EFFECTS OF SUBSTRATE CHARACTERISTICS ON THE VERTICAL DISTRIBUTION OF FOURTH INSTAR LARVAE OF *AEDES AEGYPTI* (DIPTERA: CULICIDAE)

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SHORT TITLE

Effect of substrate on the vertical distribution of mosquito larvae

Robert H. Paul

ABSTRACT

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Effects of substrate characteristics on the vertical distribution of fourth instar larvae of *Aedes aegypti* (Diptera: Culicidae)

Populations (n=25) of fourth instar *Aedes aegypti* were introduced into observation arenas that contained one of 5 types of substrates, and were sub-divided into 5 equal 1 cm horizontal zones. The larvae were videotaped to determine effects of food quality, food quantity, and nutrient deprivation on vertical distribution of larvae over time. At least two-thirds of the larvae consistently aggregated at the surface and on the bottom. The proportion depended on the nature of the substratum and was influenced by nutrient deprivation. In contrast, density of larvae in the 3 remaining zones was consistently low and was unaffected by either of these variables. Larvae were typically very active during a 15 minute period of acclimation upon introduction into the observation arena; subsequently, levels of activity declined. Most starved larvae in the presence of a high-quality food substrate fed to repletion faster than fed larvae, whereas in an arena devoid of food, they foraged on the bottom for a longer duration of time than the fed. With a substrate of a semi- or highly non-nutritive nature, foraging again appeared more intense among starved than fed individuals. Starved larvae consistently aggregated on the bottom, in contrast to fed individuals that became more evenly distributed between the surface and the bottom.

RESUME

Robert H. Paul

L'influence de la qualité et de la quantité de la nourriture, et de la restriction alimentaire sur la distribution verticale des larves de 4ième stade d'*Aedes aegypti*

Nous avons étudié l'influence de la qualité et de la quantité de la nourriture, et de la restriction alimentaire des individus, sur la distribution verticale des larves de 4ième stade d'*Aedes aegypti*. L'étude s'est réalisée dans des enceintes subdivisées en 5 zones horizontales contenant 25 larves d'*Aedes aegypti* et un des cinq types de nourriture. Nous avons observé qu'au moins les deux tiers des larves se regroupaient à la surface et au fond de l'enceinte, et que leur nombre dépendait de la nature de la diète et de l'état des individus, alors que dans les trois zones médianes, le pourcentage de larves était faible et aucunement influencé par l'une ou l'autre des variables étudiées. Les larvae ont démontré une forte activité durant la période d'acclimatation, soit 15 minutes après l'introduction dans l'enceinte, suivit par la suite d'une diminution de l'activité. En général, les larves ayant été privées de nourriture avant leur introduction dans les enceintes, et en présence d'une diète d'une valeur nutritive élevée, s'alimentaient jusqu'à satiété plus rapidement que celles n'ayant pas été privées de nourriture avant le début de l'expérience, tandis que dans une enceinte sans nourriture, elles passaient plus de temps à creuser au fond comparativement aux larves n'ayant pas été privées de nourriture. En soumettant les larvae à des diètes de nature semi ou très faibles valeurs nutritives, nous avons noté une fois de plus que le creusage était plus intense chez les individus restreint que ceux qui n'ont pas été restreint de nourriture à priori; ainsi les premiers se regroupaient au fond, tandis que les seconds se distribuaient également dans la colonne entre la surface et le fond.

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INTRODUCTION

Although studies examining the prevalence and distribution of both larval and adult mosquitoes over large geographic areas are abundant (*e.g.*, Kittayapong and Strickman 1993; Laird 1990; Toma and Miyagi 1992), those qualitatively examining localized spatial distribution of larvae are few (*e.g.*, Service 1971; Mogi and Wada 1973). In addition there is a paucity of published research specifically describing the vertical distribution of larvae within their environment in response to the presence of food. Food could be considered one of the most important determinants of habitat suitability.

For *Aedes aegypti* (L.) larvae, the air-water interface and the substratum are the two most important regions of activity, delimiting both vertical distribution, and perhaps more significantly, the zones where respiration and feeding occur (Christophers 1960). Larval feeding rates are influenced by such physical characteristics of the substratum as diameter, concentration, and nutritional quality of the constituent material (Aly 1985; Dadd 1971). Vertical distribution of *Aedes aegypti* larvae in the water column may also be governed by these same properties of the substratum.

Mosquitoes are the most significant vectors of pathogens worldwide, and consequently more research has been devoted to their control than any other group of medically important arthropods (Harwood and James 1979). The problems of resistance and pollution, arising from widespread reliance on synthetic insecticides during the past several decades have necessitated the development of biological control programmes as an

alternative to chemicals (WHO 1992). Chemical control is density-independent, inducing catastrophic mortality on the entire treated population, regardless of population number. Conversely, biological control facilitates an advantageous distribution of parasites and predators introduced into larval habitats to maximize predator-prey encounters and reduce predator energy expenditures in searching for prey (Service 1981). The rationale behind studying how characteristics of the substratum influence the natural feeding behaviour and vertical distribution of *Aedes aegypti* larvae is that a greater understanding of how to enhance the probability of encounter between the host and the potential parasite or predator could be utilized to maximize the impact of biocontrol applications in the field.

The primary objectives of this research were to examine the effects of larval food quality and quantity, as well as nutrient deprivation (*i.e., starvation*), on the vertical distribution of a population of 4th instar *Aedes aegypti*, and how percentages of larvae within horizontal strata changed over time.

LITERATURE REVIEW

Introduction

Mosquitoes have a cosmopolitan distribution ranging from temperate to tropical regions with the exception of Antarctica (Service 1980). Of the 300 described species of mosquitoes, approximately 150 occur in temperate North America (Harwood and James 1979), and of these at least 74 are found in Canada (Wood *et al.* 1979). There are 3 subfamilies: (i) ***Anophelinae***: of the 3 genera in this subfamily, *Bironella* and *Chagasia* have a restricted distribution and are of little medical importance. However the third, *Anopheles*, is an important vector of diseases, including malaria (Harwood and James 1979). (ii) ***Toxorhynchitinae***: only 1 genus with 65 species has been described. Because adults of this genus lack a piercing proboscis and therefore cannot take blood meals, they are not of medical importance (Harwood and James 1979). (iii) ***Culicinae***: this, the largest group, contains 30 genera of mosquitoes of which *Culex*, *Aedes*, *Mansonia*, *Sabethes*, *Haemogogus*, and *Psorophora* are considered the most important as vectors of disease (Harwood and James 1979).

Medical Importance of Mosquitoes

Species in the subfamily *Culicinae* are particularly important because they are vectors of two widely disparate groups of pathogens that annually affect more than 200 million people worldwide: the filarial worms which include the human filariae *Wuchereria bancrofti* (Cobbold) and *Brugyi malayi* (Brug), and viruses. The latter comprise at least 90

organisms isolated from mosquitoes (Harwood and James 1979). *Aedes aegypti* is one of the most important vectors of Yellow Fever and Dengue, and has also been implicated in the transmission of Chikungunya and Zika viruses (WHO 1984).

Biology of *Aedes aegypti*

Aedes aegypti is widely distributed globally between latitudes 45° north and 35° south, and is known to exist, or to have existed, in all countries or territories in the western hemisphere except Canada (Anonymous 1979).

Females of *Aedes aegypti* oviposit readily in aquatic habitats, both natural basins such as ponds, puddles, and water filled tree holes, as well as artificial containers, such as buckets, tires, and clogged roof gutters. The eggs are typically aggregated at or near the surface of the water, and attached to the sides of the container or basin. About 2 to 3 days are required for the embryos to develop into fully formed larvae within the egg. Adverse environmental conditions, such as low humidity which may lead to desiccation of the eggs, do not appear to affect the viability of the embryo within the egg, provided that it is fully developed. First instars hatch when the eggs become submerged in the water and are consequently subjected to decreased levels of oxygen (Anonymous 1979). The 1st instar emerges from the egg, and three successive moults give rise to 2nd, 3rd, and 4th instars with a concomitant increase in length from 1 to 8 mm. The larvae are primarily filter feeders and browsers, and under optimal conditions require 5 to 7 days to

develop to the pupal stage. The pupa does not feed. Adults emerge two to three days later. After emergence, adults disperse no more than a few hundred meters, and mate within several hours. Whereas both males and females feed on nectar, females also require a bloodmeal for the production of eggs (Anonymous 1979). Two to three days after the bloodmeal, the eggs are ready to be laid and the female seeks an oviposition site.

Behavioural Studies of Mosquito Larvae

Shannon (1931) appears to have been the first to systematically classify larval mosquito habitats according to both physical and chemical characteristics. He noted the specificity of certain species to well-defined habitat types (*e.g.*, artificial containers vs. natural basins), but more importantly demonstrated that behavioural variation in response to physical stimuli can be used to distinguish between genera and some species of mosquitoes.

While ethological studies of mosquito larvae have been ongoing for at least 60 years, only very recently has a behavioural catalogue consisting of descriptions of position, posture, and movement of larval *Aedes triseriatus* (Say) been published (Walker and Merritt 1991). This list has standardized the use of terms, some of which were applied inconsistently in past behavioural studies, and has imparted new precision to the terms used to describe the behaviour of larvae.

Detailed information describing food utilization by mosquito larvae, as well as the feeding behaviour of various species was published by

Nilsson (1987). This author calculated energy budgets for the assimilation of nutrients and, more importantly, differences in feeding time allocation.

Waldbauer and Friedman (1991) proposed that self-selection of optimal diets by insects is governed by peripheral sensilla receptive to chemosensory stimuli and by toxins or lack of nutrients that cause metabolic disruptions in metabolic routes. They suggested that the feeding behaviour of an insect can be variable as a consequence of self-selection of an optimal diet.

Mouthbrush Morphology and Methods of Feeding of *Aedes aegypti* Larvae

Recent studies investigating the mechanisms of particle retention (Dahl *et al*. 1988; Rashad and Mulla 1990), feeding currents (LaBarbera 1984; Rashad and Mulla 1990), and particle capture (Dahl *et al*. 1990; Merritt and Craig 1987) in mosquito larvae have confirmed earlier findings that feeding habits are determined by morphological specialization of mouthparts (Pucat 1965; Surtees 1959).

According to Clements (1963) and Merritt and Wallace (1981), mosquito larvae as a group may: (i) filter feed by extracting microorganisms and particulate organic debris suspended in the water column, (ii) browse using modified mouthparts allowing them to scrape food particles off the organic debris on the bottom, (iii) feed on the layer of living and non-living material floating at the surface of the water, or (iv) be predaceous, and consume small aquatic organisms including other mosquito larvae. Although species can be characterized according to their

primary mode of feeding, most may secondarily use any of the other methods.

Aedes aegypti larvae, which are primarily browsers, possess mouthparts that, relative to the mouthparts of filter feeders, have shorter labral brushes with some serrated thick hairs, rectangular maxillae with shorter thicker brushes, and moderately sclerotized mandibles (Anonymous 1979). Because they are primarily browsers, they normally feed on particulate matter present in the sediment at the bottom of their habitat (McIver and Siemicki 1977). When feeding, the larvae descend to and glide along the bottom (or less commonly the sides), propelled by their feeding brushes which detach matter from the substrate (Christophers 1960). The material is carried into the pharynx by the currents produced by the pharyngeal brushes, then ingested (Widahl 1992).

Food Preference and Nutritional Requirements of *Aedes aegypti* Larvae

Mosquito larvae survive in a wide variety of habitats which differ in pH, temperature, water depth, and surface area (Laird 1988). From these different types of habitats the larvae must acquire an abundant supply of microflora and fauna for their growth and development (Clements 1963).

Investigations attempting to describe and identify the natural foods ingested by culicid larvae date back at least 60 years. Gut content analyses of 17 species of mosquito larvae, including *Aedes aegypti*, by authors such as Hinman (1930; 1933), Howland (1930), and Rozeboom (1935) revealed their diet to consist of a diversity of material including

filamentous green algae, unicellular green algae (including desmids), diatoms, blue green algae, green flagellates, protozoans, rotifers, crustaceans, spores, and bacteria. Contemporary work by Ameen and Iversen (1978) has yielded similar conclusions regarding the types of food ingested by several species of *Aedes*. The more recent studies have placed less emphasis on larval gut content analyses, and more on ecological considerations such as feeding behaviour and mechanisms employed by larvae, including *Aedes aegypti*, to obtain food (Merritt *et al.* 1992). The role of detritivory in the growth of larvae and yield of adults has also been studied (Fish and Carpenter 1982).

Most studies on the nutritional requirements of mosquito larvae have used *Aedes aegypti* as the experimental subject. Preliminary biochemical studies by individuals like Trager (1935a; 1935b; 1936) attempted to ascertain the importance of certain growth-promoting accessory foods such as solutes (Hinman 1930) and sterile mediums of yeast and liver (Trager 1935a) to *Aedes aegypti* larvae. Later studies by DeMeillon *et al.* (1945) encompassed similar objectives but with a different approach: growth promoting foods were replaced with known substances, providing a completely synthetic diet for *Aedes aegypti* larvae. In an approach similar to Trager (1935a; 1935b; 1936) observations on *Aedes aegypti* were made to gauge the importance of dietary components such as essential water soluble factors from yeast (Golberg *et al.* 1945), lipids (Golberg and DeMeillon 1948a), proteins (Golberg and DeMeillon 1948b), and amino acids (Golberg and DeMeillon 1947) on growth and development. Subsequent research coalesced these findings by attempting to develop the best possible chemically-defined diet consisting of only necessary ingredients

in optimal amounts. This research was important because Akov (1962) and Lea and DeLong (1958) subsequently discovered that when the larval diet was suboptimal, results of experiments testing the effects of, *e.g.*, chemicals, were characteristically highly variable. Furthermore, being able to manipulate larval diet was important in studies that examined the correlation of nutritional reserves with critical weight for pupation in larval mosquitoes (Chambers and Klowden 1990).

Factors Affecting Feeding Rates of Mosquito Larvae

Factors influencing the feeding rate of mosquito larvae have been extensively investigated using *Culex pipiens* L.. Ingestion rates of non-predaceous omnivorous mosquito larvae are governed by a complex of factors (Rashad and Mulla 1989). Rates are easily measured by allowing larvae to fill their gut with readily identifiable inert material and comparing this with rates at which it is displaced upon transfer of the larvae to media containing more suitable nutrients (Dadd 1968; Dadd 1970a).

As with terrestrial larval insects (Mellanby and French 1958), mosquito larvae normally ingest small volumes of water at a "baseline" rate, and consequently displace material present in the peritrophic membrane usually in about 1 hour (Dadd 1968; 1970a). This "baseline" rate of ingestion is influenced by: phagostimulants, quality of particulate matter, and size and concentration of particles.

Phagostimulants

The presence of solutes such as the metabolites in "larval water" (water in which larvae were held overnight) (Dadd 1973), viscous colloid solutions (Dadd 1975), and water soluble yeast extracts (Dadd 1970b) modify this baseline rate in *Culex pipiens* larvae. For *Aedes vexans* (Meigen) the rate is influenced by the presence of fishmeal extracts (Aly 1985). Dadd *et al.* (1982) have suggested that strong stimulation by organic solute mixtures are an additive effect of several individually moderate and weak phagostimulants in mixture: in other words, no one phagostimulant alone will adequately elicit a strong stimulatory response.

Quality of Particulate Material

While non-selective ingestion of food may occur in some species of mosquitoes, larvae of *Culex pipiens* and *Aedes vexans* have been shown to select nutritive particulate materials for ingestion more readily than inert particles (Rashad and Mulla 1989). For example, larvae of *Aedes vexans* offered food items such as wheat flour, yeast, and fishmeal ingested these food particles on average 3 times faster than those offered inert particulates such as kaolin, pumice, or synthetic cellulose (Aly 1985). Similarly, larvae of *Anopheles albimanus* Wiedemann offered food items such as blood meal, liver powder, alfalfa flour, and wheat flour ingested these materials 6 to 9 times faster than those offered inert particulates such as kaolin, chalk, and charcoal (Aly and Mulla 1986).

Size and Concentration of Particles

Dadd (1971) found that larvae of *Culex pipiens* discriminate between food particles of varying sizes: optimal ingestion occurred with particles ranging in diameter from 0.71 μm to 1.86 μm for the first instar, and averaging 7.6 μm for the second and third instars, and 26 μm for the fourth instar. For all instars, ingestion rates declined noticeably with decreasing mean particulate diameter below 0.71 μm. Similarly, particles larger than those associated with optimal ingestion rates for each instar decreased ingestion rates. Later work on *Aedes triseriatus* by Merritt (1987) confirmed the findings of Dadd (1971), in that particulate material size affected larval ability to ingest nutrients. Using latex particles with a mean diameter of 1.01 μm, Dadd (1971) found that ingestion rates for *Culex pipiens* larvae decreased rapidly with suspensions less than or equal to 0.03% solids by weight. Dadd (1971) suggested that this threshold concentration represents a point where as ingestion proceeds, depletion lowers particle concentrations sufficiently to decrease ingestion rates. Dadd (1971) also found that ingestion rates plateaued at food concentrations equal to or higher than 0.06% solids by weight, and concluded that the presence of filtering larvae did not effectively deplete or alter food availability at higher concentrations.

Reaction of Mosquito Larvae to Physical Variables such as Shadows, Changes in Light Intensity, Mechanical Disturbance, and Parasites

Apart from feeding, grooming, and swimming, larvae exhibit the diving response when disturbed. They dive by actively swimming (*Aedes*) or

passively sinking (*Culex* and *Anopheles*) to the bottom of the habitat (Mellanby 1958). After being disturbed, individual larvae of *Aedes aegypti* return to the surface after approximately 21.4 seconds (Duhrkopf and Benney 1990), but take about 4 minutes when part of a group (Mellanby 1958).

Repetitions of shadows (Thomas 1950) and mechanical disturbances of the habitat (Folger 1946) elicit the diving response in *Culex fatigans* (Wiedemann) and *Culex pipiens*, respectively. In addition to these two stimuli, sudden changes in light intensity (Christophers 1960) elicit the diving response in *Aedes aegypti*, as well as exposure to parasites such as *Plagiorchis noblei* (Park) (Trematoda: Plagiorchiidae) (Dempster and Rau 1987; Webber and Rau 1987)

Response of *Aedes aegypti* to Intraspecific Competition

In *Aedes aegypti*, as well as other species of mosquitoes, mortality is higher in overcrowded populations resulting in densities approaching optimal, i.e., those levels affording least impediment to the survival of the population (Barbosa *et al.* 1972).

Some of the manifestations of overcrowding for *Aedes aegypti* include: (i) growth inhibition, increased larval mortality, and decreased adult size and fecundity (Bar-Zeev 1957; Wada 1965), (ii) significant drops in the metabolic activity of the mosquito larvae (Barbosa and Peters 1973), (iii) the production of growth retardant factors (GRF) - heat stable metabolites - which give the larvae a competitive advantage over rival species in habitats where they co-exist (Moore and Fisher 1969;

Bickley 1972), and (iv) distortions of the sex ratio, resulting in more males. This last effect of overcrowding was originally attributed to genetics (Hickey 1970). However, Barbosa *et al.* (1972) subsequently proved that as the density of *Aedes aegypti* larvae increased, the percentage of females produced moved closer to equalling the male percentage, suggesting that sex ratios could be dependent upon changes in stress produced by overcrowding.

Observational Techniques for Larval Mosquito Behaviour

Early studies examining larval behaviour in response to physical stimuli yielded data through qualitative and direct observation of the larvae (*e.g.*, Folger 1946; Hocking 1953; Thomas 1950). The recent development of lightweight and reliable videocassette recorders has solved one of the most important problems in observational studies: researchers cannot continuously observe and record all the behaviour of all the members of a group, and therefore obtain only a partial record (Altmann 1974). Videotapes provide a permanent record that can be reviewed repeatedly, and allow the researcher to obtain a more complete picture of the behavioural profile. The use of videotapes for documenting larval mosquito behaviour, *e.g. Aedes triseriatus* (Walker and Merritt 1991), *Aedes aegypti* (Webber and Rau 1987), and *Culiseta morsitans* (Theobald) and *Aedes communis* (De Geer) (Widahl 1992), has become more commonplace.

Mosquito Control

Biological control of mosquitoes uses alternative strategies that seek to eliminate the problems of pesticide resistance, pollution, and research and development costs. Natural enemies such as predators, parasites, and pathogens act in a density dependent manner, in contrast to insecticides which cause catastrophic density independent mortality. Successful biological control of mosquitoes requires more detailed knowledge, especially quantitative data, than is required for insecticidal measures (Service 1981).

In either case (insecticides or biological control) mosquito control agencies must continuously monitor the abundance and distribution of mosquito larvae, *e.g.*, *Aedes aegypti* (Sheppard *et al*. 1969) as indicators of when treatments are required (Walton *et al*. 1990).

Chemical Control

Prior to World War II, adult mosquito control was confined to indoor space-spraying with pyrethrum for malaria control (Fontaine 1983). The successes achieved by this approach in controlling malaria-transmitting *Anopheles* spp. effectively spurred further research which eventually led to the development of more toxic and persistent insecticides such as DDT.

Because of the medical importance of mosquitoes in vectoring several major diseases, and the considerable discomfort caused by their bites (Harwood and James 1979; Burgess 1990), literature concerning methods of chemical control of this species abounds.

(1) Adulticides: because mosquitoes undergo complete metamorphosis,

control procedures can be directed separately at larvae or adults. As only adult Culicidae pose problems to humans, control measures directed against their immature stages tend to be more desirable. The relationship between the initial number of larvae and the number of resultant adults is proportional: in other words, the larger the number of larvae, the greater the adult population (Mogi 1981).

The major groups of adulticides recommended by the United States Department of Health, Education, and Welfare (Anonymous 1979), and the World Health Organization (1984) were organophosphates such as malathion, fenthion, chlorpyrifos, and temephos, along with carbamates such as propoxur, and pyrethroids such as permethrin. The organochlorines such as DDT, though banned from North America in the early 1970's and consequently omitted from the list, are still used for mosquito control in developing countries (Brown 1986). Recent work has, however, recorded extensive resistance in populations of mosquitoes to the organophosphates (Gratz 1991; Reyes-Villanueva *et al.* 1990; WHO 1992), carbamates (Mekuria *et al.* 1991), and pyrethroids (Miller 1988) mentioned above.

(ii) Larvicides: organophosphates such as chlorpyrifos, fenthion, malathion, and temephos and the organochlorine methoxychlor were also recommended as effective larvicides (Anonymous 1979; WHO 1992).

Presently, one of the more promising groups of larvicides are insect growth regulators (IGR's), such as methoprene (WHO 1992), because of their high level of activity and efficacy against mosquitoes, including *Aedes aegypti*, and good margin of safety to non-target biota (Mulla *et al.* 1989). Unfortunately, as with the adulticides, recent research by Mekuria

et al. (1991) with chlorpyrifos, fenthion, malathion, and temephos has demonstrated a noticeable resistance to these compounds in populations of mosquitoes wherever they have been used extensively. Mekuria *et al.* (1991) concluded that the problem of resistance at their study site (Santa Domingo, Dominican Republic) warranted consideration of control measures other than the continued use of residual insecticides.

Biological Control

Investigation of biological control measures gained early popularity in the 1930's, but was largely abandoned when cheaper and more effective synthetic pesticides were developed and became available for widespread use. The ensuing total reliance on chemical pesticides produced numerous unexpected, deleterious side effects such as environmental contamination with extremely persistent chemicals, evolution of insecticide resistance in mosquitoes, increases in numbers of both old and new pests, and the exceptionally high cost of continual development of these chemicals (WHO 1992). Faced with these problems, many entomologists in the 1960's reconsidered biocontrol as a means not of eradicating target arthropods (such as mosquitoes and blackflies) but of reducing their populations to levels at which they no longer constitute a biting nuisance or pose a major health problem (Service 1981; 1983).

Biological control utilizes natural enemies (predators, parasites, or pathogens) to suppress target insect pest populations to levels assuring the minimization of pest/vector problems. Disadvantages associated with this approach (*i.e.*, the lack of marketability, difficulty in estimating reliability, and problems with the rearing, maintenance, and transport of

the biocontrol agents) are more than offset by the advantages (*i.e.*, high host specificity consequently reducing losses of beneficial insects, sustained control over a prolonged period of time, and natural recycling of the parasite). Consequently the development of biological control programs for pests, especially mosquitoes, has become desirable (Service 1981; 1983). Some of the biological control agents of mosquito larvae that have been studied for their practicality and feasibility include mermithid nematodes (Bailey and Gordon 1973; Hominick and Tingley 1984), digenean trematodes (Dempster and Rau 1987; Dempster *et al.* 1986; Rau *et al.* 1991; Webber *et al.* 1987), copepods (Brown *et al.* 1991; Rivière *et al.* 1987), fungi (Frederici 1981), ciliated Protozoa (Barrett 1968; Grassmick and Rowley 1973), larvivorous fish (Gerberich and Laird 1985; Lardeux 1992), predatory mosquito larvae (Gerberg 1985), and bacteria particularly *Bacillus thuringiensis* var. *israelensis* (Ali *et al.* 1981; Khawaled *et al.* 1988; Becker and Margalit 1993; Becker *et al.* 1992). Chemical derivatives from plants, otherwise known as botanicals, also show great promise for the control of both larval and adult mosquitoes because their novel modes of bioactivity act as ovipositional deterrents, repellents, growth inhibitors, or general toxicants (Green *et al.* 1991; Sukumar *et al.* 1991).

MATERIALS AND METHODS

Rearing *Aedes aegypti*

A colony of *Aedes aegypti* mosquitoes was maintained at 27°C, with a 14:10hr (light:dark) photoperiod, at the McGill University Institute of Parasitology. Because females of *Aedes aegypti* are anautogenous, a blood meal was provided at least once a week. Adults were provided with a 10% sucrose solution as a supplemental source of nutrients.

Oviposition sites were plastic containers (15 cm x 15 cm x 8.5 cm) lined with filter paper and filled with distilled water to a depth of 6.5 cm. Filter papers containing eggs were removed from the colony weekly, allowed to air dry, and stored in a sealed container at 4°C.

To deoxygenate the water, approximately 200ml of distilled water was placed in a 500ml Erlenmeyer flask, boiled for 30 minutes, stoppered immediately after removal from the heat, and allowed to cool to room temperature. The stopper was then removed, a section of filter paper with adhering eggs placed in the flask, and then the flask resealed. The contents were swirled to ensure that all eggs were moistened.

Eggs hatched approximately 2 to 4 h later. The flask containing first instar *Aedes aegypti* larvae ("rearing flask") was placed in an incubator at 25°C with a 14:10hr (light:dark) photoperiod. Fish food (Nutra Fin™ Livebearer Food for Guppies, Mollies and Swordtails) (refer to Appendix 1 for ingredients and composition) was finely pulverized in a mortar, and provided *ad libitum* in the rearing flask as a source of nutrients until the larvae reached the fourth instar.

Description of Experimental Apparatus

A Plexiglass arena (10.0 cm x 10.0 cm x 1.5 cm) was used to observe the behaviour and assess the pattern of vertical distribution of the introduced larvae (Fig. 1). The arena was filled to a depth of 5 cm with distilled water (25°C), and secured to a base sitting on a layer of polyethylene foam. The observation arena was divided into five horizontal 1 cm zones with zone 1 corresponding to the surface zone, zones 2, 3, and 4 in the middle of the water column, and zone 5 corresponding to the bottom zone. A Javelin Electronics Colour Television Camera (Model #:JE3012) with a 18-108/2.5 Japan Lens No.4511719 zoom lens was mounted opposite the observation arena, and adjusted until the image of the arena filled the entire screen. The camera was connected to an external Panasonic Time Lapse Videocassette Recorder (Model #:AG-6720) and black and white video monitor. The base with the mounted camera and observation arena were placed in an incubator set at 25°C, and illuminated with fluorescent light tubes (Fig. 2). The videotapes of data were viewed on a Hitachi Colour Display Monitor (Model #: CM-1481) using a General Electric Video Cassette Recorder (Model #:1CVD5025).

Description of Treatments and Introduction of Larvae into the Observation Arena

For the treatments, commercially available fish food (Nutra Fin™) and aquarium charcoal (Hagen™ Activated Carbon) were used in various mixtures or quantities as constituents of the foraging substratum in the observation arena. Fish food and aquarium charcoal were

Figure 1. Frontal view of the observation arena containing water to a depth of 5 cm, and 25 4th instar *Aedes aegypti*.

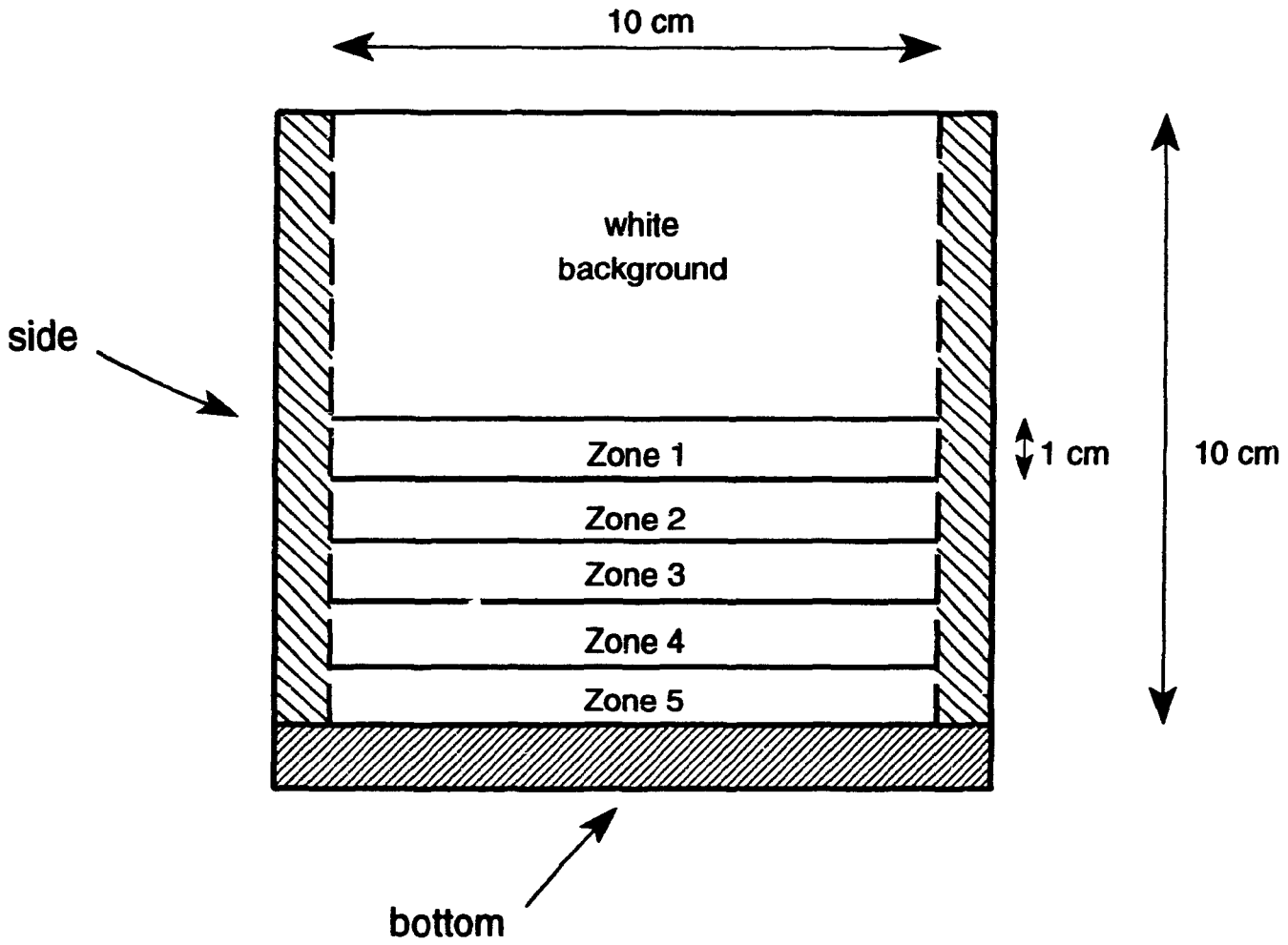
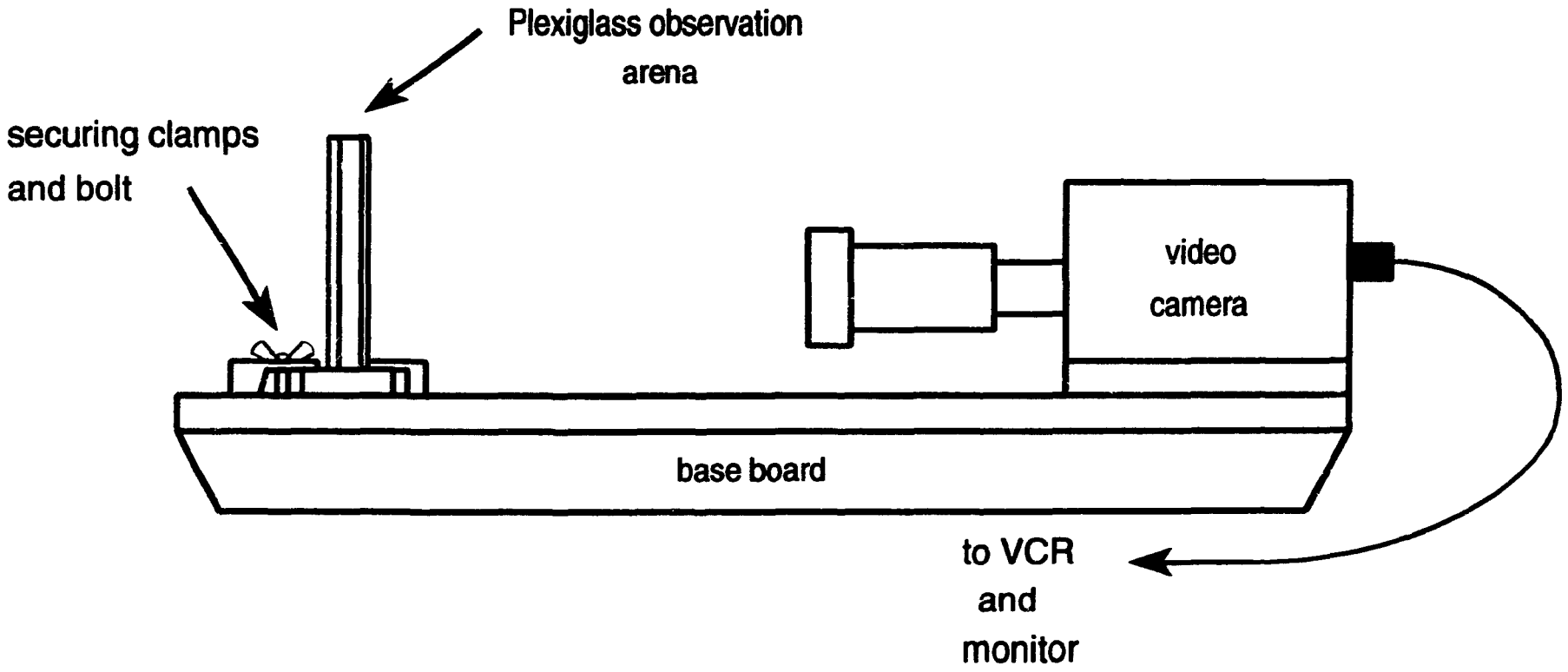

10 cm
white
background
side
Zone 1
Zone 2
Zone 3
Zone 4
Zone 5
1 cm
10 cm
bottom

Figure 2. Side view of the experimental apparatus used for filming the larvae.


Plexiglass observation
arena
securing clamps
and bolt
video
camera
base board
to VCR
and
monitor

finely ground in separate mortars for about 10 minutes to produce the particulate material that was used to form these different substrates. To ensure the consistency of the treatments among replicates and between the starved and fed larvae, quantities of substrate material sufficient to repeat any treatment 15 times was prepared and used. To determine the abundance of the various sizes of resulting particles, approximately 0.5g each of the fish food and inert charcoal were first placed in separate stoppered vials which were then topped off with distilled water and allowed to sit for 2 hours. Subsequently, a pipette was used to agitate the contents of each vial, and a 0.05ml sample was aspirated, placed on a slide, and covered with a coverslip. The slide was examined under 400x magnification on a Wild Heerbrugg stereoscopic compound microscope. Using an ocular micrometer, all the particulate material present in a transect 1353.6μm long by 42.3μm wide (corresponding respectively to the diameter of the field of view and the length of the hash marks on the micrometer) was measured (refer to Appendix 2 for dimensions of particle sizes); a total of 10 samples was used. For a comparison of the mean diameter and the range of particle sizes between the two types of ground material, see Appendix 3.

Approximately 25 fourth instars were aspirated from their rearing flask into a plastic isolation chamber (15 cm x 15 cm x 8.5 cm) filled to a depth of 5 cm with distilled water at 25°C. Fishfood was either present to excess or absent in the isolation chamber. Larvae kept in the isolation chamber in the absence of ground food for 1 hour before being introduced into the observation arena were considered "starved"; conversely those kept in the presence of an excess amount of food for 1

hour prior to introduction were considered "fed".

Both the "fed" and "starved" groups of larvae were placed into observation arenas where treatments consisted of allowing the larvae to "feed" on substrates of varying food qualities and quantities as follows:

- **Treatment OF:** Overabundance (*i.e.*, high density) of highly nutritious particulate material (0.35g of food).
- **Treatment RE:** Reduced quantity (*i.e.*, low density) of highly nutritious particulate material (0.01g of food).
- **Treatment NF:** Particulate material absent from the observation arena.
- **Treatment MX:** A mixture of equal volumes of highly nutritious particulate material and inert carbon present to excess (*i.e.*, high density) (0.35g mixture of food and charcoal in total), providing a substrate of mediocre nutritional quality present to overabundance.
- **Treatment CH:** Substrate consisting of particulate material of low nutritional quality, but at an overabundance (*i.e.*, high density) (0.35g of charcoal).

The particulate materials used in treatments OF, RE, MX, and CH were introduced into the observation arena 2 hours before the start of videotaping, to allow the material to become saturated and to settle to the bottom. After this 2 hour period, either 25 "fed" or "starved" fourth instar *Aedes aegypti* were transferred from their isolation chamber to an observation arena. Five replicates of each treatment were performed for both the fed and starved larvae.

Methods of Data Collection

After transferral from the isolation chamber to the observation arena, the larvae were videotaped for 2 hours. To eliminate any diurnal variation in larval behaviour, videotaping was consistently started at 14h00. Observations were taken over the first hour of each 2 hour videotaping period as follows:

Four minutes after the videotaping had begun, and at each subsequent two minute interval, the videotape was stopped and the vertical distribution of the population was determined by counting the larvae present (i) at the surface (zone 1) of the water in the uppermost 1 cm zone of the water column, (ii) in the middle three 1 cm zones (Zones 2, 3, and 4) of the observation arena, and (iii) in the bottom 1 cm zone (zone 5) foraging on particulate material present there.

The data that were collected over the full 1 hour observation period were divided into 4 time intervals of approximately 15 minutes each, to simplify the analysis. Each one of these "time intervals" consequently consisted of eight observations taken within this quarter-hour time frame. The observations, as previously mentioned, were made two minutes apart.

Statistical Analyses

The data were analyzed using the following statistical procedure: the number of larvae counted in a zone for any combination of time, treatment, and pretreatment conditions were divided by the total number of larvae in the observation arena, to obtain the percentage of larvae present in a specific zone for specific conditions. Arcsine transformations were then used to transform these percentage values. A

multivariate repeated measures analysis of variance (MANOVAR) was performed as an overall analysis on the entire set of data to identify the overall trends or specific highly significant effects at the 0.05 level of statistical significance. To examine more closely the patterns of significant effects, means were compared using Tukey's Studentized Range Test. The analyses were performed using SAS (ver. 6.03) (SAS/STAT User's Guide 1988).

RESULTS

The introduction of fourth instar *Aedes aegypti* into the observation arena typically elicited the diving response: larvae rapidly wriggled to the bottom of the chamber. Larval movement along the bottom substrate appeared to be random for approximately 1 minute following introduction. Activity was generally vigorous, with periodic accumulation in the corners of the arena. Thereafter, larval activity gradually diminished, and individuals began slowly floating to the surface. A proportion of the population, however, was consistently found at the bottom. Larvae at the surface were motionless for intervals of several seconds to minutes. Periodically individual larvae would swim to the bottom and sift through particulate material for short periods of time. During their descent, larvae at times momentarily ceased wriggling for 1 to 2 seconds, only to resume and continue downwards. Similarly, while some larvae floated directly from the bottom to the surface, others exhibited brief episodes of downward swimming that delayed their ascent. No filter feeding, grooming, or exploratory behaviours were evident during these pauses which occurred primarily in the mid-regions of the water column (zones 2, 3, and 4). The data obtained are presented in Tables 1 through 7, as follows.

DISTRIBUTION OF LARVAE BETWEEN ZONES

Differences between zones in the abundance of larvae was highly significant (MANOVAR, $F=684.630$, $df=4,160$ $p<0.05$), regardless

TABLE 1. Effects of treatments OF, RE, NF, MX and CH on the distribution of fed larvae between zones 1, 2, 3, 4 and 5.

Time Interval	Zone	OF Mean % Larvae	RE Mean % Larvae	NF Mean % Larvae	MX Mean % Larvae	CH Mean % Larvae
1	1	30.49a	30.98a	25.10b	25.23b	28.33b
	2	5.71b	10.88b	10.24c	7.90c	7.38c
	3	11.79b	11.96b	8.83c	9.03c	9.69c
	4	10.90b	11.92b	15.71c	12.06c	10.52c
	5	41.12a	34.26a	40.13a	45.79a	44.08a
2	1	43.84a	36.00a	32.82a	27.04b	30.10b
	2	7.97 c	11.11b	9.39b	8.61c	10.01c
	3	9.02 c	10.74b	10.82b	11.69c	8.44c
	4	14.44bc	11.28b	13.53b	13.41c	11.02c
	5	24.73b	30.88a	33.44a	39.25a	40.43a
3	1	46.58a	40.09a	32.41a	30.31a	34.61a
	2	8.94c	11.95c	9.57b	9.09b	8.61b
	3	9.41c	7.46c	10.03b	9.99b	10.32b
	4	11.86c	9.37c	11.22b	12.73b	11.11b
	5	23.20b	31.13b	36.76a	37.89a	35.35a
4	1	52.00a	43.23a	35.10a	31.02a	31.94a
	2	8.41c	8.31c	10.29b	8.43b	10.12b
	3	9.43c	8.30c	10.17b	9.31b	8.97b
	4	8.61c	8.17c	9.67b	13.78b	10.57b
	5	21.55b	31.99b	34.78a	37.46a	38.41a

Means within the same column and the same time interval having the same letter are not significantly different $p>0.05$

TABLE 2. Effects of treatments OF, RE, NF, MX and CH on the distribution of starved larvae between zones 1, 2, 3, 4 and 5.

Time Interval	Zone	OF Mean % Larvae	RE Mean % Larvae	NF Mean % Larvae	MX Mean % Larvae	CH Mean % Larvae
1	1	30.29a	35.85a	23.19b	16.93b	17.55b
	2	7.19b	10.96b	7.49c	4.85c	5.66c
	3	9.47b	12.41b	11.00c	7.96c	8.71c
	4	12.07b	10.67b	12.88c	14.37b	10.75c
	5	40.98a	30.12a	45.44a	55.83a	57.33a
2	1	44.16a	42.22a	27.17b	23.55b	25.32b
	2	9.84c	9.93c	8.46c	6.81c	7.97c
	3	9.73c	10.78c	10.61c	8.17c	9.63c
	4	8.45c	9.45c	10.97c	11.83c	9.62c
	5	27.82b	27.62b	42.80a	49.64a	47.45a
3	1	42.35a	43.56a	31.07b	24.93b	26.84b
	2	9.62c	9.15c	8.38c	7.48c	6.80c
	3	9.04c	9.42c	7.44c	8.78c	7.13c
	4	10.98c	9.42c	8.41c	10.65c	11.57c
	5	28.00b	28.45b	44.70a	48.17a	47.66a
4	1	45.46a	46.37a	30.46b	23.84b	23.50b
	2	7.80c	8.08c	7.15c	7.72c	6.81c
	3	10.28c	6.68c	8.89c	7.92c	9.28c
	4	8.66c	9.68c	11.65c	10.37c	7.89c
	5	27.79b	29.19b	41.85a	50.16a	52.53a

Means within the same column and the same time interval having the same letter are not significantly different $p>0.05$

TABLE 3. Changes over time in fed larvae in zones 1, 2, 3, 4 and 5 in response to treatments OF, RE, NF, MX and CH.

Zone	Time Int.	OF Mean % Larvae	RE Mean % Larvae	NF Mean % Larvae	MX Mean % Larvae	CH Mean % Larvae
1	1	30.49b	30.98 b	25.10 b	25.23a	28.33 b
	2	43.84a	36.00ab	32.82ab	27.04a	30.10ab
	3	46.58a	40.09a	32.41ab	30.31a	34.61a
	4	52.00a	43.23a	35.10a	31.02a	31.94ab
2	1	5.71a	10.88a	10.24a	7.90a	7.38a
	2	7.97a	11.11a	9.39a	8.61a	10.01a
	3	8.94a	11.95a	9.57a	9.09a	8.61a
	4	8.41a	8.31a	10.29a	8.43a	10.12a
3	1	11.79a	11.96a	8.83a	9.03a	9.69a
	2	9.02a	10.74ab	10.82a	11.69a	8.44a
	3	9.41a	7.46c	10.03a	9.99a	10.32a
	4	9.43a	8.30cb	10.17a	9.31a	8.97a
4	1	10.90ab	11.92a	15.71a	12.06a	10.52a
	2	14.44a	11.28ab	13.53ab	13.41a	11.02a
	3	11.86ab	9.37ab	11.22 b	12.73a	11.11a
	4	8.61 b	8.17 b	9.67 b	13.78a	10.57a
5	1	41.12a	34.26a	40.13a	45.79a	44.08a
	2	24.73b	30.88a	33.44a	39.25a	40.43ab
	3	23.20b	31.13a	36.76a	37.89a	35.35 b
	4	21.55b	31.99a	34.78a	37.46a	38.41ab

Means within the same column and the same zone having the same letter are not significantly different $p>0.05$

TABLE 4. Changes over time in starved larvae in zones 1, 2, 3, 4 and 5 in response to treatments OF, RE, NF, MX and CH.

Zone	Time Int.	OF Mean % Larvae	RE Mean % Larvae	NF Mean % Larvae	MX Mean % Larvae	CH Mean % Larvae
1	1	30.29b	35.85 b	23.19 b	16.93b	17.55 b
	2	44.16a	42.22ab	27.17ab	23.55a	25.32a
	3	42.35a	43.56a	31.07a	24.93a	26.84a
	4	45.46a	46.37a	30.46a	23.84a	23.50ab
2	1	7.19a	10.96a	7.49a	4.85a	5.66a
	2	9.84a	9.93a	8.38a	6.81a	7.97a
	3	9.62a	9.15a	8.46a	7.48a	6.80a
	4	7.80a	8.08a	7.15a	7.72a	6.81a
3	1	9.47a	12.41a	11.00a	7.90a	8.71a
	2	9.73a	10.78ab	10.61a	8.17a	9.63a
	3	9.04a	9.42 b	7.44 b	8.78a	7.13a
	4	10.28a	6.68c	8.89ab	7.92a	9.28a
4	1	12.07a	10.67a	12.88a	14.37a	10.75ab
	2	8.45a	9.45a	10.97ab	11.83a	9.62ab
	3	10.98a	9.42a	8.41 b	10.65a	11.57a
	4	8.66a	9.68a	11.65ab	10.37a	7.89 b
5	1	40.98a	30.12a	45.44a	55.83a	57.33a
	2	27.82b	27.62a	42.80a	49.64a	47.45 b
	3	28.00b	28.45a	44.70a	48.17a	47.66 b
	4	27.79b	29.19a	41.85a	50.16a	52.53ab

Means within the same column and the same zone having the same letter are not significantly different $p>0.05$

TABLE 5. Comparison between the effects of treatments OF, RE, NF, MX and CH on mean percentage of fed larvae in zones 1, 2, 3, 4 and 5.

Time Interval	Treat.	Zone 1 Mean % Larvae	Zone 2 Mean % Larvae	Zone 3 Mean % Larvae	Zone 4 Mean % Larvae	Zone 5 Mean % Larvae
1	OF	30.49a	5.71 b	11.79a	10.90a	41.12a
	RE	30.98a	10.88a	11.96a	11.92a	34.26a
	NF	25.10a	10.24a	8.83a	15.71a	40.13a
	MX	25.23a	7.90ab	9.03a	12.06a	45.79a
	CH	28.33a	7.38ab	9.69a	10.52a	44.08a
2	OF	43.84a	7.97a	9.02a	14.44a	24.73 b
	RE	36.00ab	11.11a	10.74a	11.28a	30.88ab
	NF	32.82ab	9.39a	10.82a	13.53a	33.44ab
	MX	27.04 b	8.61a	11.69a	13.41a	39.25a
	CH	30.10ab	10.01a	8.44a	11.02a	40.43a
3	OF	46.58a	8.94a	9.41a	11.86a	23.20 b
	RE	40.09ab	11.95a	7.46a	9.37a	31.13ab
	NF	32.41 b	9.57a	10.03a	11.22a	36.76a
	MX	30.31 b	9.09a	9.99a	12.73a	37.89a
	CH	34.61ab	8.61a	10.32a	11.11a	35.35a
4	OF	52.00a	8.41a	9.43a	8.61 b	21.55 b
	RE	43.23ab	8.31a	8.30a	8.17 b	31.19ab
	NF	35.10cb	10.29a	10.17a	9.67ab	34.78a
	MX	31.02c	8.43a	9.31a	13.78a	37.46a
	CH	31.94cb	10.12a	8.97a	10.57ab	38.41a

Means within the same column and the same time interval having the same letter are not significantly different $p>0.05$

TABLE 6. Comparison between effects of treatments OF, RE, NF, MX and CH on mean percentage of starved larvae in zones 1, 2, 3, 4 and 5.

Time Interval	Treat.	Zone 1 Mean % Larvae	Zone 2 Mean % Larvae	Zone 3 Mean % Larvae	Zone 4 Mean % Larvae	Zone 5 Mean % Larvae
1	OF	30.29ab	7.19ab	9.47a	12.07a	40.98cb
	RE	35.35a	10.96a	12.41a	10.67a	30.12c
	NF	23.19cb	7.49ab	11.00a	12.88a	45.44ab
	MX	16.98c	4.85 b	7.96a	14.37a	55.83ab
	CH	17.55c	5.66ab	8.71a	10.75a	57.33a
2	OF	44.16a	9.84a	9.73a	8.45 b	27.82b
	RE	42.22a	9.93a	10.78a	9.45ab	27.62b
	NF	27.17b	8.46a	10.61a	10.97ab	42.80a
	MX	23.55b	6.81a	8.17a	11.83a	49.64a
	CH	25.32b	7.97a	9.63a	9.62ab	47.45a
3	OF	42.35a	9.62a	9.04a	10.98a	28.00b
	RE	43.56a	9.15a	9.42a	9.42a	28.45b
	NF	31.07b	8.38a	7.44a	8.41a	44.70a
	MX	24.93b	7.48a	8.78a	10.65a	48.17a
	CH	26.84b	6.80a	7.13a	11.57a	47.66a
4	OF	45.46a	7.80a	10.28a	8.66a	27.79b
	RE	46.37a	8.08a	6.68a	9.68a	29.19b
	NF	30.46b	7.15a	8.89a	11.65a	41.85a
	MX	23.84b	7.72a	7.92a	10.37a	50.16a
	CH	23.50b	6.81a	9.28a	7.89a	52.53a

Means within the same column and the same time interval having the same letter are not significantly different $p>0.05$

TABLE 7. Effects of pretreatment conditions (starved vs. fed) on the mean percentage of larvae in zones 1, 2, 3, 4 and 5.

Treatment	Zone	Pre-Treatment	Time Int. 1	Time Int. 2	Time Int. 3	Time Int. 4
OF	1	starved	30.29a	44.16a	42.35a	45.46a
		fed	30.49a	43.84a	46.58a	52.00a
	2	starved	7.19a	9.84a	9.62a	7.80a
		fed	5.71a	7.97a	8.94a	8.41a
	3	starved	9.47a	9.73a	9.04a	10.28a
		fed	11.79a	9.02a	9.41a	9.43a
	4	starved	12.07a	*8.45b*	10.98a	8.66a
		fed	10.90a	*14.44a*	11.86a	8.61a
	5	starved	40.98a	27.82a	*28.00a*	*27.79a*
		fed	41.12a	24.73a	*23.20b*	*21.55b*
RE	1	starved	35.85a	42.22a	43.56a	46.37a
		fed	30.98a	36.00a	40.09a	43.23a
	2	starved	10.96a	9.93a	*9.15b*	8.08a
		fed	10.88a	11.11a	*11.95a*	8.31a
	3	starved	12.41a	10.78a	9.42a	6.68a
		fed	11.96a	10.74a	7.46a	8.30a
	4	starved	10.67a	9.45a	9.42a	9.68a
		fed	11.92a	11.28a	9.37a	8.17a
	5	starved	30.12a	27.62a	28.45a	29.19a
		fed	34.26a	30.88a	31.13a	31.99a
NF	1	starved	23.19a	27.17a	31.07a	30.46a
		fed	25.10a	32.82a	32.41a	35.10a
	2	starved	7.49a	8.46a	8.38a	*7.15b*
		fed	10.24a	9.39a	9.57a	*10.29a*
	3	starved	11.00a	10.61a	*7.44b*	8.89a
		fed	8.83a	10.82a	*10.03a*	10.17a
	4	starved	12.88a	10.97a	8.41a	11.65a
		fed	15.71a	13.53a	11.22a	9.67a
	5	starved	45.44a	42.80a	44.70a	41.85a
		fed	40.13a	33.44a	36.76a	34.78a

Paired means within the same column and the same treatment having the same letter are not significantly different $p>0.05$

TABLE 7.*(continued)* Effects of pretreatment conditions (starved vs. fed) on the mean percentage of larvae in zones 1, 2, 3, 4 and 5.

Treatment	Zone	Pre-Treatment Conditions	Time Int. 1	Time Int. 2	Time Int. 3	Time Int. 4
MX	1	starved	*16.93b*	23.55a	24.93a	23.84a
		fed	*25.23a*	27.04a	30.31a	31.02a
	2	starved	*4.85b*	6.81a	7.48a	7.72a
		fed	*7.90a*	8.61a	9.09a	8.43a
	3	starved	7.96a	*8.17b*	8.78a	7.92a
		fed	9.03a	*11.69a*	9.99a	9.31a
	4	starved	14.37a	11.83a	10.65a	10.37a
		fed	12.06a	13.41a	12.73a	13.78a
	5	starved	55.83a	*49.64a*	*48.17a*	*50.16a*
		fed	45.79a	*39.25b*	*37.89b*	*37.46b*
CH	1	starved	*17.55b*	25.32a	*26.84b*	*23.50b*
		fed	*28.33a*	30.10a	*34.61a*	*31.94a*
	2	starved	5.66a	7.97a	6.80a	*6.81b*
		fed	7.38a	10.01a	8.61a	*10.12a*
	3	starved	8.71a	9.63a	*7.13b*	9.28a
		fed	9.69a	8.44a	*10.32a*	8.97a
	4	starved	10.75a	9.62a	11.57a	7.89a
		fed	10.52a	11.02a	11.11a	10.57a
	5	starved	57.33a	47.46a	*47.66a*	*52.53a*
		fed	44.08a	40.43a	*35.35b*	*38.41b*

Paired means within the same column and the same treatment having the same letter are not significantly different $p>0.05$

Concomitantly, significant changes over time in abundance within zones occurred (MANOVAR, $F=25.330$, $df=12,480$, $p<0.05$).

Regardless of the pre-treatment or type of substratum offered, two-thirds to slightly more than three-quarters of the larvae consistently occupied regions both at the surface (zone 1) of the water column and on the bottom of the observation arena (zone 5) (**Table 1 and Table 2**). As a consequence, larvae found within the three middle zones generally comprised less than one-third of the total population. Not only were the larval numbers relatively uniform between these zones throughout the entire period of observation, but their abundance within them was essentially static over time, and averaged 10.2% per zone (*i.e.*, less than 3 larvae) (**Table 3 and Table 4**).

An overabundance of high-quality food ***(Treatment OF)*** produced no significant differences between the percentage of larvae at the surface and on the bottom during the first 15 minutes of observation (time interval 1), for either of the pre-treated (**Table 1 and Table 2**) groups. Subsequently (time intervals 2, 3, and 4), there was a significant accumulation of both starved as well as fed larvae at the surface; during this time, there were no significant fluctuations in the proportions of larvae present at the surface (**Table 3 and Table 4**).

When starved larvae were subjected to small amounts of high-quality food ***(Treatment RE)*** the pattern of distribution over time was similar to that elicited by treatment OF (**Table 1 and Table 2**), except that the shift of fed larvae to the surface was slightly delayed (**Table 3 and Table 4**).

In the absence of food ***(Treatment NF)***, both starved (**Table 2**) and fed (**Table 1**) larvae were found on the bottom in significantly higher

numbers than at the surface during the first 15 minutes of observation. Thereafter, the fed larvae became more uniformly distributed between these two zones even though their numbers increased and peaked at the surface during time interval 4 (Table 3). Conversely, the starved larvae occupied the bottom in significantly higher numbers during the corresponding period; nevertheless, their abundance at the surface increased over time and plateaued during the second half of the observation period (Table 4).

In the presence of either a high-density but mediocre *(Treatment MX)* or low-quality *(Treatment CH)* substrates, significantly higher percentages of starved larvae were found on the bottom than at the surface (Table 2) throughout the observation period. Nevertheless, for both treatments, proportions at the surface increased significantly after time interval 1 and subsequently plateaued, while it concomitantly decreased and eventually stabilized on the bottom (Table 4). Fed larvae responded similarly to treatments MX and CH and although a significantly higher percentage foraged on the bottom than were found at the surface during time intervals 1 and 2 (Table 1); subsequent observations showed no significant differences. This coincides with changes in their proportions at the bottom over time, *i.e.*, it was highest during time intervals 1 and 2, but generally declined over the following 2 intervals (Table 3).

PERCENTAGE OF LARVAE WITHIN ZONES

In general, both pre-treatment (MANOVAR, $F=8.063$, $df=4,160$, $p<0.05$) and treatments (MANOVAR, $F=15.092$, $df=16,160$, $p<0.05$) significantly affected the percentage of larvae present within the zones.

Although no significant differences between proportions of fed and starved larvae occurred in an environment that contained either little *(Treatment RE)* or no food *(Treatment NF)*, significant differences for the remaining treatments *(OF, MX, and CH)* were evident (primarily on the bottom) during the second half-hour of observation and were marked by consistently higher percentages of starved than fed larvae (Table 7).

With respect to treatments, fed larvae were found at the surface in similar proportions (Table 5) during time interval 1, regardless of substrate type. During subsequent observations, significantly greater proportions of larvae occupied the surface zone in the presence of an overabundance of high-quality food *(Treatment OF)*, than for any other treatment. Any differences between treatments on the percentage of larvae at the bottom initially mirrored those occurring at the surface (Table 5); during the subsequent three intervals, however, abundance was lowest in treatment OF, and generally higher for treatments NF, MX, and CH.

Starved larvae provided with high quality food, either in a surfeit *(Treatment OF)* or reduced quantity *(Treatment RE)*, were consistently abundant in similar proportions in the surface and bottom zones (Table 6). Similarly, larvae not provided with food in the arena *(Treatment NF)*, or provided with food that was overabundant but mediocre *(Treatment MX)* or poor *(Treatment CH)* in quality aggregated in the same zones, in similar proportions. Nevertheless, starved individuals treated with high quality food *(Treatment OF and RE)* were consistently present in smaller proportions at the bottom than those exposed to low quality food *(Treatments NF, MX, CH)* (Table 6). With few exceptions, proportions of starved (Table 6) or fed (Table 5) larvae in the mid-region zones 2, 3, and 4 generally showed no significant variation.

DISCUSSION

Although the larvae typically accumulated at the surface of the water column and on the bottom of the observation arena, their abundance within these zones was influenced by the nature of the substratum. Changes over time in the percentage of larvae within the zones was not as important as fluctuations in their relative proportions between the zones, as the percentage of larvae in the former generally was static during the last 45 minutes of observation. The effects of food deprivation on larval distribution were most obvious within the bottom 1 cm region of the observation chamber, and were evident even 1 hour after the larvae had been introduced.

Because *Aedes aegypti* larvae breathe at the air-water interface and feed on particulate matter on the bottom (Christophers 1960), these regions are consequently natural foci of activity, explaining why at least two-thirds of the larvae consistently occupied the surface or bottom regions. Therefore, larval abundance in the three middle zones was characterized by consistently low percentages within the zones and uniform proportions between the zones. Direct observation confirmed that the larvae were primarily in transit between the surface and bottom zones; consequently, shifts in larval distribution as a function of varying conditions characteristically involved only these zones *i.e.*, surface and bottom. Generally, within each of the mid-region zones, no significant changes over time in abundance, or differences between densities of starved and fed individuals in response to the treatments were observed. Any deviations from this pattern is suggestive of slight individual

variation in larval response to the different substrates. Because, on average, fewer than 3 individuals were present per mid-region zone, any such variation in behaviour between larvae would consequently become conspicuous. Consequently, no biological significance should be associated with these fluctuations since only a small proportion of the population ever occupied these zones.

Differences between zones

TREATMENT OF. Access to an abundance of highly nutritious food may have provided ideal conditions for larval feeding and consequently, growth. Indeed, within 15 minutes after their introduction, a majority of both starved and fed groups of larvae shifted from **(a)** being equally distributed between the surface and bottom **(b)** to accumulation at the surface. This rapid re-distribution and subsequent stabilization suggests that they fed to repletion in less than 15 minutes, and consequently spent minimal time foraging. In spite of this similarity, significantly more starved than fed individuals aggregated on the bottom towards the end of the observation period, perhaps indicative of their requirement for slightly more time to feed to satiation. Dadd (1968) found that *Aedes aegypti* larvae readily displaced material from their gut in less than one hour by ingestion of particles of broadly nutritive values; consequently, differences between starved and fed groups of larvae should have been evident during the first half-hour of observation, and not the last, since under conditions of food overabundance a "starved" larva could easily become "fed", unless the starved larvae are too weak to ingest. Larvae deprived of nutrients for extended periods apparently feed more slowly

than fed individuals, even after prolonged exposure to an overabundance of highly nutritious food. Ultimately, these different reactions may represent some compensatory reaction of the larvae to the effects of nutritional deprivation, possibly a mechanism allowing them to take advantage of their nutrient-rich environment.

TREATMENT RE. The second treatment differed from the previous one in that a reduced quantity rather than an overabundance of food was offered to the larvae. Nevertheless, a similar pattern of dispersal over time (*i.e.*, the larvae were evenly distributed between the surface and the bottom during the first 15 minutes of observation, but subsequently aggregated at the surface) was observed between starved larvae exposed to either a low abundance or an overabundance of food, again suggesting that even when nutrients were scarce, feeding to satiation occurred rapidly. This overall shift towards the surface might also have resulted from the gradual depletion of the small quantity of food present in the observation arena through continuous larval feeding: as the amounts decreased, the incentive to forage declined since the energy expenditure required to forage may have eventually superseded the energy obtained through feeding. Consequently, greater proportions of larvae eventually occupied the surface zone.

The fed larvae, like the starved, were initially equally numerous at the surface and on the bottom when offered reduced amounts of food; however, their subsequent aggregation at the surface occurred later than with the starved individuals, suggesting that their feeding rates were slower or simply more prolonged and copious.

Larvae would conceivably spend more time searching in an environment

where food is sparse than in one where it is overabundant, because feeding to satiation under impoverished conditions would presumably take longer. This behaviour was observed, but exhibited only by the fed individuals. If one considers that a minimum critical weight must be attained during the larval stage for pupation to occur (Chambers and Klowden 1990), and that adults from pupae with low nutrient reserves need to locate a food source sooner upon emergence than those with adequate reserves (suggesting some type of competitive advantage) (van Handel 1988), then one would expect the contrary to occur, since starved larvae theoretically carry reduced nutrient reserves. It may simply be that the fed larvae with larger corporeal reserves of nutrients were able to allocate more time and energy for foraging.

TREATMENT NF. For the starved larvae, the lack of ingestible material in the observation arena along with the complete deprivation of food prior to introduction probably constituted a "worst-case scenario", because not only would their nutrient reserves have been depleted, but their foraging efforts would have been fruitless. Nevertheless, significantly more starved than fed larvae aggregated on the bottom throughout the duration of the observation period, suggesting that a relatively high level of foraging activity was sustained among the starved individuals even under these adverse conditions. Ultimately, the complete absence of particulate matter in any environment makes the presence of mature mosquito larvae in that habitat unlikely because their growth depends on the availability of nutrients. Although particulates are, in nature, the major food source of many mosquito larvae (Merritt 1987), some species will develop to adulthood in sterile synthetic media containing

most of the essential nutrients (Akov 1962; Nayar 1966). The absence of solids in the larval diet has however been demonstrated to reduce feeding and growth rates (Dadd 1975; Nayar 1966) unless low concentrations of colloids are incorporated into the dietary medium (Dadd and Kleinjan 1976). Therefore, if the feeding rate of the starved larvae was reduced by the absence of particulate material in the arena, perhaps accumulation of the starved larvae near the bottom was a function of "searching by expectation" whereby they relied only on innate responses to find optimal regions of exploitation in their habitat, and thus were not necessarily dependent upon information gained from previous experience (Bell 1990).

The majority of fed individuals also initially aggregated on the bottom. However, unlike the starved larvae, they subsequently became equally abundant at the surface and on the bottom, suggesting that the overall intensity of feeding within the population diminished quickly with time. Dethier (1976) found that searching behaviour for food in the blowfly *Phormia regina* (Meigen) was probably regulated by the animal's "physiological state", *i.e.*, the quantity ingested is affected by the time since the previous meal, the quantity and quality of the previous meal, and the amount of energy expended in the interim. Assuming the same principles are applicable to mosquito larvae as a mechanism of nutrient regulation, the fed larvae would not be expected to accumulate (nor, in this study, were they actually observed *to* accumulate) near the bottom for as long as the starved individuals, because presumably they had greater nutrient reserves.

TREATMENTS MX and CH. It is unlikely that *Aedes aegypti* larvae would encounter food sources as homogeneous as those presented in these

experiments under normal field conditions. Laird (1988) observed that both artificial and natural containers provide highly heterogeneous environments containing a wide variety of taxa and organic material which the larvae may potentially ingest. A mixture of highly-nutritious food and inert charcoal particles (Treatment MX) might therefore more closely approximate a mixed-type substrate that the larvae would typically encounter in the field. When food was mixed with charcoal particles, fed and starved larvae reacted similarly to this mixture and charcoal alone: in the presence of either substrate, starved larvae consistently aggregated at the bottom whereas the fed only occupied this zone during the first half-hour, and subsequently dispersed.

Response to the first three treatments (OF, RE, and NF) indicates that the presence or absence of food influenced the vertical distribution of fed and starved larvae within the arena. Thus, the response of the larvae to the remaining two treatments (MX and CH) should have differed because only one contained food particles. Charcoal particles, of negligible nutritional value (Aly and Mulla 1986), appeared to serve primarily as a phagostimulant, because starved larvae in an environment containing a high-density, high-quality substrate (treatment OF) soon aggregated at the surface, whereas in the presence of a substrate of similar density, but of mediocre (MX) or poor nutritional quality (CH), they continuously foraged on the bottom. This pattern suggests that the high-quality food items were ingested faster than those of lesser quality: these results support the findings of Aly (1985) and Aly and Mulla (1986) who found that larvae of *Aedes vexans* and *Anopheles albimanus* ingested food items (*e.g.*, yeast, wheat flour, and fishmeal) between 3 to 9 times

faster than inert particulates (*e.g.*, kaolin, chalk, and charcoal). Apparently ingesting poor quality food does not diminish larval drive to forage, but it does affect vertical distribution in the environment.

Acquiring food would presumably be more critical for starved larvae than fed; consequently, foraging by the latter may be expected to cease earlier in situations where nutritive material is not readily available (*i.e.*, treatments NF, MX, and CH). The data support this hypothesis since a majority of starved larvae continuously foraged on the bottom regardless of the treatments (NF, MX, and CH), whereas the fed individuals, also initially aggregated in this region, eventually became equally abundant at the surface and the bottom. If as Dadd (1971) stated, ingestion rate is regulated by an endogenous feedback mechanism dependent upon the degree of nutritional repletion brought about by feeding ("nutrient dependent regulation"), then a threshold of nutritional repletion may stimulate an "on/off" feeding switch in the larvae. More effort is required by starved individuals than fed which results in prolonged and more intense foraging by the former. Although treatment MX was defined as a mixture, by weight, of ground food and charcoal, treatment CH was different because it consisted of ground charcoal alone. Regardless of the distinct differences between these two treatments, all larvae responded consistently to either substrate, possibly because differential rates of dissolution of the two constituents increased the relative proportion of one in the mixture, or because differential sedimentation rates of ground food and charcoal occurred because a larger proportion of the ground charcoal (45%) than ground food (30%) consisted of particles less than 14.1 μm in diameter. Assuming that smaller particles remain in suspension

for a longer periods of time than the larger, heavier ones, the food would presumably settle to the bottom more rapidly and in greater quantities than the charcoal. Subsequently, the food may have become covered by a thin layer of charcoal, rendering it almost indistinguishable to the foraging larvae from a substrate entirely of ground charcoal. Merritt (1987) and Dadd (1971) found with *Aedes triseriatus* and *Culex pipiens*, respectively, that particle sizes larger or smaller than those associated with optimal ingestion rates for each instar (*e.g.*, particles averaging 26μm for 4th instar *Culex pipiens* larvae) clearly decreased ingestion rates. It is possible that the slightly larger average diameter of the food particles may have put them beyond the optimal food size range for 4th instar *Aedes aegypti*. Inappropriate food particle size may have ultimately influenced larval selection for ground charcoal particles only, thus resulting in a response to the mixed substrate as if it were charcoal alone, *i.e.*, both substances equally nutritionally insubstantive.

Changes Over Time

The largest fluctuations over time in larval abundance, either at the surface or on the bottom, were observed in transition from the first to the second observation interval, each interval being 15 minutes in duration. Subsequently, because larval abundance remained virtually static regardless of the type of substrate or pre-treatment conditions they were provided with, overall activity was most vigorous within the first 15 minutes after the larvae were introduced into their new environment. The transfer of the larvae from the isolation chamber to the observation arena was performed using a pipette allowing rapid withdrawal

of larvae from one habitat and their discharge into another. This mode of transferral appears to have elicited the diving response among the larvae, and may have initially increased their levels of activity, as well as possibly magnifying exploratory behaviour as they searched for food, regions of optimal water temperature, light intensity, and protection from predators in their novel environment (Christophers 1960). The initial flurry of activity suggests that the diving response remained unabated even 15 minutes after larval introduction into the observation arena. Consequently, a 15 minute delay before videotaping would arguably eliminate any masking properties of the diving response over other natural larval behaviours *e.g.*, foraging. However, because differences in dispersal between larvae that were subjected to the various treatments were evident, it can be concluded that the effect of the diving response was negligible within the first quarter-hour time frame, and thus no delay was necessary.

Although the overall stability of larval numbers within zones during the last 45 minutes of observation suggests that the population had reached an equilibrium state with respect to their activity or vertical distribution in the habitat, it is possible that a "natural equilibrium" was never attained since the larvae were not purposefully agitated following their introduction into the observation arena. A natural equilibrium *may* only be precipitated under field conditions where disruptive stimuli caused by rain, wind, passing shadows, presence of predators, fluctuations in air and water temperatures, and vibrations resulting from passing people or vehicles, are a common occurrence (Thomas 1950; Folger 1946).

Comparison Between Treatments

Starved larvae exhibited a similar response to the presence of high-quality foods (*i.e.*, treatments OF and RE), and to the absence of high quality foods (*i.e.*, treatments NF, MX, and CH), gauged by the presence of approximately the same number of individuals either at the surface or on the bottom throughout the entire period of observation. The first two treatments, disparate only in quantity, may not have been distinct enough to elicit a different response from the starved larvae. The suggestion that the homogeneity of larval response to the remaining three treatments was a consequence of similarities between treatments would be discordant with the nature of the treatments: two contained particles of ground food and charcoal (MX) or ground charcoal alone (CH), whereas the third (NF) contained no particulates at all. In contrast to the starved larvae, no distinction was evident between the response of fed individuals provided with high-quality foods or denied high-quality foods. In fact, the distribution of fed larvae in an arena devoid of food (NF) *was* as different from one containing either a mixture of food and charcoal (MX), or ground charcoal (CH) alone, as it was between the two arenas containing high quality food but in different quantities (OF and RE). Thus, a slightly greater variation in response to the various treatments was noted among the fed larvae than the starved, since the starved responded similarly to substrates present in widely differing amounts (*i.e.*, treatments OF and RE), and of diverse qualities (*i.e.*, their response to treatments NF, MX, and CH was the same).

Assuming that (a) foraging activity among the starved larvae was sustained at similarly high levels in the presence of two different

amounts of food to satisfy the requirement for highly nutritious material, and (b) the similarity of their reaction to the remaining three treatments is attributable to the inability of the larvae to obtain adequate high-quality food ("nutrient dependent regulation") (Dadd 1971) as well as an innate behaviour to find optimal regions of exploitation (Bell 1990), the question as to why fed larvae apparently forage less discriminately than starved larvae arises.

If the mechanism used by mosquito larvae to locate resources are multi-modal (*e.g.*, olfactory and visual), as they are with other insects (Bell 1985), then presumably the quality of the sensory input (and resultant behaviour) varies with the physiological status of the larvae. The observation that starved larvae distinguished more readily between nutritive and non-nutritive substrates than did the fed, and apparently modified their behaviour accordingly, seems to support this hypothesis. Food deprivation in insects increases the probability of responding to resource related cues (Bell 1990). Thus it might be concluded that the starved larvae were actually more sensitive to subtle differences between treatments than were the fed.

Practical Applications of This Study

Mosquito larvae in diverse habitats become aggregated at food-rich foci to optimize feeding on nutritive organic material (Merritt *et al.* 1992). It is therefore important that the distribution of parasites and predators introduced into larval habitats for biological control purposes closely resembles that of their hosts or prey. This enables the parasites/predators to maximize the frequency of encounters with potential

prey and concomitantly reduce search effort, with the consequence of increasing efficacy in mosquito population reduction (Service 1981). The idea of a close host-parasite association being necessary for the effective use of a biological control agent is neither novel nor recent. Petersen (1973) clearly demonstrated that the incidence of parasitism in *Culex pipiens quinquefasciatus* Say by the mermithid nematode *Reesimermis nielseni* Tsai and Grundmann was highest when larvae were situated at the surface in the corners of a square pond and lowest when they were located near the center. He concluded that contact between the host and parasite is not a result of chance collision, but rather because of similar behavioural responses of the host and parasite that increase the probability of contact. Similarly, many species of mosquitoes with varied feeding behaviours exhibit different levels of susceptibility to a given strain or preparation of ingested, bacterially-derived stomach toxins such as *Bacillus thuringiensis* var. *israelensis* (Lacey and Undeen 1986).

The relationship between *Aedes aegypti* larvae and digenean trematodes, demonstrated to impair the survival and development of mosquito larvae (Dempster 1988), exemplifies the ideal spatial distribution between host and parasite. *Aedes aegypti* larvae are primarily browsers and spend considerable time feeding on organic material at the bottom of their habitat (McIver and Siemicki 1977). Similarly, cercariae of *Plagiorchis noblei* (Trematoda: Plagiorchiidae) (Webber 1986) and *Plagiorchis elegans* (Trematoda: Plagiorchiidae) (Bock 1984) sink towards the bottom soon (>80% after 4 hours) after emerging from their snail intermediate host, during an interval when the cercariae are most infective (C. Lowenberger 1993, pers. comm.). Consequently, larval

distribution and the coincidence that most of the cercariae eventually sink to the bottom during peak infectivity provides an advantage to the parasite - proximity to potential hosts.

Ultimately, the frequency of host-parasite encounters depends on the distribution and mobility of both the target organism and the parasite (Kennedy 1975). Assuming that containers in the field infested with *Aedes aegypti* larvae contained sufficient quantities of organic material to sustain growth and development, the addition of highly nutritious material (*i.e.*, treatments OF and RE) would probably decrease larval contact with the bottom. Consequently, encounters with introduced cercariae would be limited, because when provided an enriched substratum on which to forage, feeding and subsequently aggregation at the surface occurred rapidly. Removal of organic material from larval habitats (*i.e.*, treatment NF) as well as measures to prevent its introduction, such as the addition of metal covers or wire mesh over potential breeding sites (Laird 1988) would also be ineffective at increasing larval foraging time because in the absence of any particulate material the fed larvae briefly formed an aggregate on the bottom (<15 minutes), before becoming evenly dispersed between the top and the bottom. Therefore, the best strategy to increase larval contact with cercariae on the bottom might be the addition of inert particulate material such as charcoal into the larval habitat, because in the presence of this type of substrate, the larvae aggregated on the bottom in significantly higher densities than at the surface, and for longer periods of time than for any other treatment.

Past research by Webber (1986) supports this hypothesis, because he found that: (1) the distribution of *Aedes aegypti* larvae within a habitat

influenced their susceptibility to the entomophilic digenean trematode *Plagiorchis noblei*; and, (ii) the behaviour of *Aedes aegypti* larvae compared to that of *Anopheles quadrimaculatus* Say larvae significantly increased their relative susceptibility to the cercariae - the former were more active than the latter, and consequently spent more time in the lower half of the observation chamber where a higher proportion of infectious cercariae were present.

In conclusion, this research was conducted primarily to obtain a understanding of how nutrient deprivation, as well as food quantity and quality affects foraging behaviour in *Aedes aegypti* larvae, and consequently how this influenced their spatial distribution over time. Although larval habitats in the field are inherently more complex than those created under lab conditions, these results provide a simple picture of larval response to extreme conditions, and thus might allow for the prediction of their distribution in habitats that are of an intermediate nature. This information might theoretically be applied in making more accurate predictions of the efficiency of certain entomopathogens in controlling mosquitoes, because it describes in general terms when and where most of the larval population is likely to be found, and under what conditions. Acquiring a clearer understanding of the spatial and temporal dispersal of *Aedes aegypti* larvae (as well as other species) within their habitat in response to food might facilitate the selection of an appropriate biological control agent whose behaviour and distribution sufficiently parallels that of the mosquito larvae.

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APPENDICES

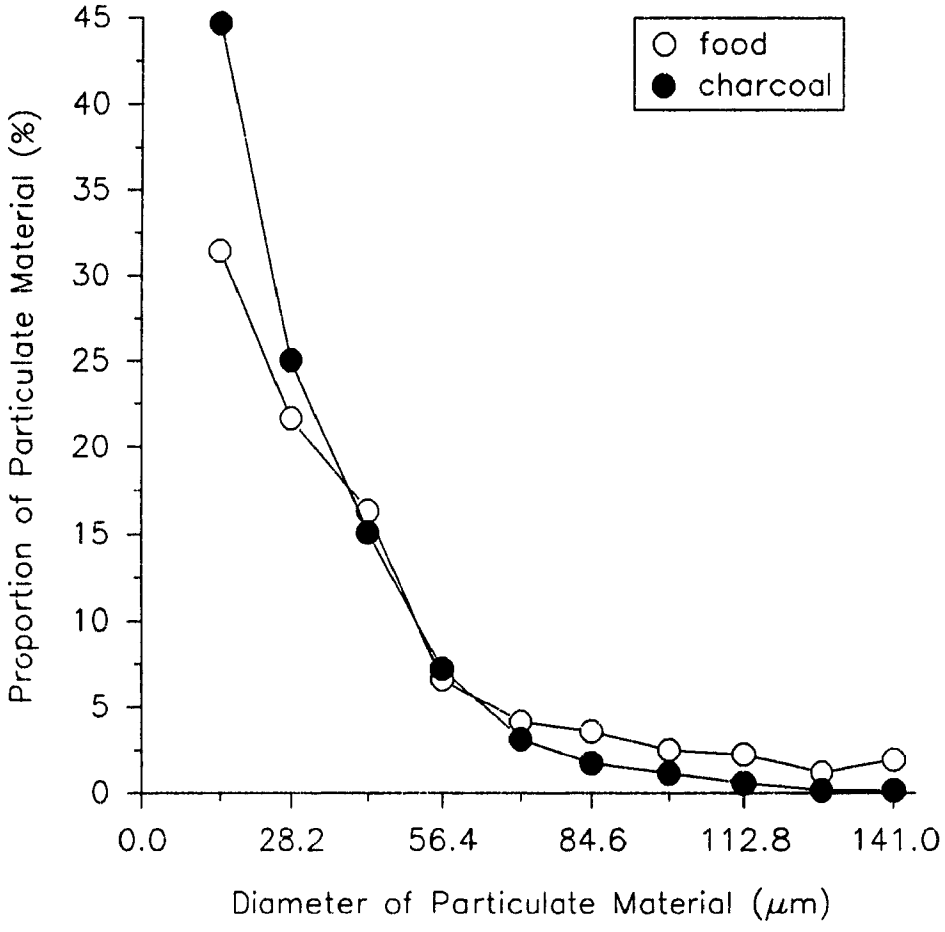
Appendix 1.

Ingredients and composition of Nutra Fin™ Livebearer Food for Guppies, Mollies, and Swordtails.

Ingredients	*Composition*
Fish meal, soy flour, plankton, mosquito larvae, shrimp meal, dried daphnia, torula dried yeast, wheat feed flour, fish liver, spinach powder, chlorophyll.	Minimum crude protein.....42% Minimum crude fat.....5% Maximium crude fiber.....2% Maximum moisture.....8%

Appendix 2.

Particle size distribution of the ground charcoal and food



Appendix 3.

Dimensions of ground charcoal and food particles used as constituents of the substrates that were present in the observation arena.

Material	Average diameter (μm)	Maximum diameter (μm)	Minimum diameter (μm)	Percentage particulate material less than 141μm
charcoal	30.8	282.0	3.5	98.9
food	53.9	465.3	7.1	91.0