	Gut contractions/ min (10)	Wet weight mg (5)	Dry weight mg (5)	Wet weight/ Dry weight (5)
Starved	23.42 ± 3.90 b	27.74 ± 8.48 b	3.86 ± 0.08 b	0.83 ± 0.02 b	4.6 ± 0.21 b
Crowded	10.24 ± 3.01 c	13.74 ± 4.04 c	3.97 ± 0.10 b	0.81 ± 0.01 b	4.9 ± 0.01 c
Parasitized	2.50 ± 1.64 d	8.45 ± 2.11 c	3.57 ± 0.15 c	0.71 ± 0.04 b	5.0 ± 0.49 c
Control	41.05 ± 4.66 a	45.50 ± 4.15 a	4.49 ± 0.13 a	1.05 ± 0.03 a	4.3 ± 0.13 a
r [b]	0.97	0.90	-	1.0	0.93
LSD	3.83	6.12	0.20	0.18	0.33

Means in columns bearing the same letter are not significantly different ($P > 0.05$).

[a] Replicates in parentheses.

[b] Linear correlation coefficient.

Table 2. Mean total protein, amino acid and carbohydrate levels in the serum of starved, crowded, *Plagiorchis elegans*-infected and control fourth instar *Aedes aegypti* larvae.

Treatment groups	Total protein (μg/μl)	Total amino acids (μg/μl)	Total carbohydrate (μg/μl)
Starved	20.0 ± 0.2 b	0.12 ± 0.02 b	8.0 ± 0.5 b
Crowded	20.4 ± 0.2 b	0.13 ± 0.03 b	7.2 ± 0.7 bc
Parasitized	15.0 ± 0.5 c	0.14 ± 0.02 b	6.5 ± 0.8 c
Control	53.7 ± 0.5 a	0.23 ± 0.02 a	10.2 ± 0. a
r [a]	0.85	0.74	0.94
LSD	0.65	0.04	1.2

Means in columns bearing the same letter are not significantly different ($P > 0.05$).

[a] Linear correlation coefficient.

Table 3. Mean number of eggs (± SE) laid by *Aedes aegypti* females on D-glucose solutions in which crowded, starved, *Plagiorchis elegans*-infected or control larvae had been incubated for 24 h, and the number of eggs laid on corresponding waters in which larvae had been incubated in the absence of D-glucose but to which D-glucose was added just prior to the oviposition bioassay.

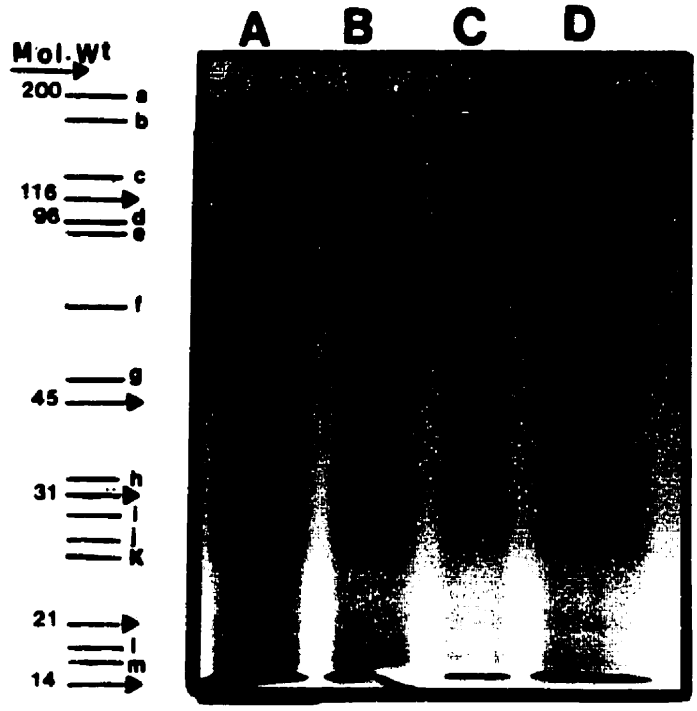
Treatment groups	Media from larvae incubated in D-glucose solution (0.01 g/L)	Larval waters with D-glucose added after incubation (0.01 g/L)	*t*-value
Starved	187.39 ± 11.34 c	84.69 ± 2.99 c	0.001 *
Crowded	177.45 ± 9.52 c	72.33 ± 6.92 cd	0.001 *
Parasitized	110.76 ± 15.33 d	63.03 ± 9.38 d	0.011 *
Control Larvae	398.00 ± 10.61 a	392.71 ± 9.28 a	0.242 NS
Medium Controls	310.05 ± 40.06 [1] b	220.02 ± 16.54 [2] b	0.031 *

Means in columns bearing the same letter are not significantly different ($P > 0.05$); *, means within rows are significantly different at the 0.05 level; NS, not significant.

[1] D-glucose solution (0.01 g/L).

[2] Distilled water (no D-glucose).

Fig. 1. SDS-PAGE of 23 μg of total serum proteins from fourth instar *Ae. aegypti.* (A) control larvae, (B) larvae starved for 7 d, (C) larvae parasitized by *P. elegans* for 2 d, (D) larvae crowded for 2 d. The molecular weights of the protein standards are indicated by the arrow [myosin (200-KDa), galactosidase (116-KDa), phosphorylase (97-KDa), bovine serum albumin (66-KDa), ovalbumin (45-KDa), carbonic anhyrase (31-KDa), trypsin inhibitor (21-KDa), and lysozyme (14-KDa)]. Lower case letters "a" to "m" represent protein bands discussed in the text.


A
B
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Mol.Wt
200
116
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31
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CONNECTING STATEMENT 5

Evidence is presented in Chapter 5 that crowded larvae, starved larvae and larvae infected with *Plagiorchis elegans* are nutritionally stressed and that the level of oviposition repellency is proportional to the severity of this stress. This is particularly acute in the case of infection where the parasite appears to block the conversion of stored trehalose to glucose. This effect can be ameliorated experimentally by the addition of glucose to the larval environment. The chapter underscores the deleterious effects of starvation, crowding and infection. Natural selection would favor females which avoid ovipositing in sites deleterious to larval survival. Many mosquito species have very similar environmental requirements. It would follow that females sensitive to heterospecific chemical messages would have an adaptive advantage, particularly if the negative message is sent by larvae infected with a pathogen of low host specificity. In Chapter 6, I address the question of whether the chemical messages associated with oviposition attraction/repellency are perceived across species barriers and I discuss the findings in the light of intra- and interspecific competition for limited resources and pathogen avoidance.

CHAPTER 6

OVIPOSITION RESPONSES OF *AEDES AEGYPTI* AND *AEDES ATROPALPUS* (DIPTERA: CULICIDAE) FEMALES TO WATERS FROM CONSPECIFIC AND HETEROSPECIFIC NORMAL LARVAE AND FROM LARVAE INFECTED WITH *PLAGIORCHIS ELEGANS* (TREMATODA: PLAGIORCHIIDAE)

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ABSTRACT Ovipositing *Aedes aegypti* (L.) females were attracted to waters in which uninfected, conspecific *Aedes atropalpus* (Coquillett) larvae had been reared, but were repelled by waters from larvae of either species infected with the entomopathogenic digenean, *Plagiorchis elegans* (Rudolphi). In contrast, *Ae. atropalpus* females were attracted to or repelled by waters from conspecific uninfected and infected larvae, respectively, but did not respond to corresponding waters from *Ae. aegypti* larvae. The sensitivity of ovipositing females of both species to repellents and attractants is discussed in terms of possible selective pressures exerted by unstable breeding environments.

KEY WORDS *Aedes aegypti*, *Aedes atropalpus*, *Plagiorchis elegans* Infection, Oviposition, Attractant, Repellent

INTRODUCTION

Larvae of several mosquito species produce chemical signals used by gravid conspecific (Soman and Reuben 1970, Ikeshoji and Mulla 1970, Kalpage and Brust 1973, Roberts and Hsi 1977, Trimble and Wellington 1980, Bentley *et al.* 1981, Maire 1984) and heterospecific (Gubler 1971, Bentley *et al.* 1976, McDaniel *et al.* 1979, Maire and Langis 1985) females. The chemical message released into the aquatic environment by such larvae may be modulated to reflect the nutritional (Zahiri *et al.* 1997a) and parasitological (Lowenberger and Rau 1994) status of the sender. For example, larvae of *Aedes aegypti* (L.) characteristically render their aquatic environment attractive to gravid, conspecific females (Soman and Reuben 1970, Roberts and Hsi 1977). However, if such larvae are starved (Zahiri *et al.* 1997a) or are infected with the entomopathogenic stages of the digenean parasite *Plagiorchis elegans* (Rudolphi) (Lowenberger and Rau 1994), these waters become strongly repellent. The effects are directly proportional to the duration of starvation and the intensity and site of infection (Zahiri *et al.* 1997b). Infection of the larvae renders waters repellent more quickly than does starvation (Zahiri *et al.* 1997a). We suspect that the induction of repellency represents a general response to stress.

The phenomenon of oviposition attractancy has been studied in several mosquito species, including *Aedes atropalpus* (Coquillett) (Bentley *et al.* 1976, Maire 1984). However, to date oviposition repellency has been studied only in *Ae. aegypti* (Lowenberger and Rau 1994). Although the metabolites and physical presence of

bacteria render waters from uninfected *Ae. aegypti* larvae attractive to ovipositing conspecifics (Benzon and Apperson 1988), such waters remain highly attractive following filter sterilization (Lowenberger and Rau 1994, Zahiri *et al.* 1997b) so the larvae also contribute to the attractive effect. Similarly, uninfected axenically reared *Ae. atropalpus* larvae render waters attractive to their conspecifics (Maire 1985). Such similarities between species allow more reliable comparisons, first of heterospecific oviposition responses to attractant waters and subsequently conspecific and heterospecific response to repellent waters. We also chose these 2 very different species, one a circumtropical, peridomestic container breeder and the other a temperate, feral, rock-pool breeder, because of the enhanced probability that they would respond differently to heterospecific oviposition signals. The current work is a report on the conspecific and heterospecific oviposition responses of *Ae. aegypti* and *Ae. atropalpus* to waters from uninfected larvae and waters from larvae infected with metacercariae of *P. elegans.*

MATERIALS AND METHODS

Colonies of ≈ 100 females and 100 males of *Ae. aegypti* and *Ae. atropalpus* were maintained in cages (30 by 30 by 50 cm) at 27 ± 2°C, 75% RH, and a 16:8 (L:D) h photoperiod. Colonies were provided with a 10% sucrose solution ad libitum, and the anautogenous females of *Ae. aegypti* were fed on the blood of newly born rat pups twice weekly (Animal Use Protocol No. 3164, McGill University, Canada). Groups of

50 larvae of either species were reared in clear plastic containers (13.5 cm diameter, 12 cm depth) filled with 100 ml of distilled water. Larvae of both species were reared under the same temperature and light conditions and were fed finely ground Tetramin fish food (Tetra Werke, Melle, Germany) ad libitum at a concentration of 0.25g of dry powder per liter of distilled water. The digenean, *P. elegans,* was reared in the laboratory following the procedure of Lowenberger and Rau (1993). Freshly emerged cercariae of *P. elegans* were obtained according to the method of Webber *et al.* (1986).

Groups of fifty 3rd-instars of both species were exposed to 500 freshly emerged cercariae of *P. elegans* in 100 ml of distilled water (depth, 8 cm) for 20 min. The larvae were rinsed once in 100 ml of distilled water and each larva was transferred to individual tissue culture wells (2.2 cm, depth 1.8 cm, diameter) containing 2 ml of distilled water. The larvae were incubated at 27 ± 2°C for 48 h then were transferred to microscope slides, crushed lightly under a coverslip in a drop of water, and examined under a compound microscope (40 ×) to determine the intensity of infection with *P. elegans* metacercariae. Waters from larvae infected with >3 metacercariae (3.83 ± 0.78, mean ± SE) were sterilized by filtration (2μm), and stored individually in sealed serum vials at 4°C until used. Waters from uninfected, sham-exposed larvae of both species were used as uninfected larval controls.

Waters from individual larvae were tested for repellency / attraction and compared with distilled water controls. Samples were arranged randomly on 18-well tissue culture plates, each well separated from the next by a distance of 2.2 cm. The

18-well test arrays held 3 waters each from infected *Ae. aegypti* and *Ae. atropalpus* larvae (n = 3 each), a corresponding number of wells from uninfected, sham-exposed larvae, and 3 distilled water controls for each species. The oviposition responses of females to such arrays of larval waters were tested separately in flight cages (30 x 40 x 55 cm) each containing 100 gravid females of either *Ae. aegypti* or *Ae. atropalpus*. Each trial extended over a 26-h period in a photoperiod of 8:10:8 (L:D:L) h and was replicated four times. The number of eggs laid in each well was recorded. Numbers of eggs from each of the triplicate wells per plate were summed and expressed as a percentage of the highest egg count among all 4 replicates of the same species. Comparisons among these values were made by analysis of variance (2-way ANOVA). The LSD procedure was used to separate the means when F statistics were significant. All statistical procedures were performed using the SAS statistical package (SAS Institute 1994).

RESULTS

The percentage of eggs laid by *Ae. aegypti* on waters derived from uninfected, conspecific 3rd instars was similar to the percentage laid on water from uninfected *Ae. atropalpus* larvae (Table. 1). Significantly greater proportions of *Ae. aegypti* eggs were laid on water that had contained uninfected *Ae. aegypti* larvae than on distilled-water controls, whereas significantly lower proportions of eggs were laid on water from larvae of *Ae. aegypti* and *Ae. atropalpus* infected with metacercariae of *P. elegans*

(Table. 1). Females of *Ae. atropalpus* deposited significantly larger proportions of eggs on waters derived from conspecific, uninfected larvae than on waters from corresponding *Ae. aegypti* heterospecifics. Indeed, the percentage of eggs laid on waters from such heterospecific larvae was statistically indistinguishable from the that laid on distilled water controls. In contrast, waters from *Ae. atropalpus* larvae infected with metacercariae of *P. elegans* attracted a significantly lower proportion of eggs from conspecific adults. Water from infected *Ae. aegypti* larvae received proportions of *Ae. atropalpus* eggs similar to those of the distilled water controls.

DISCUSSION

Our data confirmed earlier findings that waters from late instars of *Ae. aegypti* and *Ae. atropalpus* strongly attracted conspecific gravid females (Soman and Reuben 1970, Kalpage and Brust 1973, Maire and Langis 1985). Similar responses to conspecific larvae have been recorded for other *Aedes* species, including *Aedes triseriatus* (Say) (Bentley *et al.* 1976), *Aedes togoi* (Theobald) (Trimble and Wellington 1980) and *Aedes communis* (DeGeer) (Maire and Langis 1985).

Heterospecific oviposition attractancy within the genus *Aedes* is less well documented. In the present study, *Ae. aegypti* was attracted to waters from *Ae. atropalpus* larvae. This parallels the behavior of *Ae. triseriatus* (Bentley *et al.* 1976, McDaniel *et al.* 1979) and *Ae. communis* adults (Maire and Langis 1985), both of which were attracted to *Ae. atropalpus* waters. It also parallels the behavior of *Ae.*

aegypti females in response to uninfected *Ae.* (*Stegomyia*) *albopictus* larvae (Sucharit *et al.* 1980). In the current study, attraction was not mutual; *Ae. atropalpus* females were not attracted by waters from *Ae. aegypti* larvae. It is not known if this also applies to waters derived from *Ae. communis* and *Ae. triseriatus* larvae; however, *Ae. albopictus* females are not as attracted to *Ae. aegypti* waters as to their own (Sucharit *et al.* 1980).

Aedes aegypti is a widely distributed tropical and subtropical species and the larvae exploit a wide variety of container habitats including treeholes and artificial containers (Welch and Long 1984). In contrast, north-temperate zone *Ae. atropalpus* larvae use rock pools, a more limited habitat (Kalpage and Brust 1973). Conceivably it may be of adaptive significance for gravid females of species with a relatively broad environmental tolerance to breeding sites to be attracted to the waters of other species, whereas species with more restrictive environmental requirements might be served best by a behavioral insensitivity to waters from heterospecifics.

Sensitivity of gravid females of both *Ae. aegypti* and *Ae. atropalpus* to the respective attractant and repellent properties of waters derived from uninfected and *P. elegans*-infected conspecific or heterospecific larvae was linked strongly. There may be a common receptor for the attractant and repellent compounds and also may reflect adaptation of mosquitoes to breeding habitats. The habitats in which many mosquito species develop are ephemeral (Bentley and Day 1989). Because larvae developing in less than optimal environments have a reduced probability of survival, selection would

favor females that are able to assess the quality of a potential larval development site (Macan 1961, Petranka and Fakhoury 1991). Females are aided in this process by larvae whose presence in the environment strongly encourages oviposition. Kin selection may favor this behavior further by enhancing the inclusive fitness of such larvae (Smith Trail 1980). However, unless the content of this chemical message strongly reflects environmental quality and unless the attraction wanes as the environment of the larvae changes for the worse, attraction could become counteradaptive.

When the uninfected, healthy larvae and the ovipositing females which their waters attract are closely related, both the larvae and the females derive a reproductive advantage according to the theory of kin selection. As the degree of relatedness declines, proportionally fewer benefits accrue to the larvae, whereas the sensitive females retain their advantage. When the sensitivity to attraction involves heterospecifics the process may enhance interspecific competition and may have a negative impact on the larval population.

Waters rendered unsuitable by a pathogen at least should be neutral, and at best repellent, to maintain an adaptive advantage for ovipositing adults and closely related, conspecific larvae. If the larvae are conspecific but not closely related, only the sensitive females derive benefits. This also should hold true when sensitivity to a specific repellents involves heterospecifics.

When repellency is induced by starvation, a condition frequently associated with high densities of larvae, both sensitive females and larvae, whether conspecific or heterospecific and whether closely related or not, may derive reproductive benefits. Females do not lose their reproductive investment and resident larvae avoid an increase in the level of competition by excluding others. This may ensure that limited food resources are not partitioned to the point where eventually no larvae are able to reach the adult stage.

If we can extend the parallelism between oviposition attraction and repellency, as observed in the current study, to other combinations of species, we may be able to make some predictions as to how these might interact competitively. Therefore, *Ae. aegypti* females are sensitive to the attractants produced by larvae of *Ae. albopictus* but the attraction is not mutual. This may attract *Ae. aegypti* females to lay eggs in suitable *Ae. albopictus* habitats but not the reverse (Sucharit *et al.* 1980). It would follow that *Ae. aegypti* females would not lay eggs into habitats rendered unsuitable for *Ae. albopictus* larvae by the presence of a pathogen such as *P. elegans* or insufficient food. In contrast, whereas *Ae. albopictus* would avoid ovipositing in habitats rendered unsuitable by conspecific larvae, it nevertheless would enter such habitats rendered unsuitable by *Ae. aegypti*. This may place *Ae. albopictus* at a disadvantage in its competition with *Ae. aegypti* (Sucharit *et al.* 1980).

Oviposition attractants and repellents may be common in natural mosquito breeding sites. The interaction between these two factors may have far-reaching effects on the distribution and abundance of many container-breeding mosquito species.

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Table 1. Proportions of eggs (mean ± SE) laid by females of *Aedes aegypti* and *Aedes atropalpus* on waters derived from *Plagiorchis elegans*-infected larvae or uninfected larvae, both conspecific and heterospecific, as well as on distilled water controls (n = 4 per species).

	% eggs laid [a]		
Larval waters	*Ae. aegypti*	*Ae. atropalpus*	*F*
Ae. aegypti (uninfected)	91.93 ± 5.46 a	69.41 ± 3.13 b	51.15*
	(430.34 25.57)	(320.01 14.44)	
Ae. atropalpus (uninfected)	88.62 ± 2.44 a	96.04 ± 4.42 a	8.63*
	(414.66 11.44)	(442.65 20.37)	
Distilled water control	67.90 ± 3.49 b	68.66 ± 1.51 b	0.16
	(317.57 16.32)	(316.50 6.95)	
Ae. aegypti (infected)	58.23 ± 3.31 c	70.55 ± 4.64 b	18.70*
	(277.33 6.50)	(325.32 21.39)	
Ae. atropalpus (infected)	59.24 ± 1.39 c	59.27 ± 2.13 c	0.01
	(272.50 15.50)	(273.25 9.81)	
Distilled water control	67.09 ± 2.34 b	69.85 ± 2.71 b	2.36
	(314.00 ± 10.98)	(322.10 ± 12.52)	

Means in a column with the same letter are not significantly different at the 0.05 level; *, means within rows are significantly different at the 0.05 level.

[a] Values in parentheses are numbers of eggs (sum of triplicate wells) per replicate.

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CHAPTER 7

GENERAL DISCUSSION AND CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

GENERAL DISCUSSION

Gravid females of many species of mosquitoes show a high degree of preference in selecting oviposition sites. This preference may be due to the presence of oviposition pheromones or other oviposition attractants and repellents in natural habitats (Kramer and Mulla 1980). I have dealt with the twin phenomenon of oviposition attraction and repellency exhibited by gravid females of several mosquito species. The selection by such females of oviposition sites suitable for the development of their progeny is crucial to the survival of the species and is of considerable significance in mosquito population dynamics and control (Maire 1982, 1983). The females must chose among a complex array of physically and temporally diverse habitats (Bentley and Day 1989). A wrong choice may spell the death of their offspring and the loss of their reproductive investment (Blaustein and Kotler 1993).

Females are aided in this process of selection by conspecific larvae which already occupy these habitats. Females are sensitive to substances produced by such larvae in response to their aquatic habitat (Soman and Reuben 1970, Roberts and Hsi 1977, Trimble and Wellington 1980, Bentley *et al.* 1981, Maire 1984). These messenger substances transmit information regarding environmental quality to the ovipositing females as these come into contact with the water surface (Bentley and Day 1989). Waters from normal, healthy larvae induce females to stay and oviposit (Maire 1985). However, when larvae are infected sublethally with the entomopathogenic digenean, *Plagiorchis elegans*, the chemical message is altered and females are repelled and fail to oviposit in such sites.

I have provided evidence that the repellent effect induced by *Aedes aegypti* larvae infected with *P. elegans* is proportional to the severity of the infection, both in terms of intensity and the location of the parasite in various host tissues. Larvae infected with Plagiorchiidae exhibit reduced survival and have extensive developmental changes (Dempster *et al.* 1986) and behavioral modification (Webber *et al.* 1987). Dempster *et al.* (1986) demonstrated that these same two factors also govern the virulence of infection with this parasite. Larvae become repellent to ovipositing females in response to the stress of sublethal infection and that perhaps other adverse environmental factors may also be able to induce repellency.

Indeed, two other common stressors, starvation and crowding, induced similar but less severe repellent effects. Starvation and crowding also increase larval

developmental times (Kuno and Moore 1975, Moore and Fisher 1969 respectively) and modify larval behavior (Christophers 1960). Production of growth retarding factors (GRF) by *Ae. aegypti* larvae reared under stressfull conditions has similar effects (Peters *et al.* 1969); but it remains to be established whether the repellent effects and the GRF are the same or different compounds.

The repellent effect persisted for more than one week at room temperature; the attractant effect persisted significantly longer. The stability of attractants has been assessed in different species (Kalpage and Brust 1973, Bentley *et al.* 1976, Maire and Langis 1985). Repellency remains in evidence at dilutions of 1 in 512. Such strong and persistent activity may well limit recruitment into the larval mosquito population and may thus enhance the direct lethal effects of the parasite on mosquito larvae. The final result may be a further reduction in the emergence of adult mosquitoes from such breeding sites.

Both *Plagiorchis* infection and crowding entail a decrease in the consumption of food and may therefore mimic the effects of starvation. Analysis of the body fluids of mosquito larvae subjected to parasitic infections, crowding or starvation underscored this similarity. Infected, crowded and starved larvae all experienced similar changes in sugar, lipid and protein concentrations. In the case of infection with *P. elegans*, nutritional deficits are exacerbated by the parasite's apparent ability to block the conversion of trehalose to glucose. This effect can be ameliorated experimentally by

incubating infected larvae in a dilute solution of glucose. The addition of similar concentrations of glucose to already repellent waters had no such effects.

The sensitivity to the repellent compound extends across species boundaries but not uniformly, suggesting that this phenomenon may play an important role in both intra- and interspecific competition for limited resources. The work has changed our views on competition among mosquito larvae. It has been suggested that competition among mosquito larvae is of the "scramble" type where all larvae have equal opportunity to acquire resources (Price 1984). As these resources decline, they reach a point where none of the larvae can acquire enough to attain the imago stage. As a consequence, the population crashes, only to rebound as resources are renewed. Instead, we see that competition is more of the "exclusion" type. Competition for resources is prevented from mounting, to some extent, by excluding additional competitors.

This work opens the way to deflecting ovipositing females away from sensitive areas, such as human habitations, to suboptimal breeding sites which will yield fewer adults. Sublethal effects may have a greater impact on insect control than has been realized. Researchers should address the identification and synthesis of this chemical compound(s), the effects of other stressors on various mosquito hosts and the relationships between the repellent effect and other stress-induced factors produced by mosquito larvae.

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CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

1- The oviposition repellent effect was shown to be proportional to the severity of the infection, in terms of intensity and location of the parasite in the tissues of the host.

[Zahiri, N., M. E. Rau., D. J. Lewis, and Sh. Khanizadeh. 1997. Intensity and site of *Plagiorchis elegans* (Trematoda: Plagiorchiidae) infections in *Aedes aegypti* (Diptera: Culicidae) larvae affect the attractiveness of their waters to ovipositing, conspecific females. J. Environ. Entomol. 26: 920-923].

2- Starved larvae were shown to induce oviposition repellency in conspecific, gravid females. The induction of repellency was not restricted to parasitized mosquito larvae.

[Zahiri, N., M. E. Rau, and D. J. Lewis. 1997. Starved larvae of *Aedes aegypti* (Diptera: Culicidae) render waters unattractive to ovipositing conspecific females. J. Environ. Entomol. 26: 1087-1090].

3- Other stressors such as crowding were demonstrated to produced repellent effects in conspecific females. By titrating of repellent/attractant, it was shown that suggested that *Plagiorchis elegans*- infected larvae generated the most powerful oviposition repellent effect.

[Zahiri, N., and M.E. Rau. 1997. Oviposition attraction and repellency of waters from *Aedes aegypti* (Diptera: Culicidae) larvae subjected to crowding, confinement, starvation or infection. J. Med. Entomol. (submitted for publication)].

4- *Aedes atropalpus* larvae infected with *Plagiorchis elegans* also induce oviposition repellency in conspecific gravid females, and the sensitivity to the semiochemical produced crosses species boundaries. Gravid females of *Aedes aegypti* are repelled by waters from infected *Ae. atropalpus*. The repellent effects may have a role in interspecific competition.

[Zahiri, N., M. E. Rau, and D. J. Lewis. 1997. Oviposition responses of *Aedes aegypti* and *Aedes atropalpus* (Diptera: Culicidae) females to waters from conspecific and heterospecific normal larvae and from larvae infected with the entomopathogenic digenean *Plagiorchis elegans* (Trematoda: Plagiorchiidae). J. Med. Entomol. 34: 565-568].

5- The analysis of body fluids of parasitized, starved and crowded larvae revealed similar changes in carbohydrates, proteins, and lipids in comparison to controls. The changes are characterized by a strong correlation with the oviposition repellency effect.

[Zahiri, N., G.B. Dunphy, and M.E. Rau. 1997. Effects of the entomopathogenic digenean *Plagiorchis elegans* (Trematoda: Plagiorchiidae), starvation and crowding on the serum composition of *Aedes aegypti* (Diptera:

Culicidae) larvae and the production of an oviposition repellent. J. Med. Entomol. (in press)].

6- I have developed a micro-bioassay for oviposition attractant/repellency. This allows the analysis of small samples from individual larvae. This method was used in all of the above publications.