Selection of oviposition sites by Aedes aegypti : behavior of gravid mosquitoes and mechanisms of attraction.

SELECTION OF OVIPOSITION SITES BY *AEDES AEGYPTI*: BEHAVIOR OF GRAVID MOSQUITOES AND MECHANISMS OF ATTRACTION

A Dissertation Presented

by

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Submitted to the Graduate School of the
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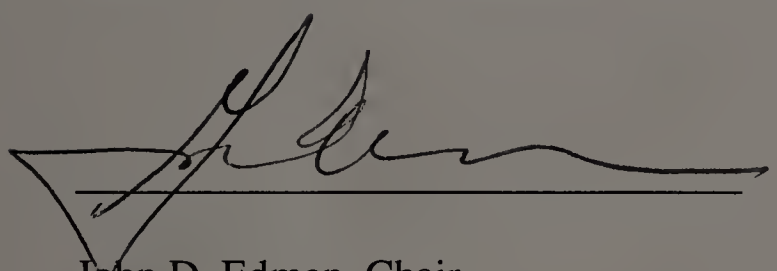

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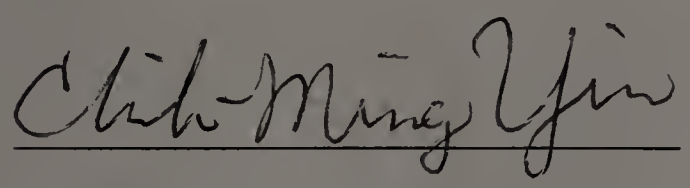

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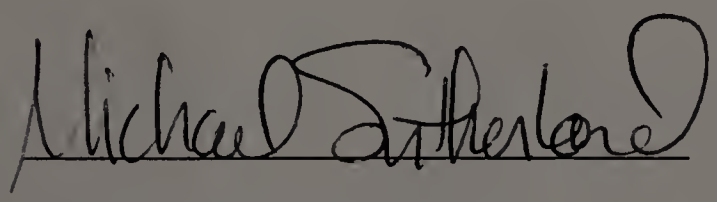

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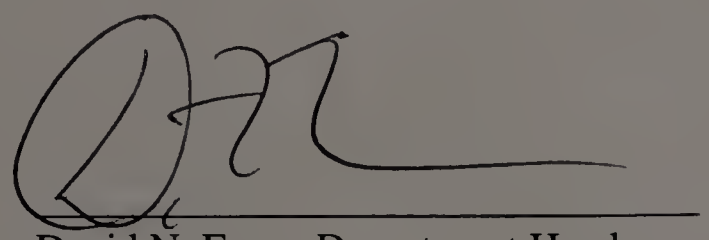

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ABSTRACT

SELECTION OF OVIPOSITION SITES BY *AEDES AEGYPTI*: BEHAVIOR OF GRAVID MOSQUITOES AND MECHANISMS OF ATTRACTION

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Gravids preferred oviposition sites that contain larvae over those that contain only water. The degree of preference increased with increasing larval instar, and also increased with increasing larval density up to a density of 2 larvae per ml. At higher densities the response became negative. Larvae act as indicators of site-suitability, and may indicate imminent competition at high densities, regardless of the presence of contaminating microfauna. Gravids rely on olfactory and chemo-tactile cues to detect the presence of larvae at oviposition sites, with a greater reliance on olfactory cues. Visual cues are unimportant in the detection of larvae.

Gravids avoided ovipositing in a site already harboring eggs. This negative response increased with increasing egg density. Eggs are indicators of direct competition in the larval habitat. This avoidance reaction is mitigated by both olfactory and chemo-tactile cues. That egg distribution did not affect subsequent oviposition suggests the chemo-tactile response is more likely due to local concentration of chemicals than due to direct contact with eggs.

Females visit more smaller sites when all available oviposition sites are identical, and lay fewer eggs in smaller treatment sites, than they do compared to larger sites. The gravid response is independent of substrate surface area and water volume. Eggs were not distributed differently in oviposition sites of different size but equal diameter, suggesting females may select different size-sites based on water surface area. Size is probably an indirect measure of available resources.

The oviposition response does not vary with either chronological age of the gravid, nor with her gonotrophic cycle. Females separated in age by 2 weeks in post-emergence age or by 2 gonotrophic cycles responded to oviposition sites in the same manner. Oviposition behavior is flexible but does not take into account a female's relative fitness.

The response to oviposition sites containing an attractive density of larvae was further investigated. Females could not discriminate between control and treatment sites in a wind tunnel, even when released but 22 cm from the attractive treatment. The olfactory cues associated with larval rearing habitat act as close range oviposition attractants.

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CHAPTER 1

LITERATURE REVIEW

Background

Chief among the human parasites transmitted by *Aedes aegypti* mosquitoes are the Yellow Fever virus and the 4 strains of Dengue virus. Although Yellow Fever epidemics are now infrequent, the incidence of Dengue and the associated Dengue Hemorrhagic Fever (DHF) continues to increase throughout the tropics and sub-tropics, especially in the Western Hemisphere. Treatment for Dengue/DHF is symptomatic; no vaccine is available (Gratz 1993). The most effective public health tactic in suppression of Dengue is control of the principal vector, *Ae. aegypti.*

Chemical control is the cheapest and most widespread form of large-scale mosquito control available. High cost, the spread of insecticide resistance and the sensitivity of the environment to many insecticides are major drawbacks in the reliance upon chemical control of mosquito populations. The latter is not as much a problem when dealing with urban populations of *Ae. aegypti* but the public perception of insecticides as an immediate health threat renders spraying regimes in urban areas undesirable (Service 1995). The relatively new control method of insecticide-impregnated bednets offers little help in control of *Ae. aegypti*, as this mosquito is day-active.

Other forms of chemical control include the use of biting-repellents such as DEET. While effective as a personal protection method among older children and adults with regular application, it is expensive and therefore unlikely to find widespread

application in developing nations. Reports of DEET sensitivity in small children, who are the main victims of dengue, indicate DEET will not be useful in protecting this segment of the population (Osimitz and Grothaus 1995). More readily available repellents (derived from natural products) are under investigation, but to date none are as effective as DEET.

The most effective control method throughout history would fall in the category of cultural control. Source reduction is the elimination of vector breeding sites. Drainage of marshes and ditches can render large unsuitable for mosquitoes. Though now limited in usage due to the irreversible damage to the local ecosystem, researchers extended the principles of source reduction to urban habitats. Water storage jars are prime *Ae. aegypti* breeding habitat in many tropical areas. In one Thai study, researchers covered these jars to prevent oviposition by gravid females (Strickman and Kittayapong 1993). The cover, a simple (and cheap) metal lid, did not form a tight seal with the clay pots, and such covered pots were more likely to harbor mosquito larvae than their uncovered counterparts.

The ability to control mosquitoes with other organisms (predators and parasites) is not yet feasible. Predators such as mosquitoes in the genus *Toxorhynchites* are adept at finding even the smallest and most discrete oviposition sites used by *Ae. aegypti*. However, *Toxorhynchites* populations are simply too small to effectively control the rapid-growing populations of *Ae. aegypti* (Gerberg 1985). This problem is common to all predators tested (insects, fish and bats) and parasites (*e.g.*, iridescent virus and entomopathic digeneans) (Marten *et al.* 1994). Although companies manufacture and use

the toxin produced by *Bacillus thruringiensis* var. *israelensis* (Bti) as a pesticide, the organism itself is not self-sustainable at levels that produce enough toxin to control larval *Ae. aegypti* (Yousten *et al.* 1992). The toxin produced by *Bacillus sphaericus* is mosquitocidal, and the organism recycles in highly polluted waters. Such waters rarely contain *Ae. aegypti* however and high application rates are required (over 1 g technical powder/m^2), so the application of *B. sphaericus* is likely to remain limited (Federici 1995).

Several traps are available that attract host-seeking mosquitoes, but these traps are costly and require regular maintenance, and in most settings cannot out-compete live hosts. Thus in urban areas, with an abundance of human and domestic animal hosts, these traps remain largely ineffective against *Ae. aegypti* (Gratz 1993).

An alternative control method involves attraction of gravid mosquitoes to oviposition sites and killing them with a residual insecticide. This approach is focal in nature, and does not allow contact of pesticides with storage jars or water intended for household use. Success of this control option requires that *Ae. aegypti* physically land in the site. Thus treated sites must be more attractive than any other nearby oviposition sites. This project examines the behavioral aspects of *Ae. aegypti* oviposition, with a focus on the abiotic and biotic stimuli that serve to orient gravid mosquitoes toward or away from oviposition sites.

Requirements for Oviposition

Mating Requirement

Female *Ae. aegypti* emerge from their pupal cases ready to mate (Clements 1992). Males of the species emerge roughly 0.5-1.0 d earlier; this gives them time to become fully competent breeders (Clements 1992). Many males will attempt to mate with a single female. Though the process of mate selection in mosquitoes is poorly understood, researchers demonstrated that males reduce the chance of sperm competition by production of matrone, an accessory gland substance (Craig 1967). Matrone inhibits the mating drive of the female, rendering her refractory to the mating attempts of subsequent males (Craig 1967).

Matrone also enables a gravid female to oviposit. Virgin females will develop eggs from a blood meal but they will not oviposit. This inhibition changes upon implantation of male accessory glands (Hiss and Fuchs 1972, Leahy and Craig 1965). Klowden (1990) indicated that increased response to oviposition site stimuli (termed pre-oviposition behavior) did not occur in gravid *Ae. aegypti* that were unmated. Pre-oviposition behavior became apparent in females implanted with male accessory glands.

Protein Requirement

Ae. aegypti egg production requires more proteins and amino acids than a female can produce endogenously. This species has adapted to feeding on the blood of vertebrate hosts to obtain these nutrients. Blood meal digestion occurs in the midgut of the mosquito. The distention of the midgut, measured by abdominal stretch receptors,

signals the female to disengage host-seeking behavior and possibly prepares her for egg development (Klowden and Lea 1978, Klowden 1983). If the female has low natural energy reserves and the initial blood meal is small, a female may require additional blood meals to initiate oogenesis. If these feedings occur within 14 h of the initial meal, the total amount of protein available for egg development increases, allowing the female to develop a greater number of eggs with her first egg batch (Lea *et al.* 1978). A blood meal taken later than this 14 h window will not contribute to egg development due to the effects of an oostatic hormone (Meola and Lea 1972).

The proteins and amino acids absorbed through the midgut wall are taken up by the ovaries, allowing a fixed number of eggs to develop (depending on the quantity of blood imbibed and its amino acid composition). As digestion proceeds, the distention of the midgut subsides. The maturing ovaries signal the fat body to produce a hemolymph-borne substance (vitellogenin) that inhibits host-seeking behavior in sugar-fed females (Klowden 1981, Klowden *et al.* 1987). This redundant message is necessary: the distended gut-mediated inhibition subsides as blood meal digestion continues (Klowden 1990). The eggs develop over the next 2-3 d, at which point the female seeks oviposition sites in which to deposit her eggs. The process by which a gravid female orients toward a site is termed "pre-oviposition behavior" (Klowden and Blackmer 1987). The act of laying eggs is termed "oviposition."

Oviposition habitat requirement

In natural settings, gravid *Ae. aegypti* seek tree-holes and other natural containers that collect water (Clements 1992). Females land and begin to deposit their eggs on the moist area above the water surface; occasionally eggs are deposited on the water surface. Eggs are laid singly, and are white when initially extruded from the female's ovipositor. They harden and darken over the next several hours, due to the process of tanning (Clements 1992). The eggs of *Ae. aegypti* require a period of dessication prior to hatch. This phenomenon reflects their adaptation to regions in which the availability of water is transient. Dried eggs may survive for months. They hatch when rains cause flooding of the site in which they were laid.

In urban, suburban and some rural areas, *Ae. aegypti* females readily oviposit in a wide variety of man-made containers. Examples include water storage jars, flower pots, plastic cups and refuse tires (Welch and Long 1984). Not all suitable containers are equally preferred, however. Under the constraints of fitness, a gravid female selects oviposition sites based on a variety of abiotic and biotic factors.

Fitness Considerations

Female mosquitoes select oviposition sites based upon considerations of fitness. Fitness is a measurement of the spread of an organism's genes throughout the population (Andrewartha and Birch 1954). Mosquito genes are only transferred through reproduction. Thus the greater the number of surviving progeny, the greater a mosquito's fitness (Charnov and Skinner 1985). Immature mosquitoes cannot emigrate from their

habitat, and thus the choice of an oviposition site directly impacts a gravid female's fitness (Blaustein and Kotler 1993). The fitness of a particular mosquito is maximized if its progeny have adequate nutrition and minimal exposure to parasites and predators.

Therefore, above and beyond the basic requirements for oviposition site suitability, gravid females tend to select sites that yield maximum survival for their progeny. Immature mosquitoes require adequate temperature, space and nutrition for development (Terzian and Stahler 1949, Christophers 1960, Moore and Fisher 1969, Livdahl 1982, Suleman 1982). As temperature in the pools in which *Ae. aegypti* breed is generally uniform, their immatures may compete for either space or food.

Eggs do not compete with each other as they are inactive and a developing larva has all nutrient requirements located within its eggshell. In some mosquito species competition begins as soon as the larvae hatch. Aedine mosquitoes hatch upon flooding of a site after their eggs have undergone a period of dessication. Not all the eggs hatch immediately however (Livdahl and Edgerly 1987). Researchers note a reduced oxygen tension in the hatching medium (water) just prior to the emergence of the first larvae (Gjullin *et al.* 1941). This drop in local oxygen content serves as a hatching stimulus. A concomitant increase in the bacterial populations just prior to hatch indicates that bacteria may consume the oxygen. Gillet *et al.* (1977) propose that hatching larvae browse the surface of other eggs, obtaining their first meal by eating bacteria. This reduces the bateria populations and causes the local oxygen tension to rise again. So the first larvae regulate the hatching of subsequent eggs, forcing the egg batch to hatch in installments.

This will prevent overcrowding and reduce competition between mosquitoes of the same species, and possibly of the same parents (Livdahl *et al.* 1984).

Mosquito larvae also influence competition as they mature. *Culex* larvae emit pheromones that affect the growth of other immature mosquitoes, including congenerics and mosquitoes such as *Ae. aegypti* (Ikeshoji and Mulla 1970). Evidence exists that a growth-retardant factor (GRF) may be produced when *Culex quinquefasciatus* larvae are food- rather than space-limited (Ikeshoji and Mulla 1970, Suleman 1982). This chemical inhibits the growth of younger instars in the same rearing medium (Ikeshoji and Mulla 1970). GRF is not produced at densities of less than 5 larvae/ml. Note that a growth-promoting factor may also exist, but is only produced at moderate larval densities with an unlimited food supply (Suleman 1982). Gravid female detection of such indicators would assist in determinations of site-suitability.

A similar phenomenon has been noted with experiments on *Ae. aegypti* larvae. A GRF is produced when food availability is low; its production is not dependent upon available space (Moore and Whitacre 1972). The authors deemed GRF capable of larval population regulation. Larval *Ae. aegypti* outcompete mosquitoes of different species (*Aedes albopictus*) and genera (*Cx. quinquefasciatus*) in the laboratory (Black *et al.* 1989, Peters *et al.* 1969). Moore and Whitacre (1972) suggested GRF may be the factor that confers this interspecific competitive advantage to *Ae. aegypti*.

Mosquito pupae do not feed, and thus competition is not related to nutrition. There is no evidence that pupae compete for space. Pupal mortality in the absence of predation reflects the degree of competition they faced as active larvae. This is because

their survival depends upon the nutrient reserves they accumulated while in the larval stages (Shannon and Putnam 1934).

Predation and parasitism affect all mosquito immature stages. Predators may be non-related organisms or may be conspecifics. Conspecifc predation (cannibalism) occurs in a few mosquito species, but *Ae. aegypti* mouthparts limit their predation to organisms the size of bacteria and protozoa. They are susceptible to predation by many other organisms, including insects, amphibians and fish. There are numerous parasites that infect mosquito larvae. Though field studies show these viruses, bacteria, protozoa, fungi or nematodes are seldom successful in naturally controlling mosquito populations, parasite influence upon a single oviposition site may be considerable (Service 1995). Therefore gravid *Ae. aegypti* should avoid sites containing predators or parasites. There is evidence that ovipositing females may detect cues from predators and parasites (Blaustein and Kotler 1993, Lowenberger and Rau 1994).

Given these fitness concerns, ovipositing *Ae. aegypti* must detect and absorb information from a variety of sources. While the biology of the site plays an important role in oviposition site choice, the abiotic factors affect initial orientation to and acceptance of suitable oviposition sites. Females detect these abiotic factors by visual, olfactory and chemo-tactile sensing apparati.

Attraction/Stimulation versus Repellency/Deterrency

The orientation of any organism towards or away from a particular object (in this study, oviposition sites) may be described as either positive (towards the object) or

negative (away from the object) (Dethier *et al.* 1960). If the initial response is towards the object, that object is defined as "attractive." If the initial response is away from the object, that object is defines as "repellent." The initial attraction to an object may be based on several long-range cues. However, short-range cues may come into play once the organism is at the object. A mosquito may find an oviposition site attractive based upon visual and long-range chemosensory inspection, but upon arrival at the site receive cues (such as chemo-tactile cues) that initiate a further positive (stay at the site) or negative (leave the site immediately) reaction (Dethier *et al.* 1960). A localized positive reaction is termed "stimulation," while a similarly focal negative reaction is defined as "deterrency." The distinctions between attraction and stimulation, and repellency and deterrency, is mainly based upon the range at which the directed response may be measured (Day, personal communication).

Abiotic Factors that Influence Oviposition Site Choice

Gravid female mosquitoes use a wide variety of cues based on environmental factors to determine the suitability of an oviposition site. The incorporation of visual and olfactory cues assist in orientation towards a site, while olfactory and chemo-tactile cues present within the site serve as oviposition stimulants or deterrents. Studies reveal that *Ae. aegypti* females rely on all their sensory modalities to detect physical factors such as shade, reflected light, temperature, humidity, salinity, substrate moisture and substrate texture.

Visual Cues

Aedes aegypti are day-active mosquitoes, and as such utilize visual cues in the initial orientation toward both hosts and oviposition sites. Suction-trap experiments revealed that the maximum range at which mosquitoes of the genera *Aedes*, *Anopheles*, *Culex*, *Culiseta*, *Psorophora* and *Uranotaenia* may perceive objects is 15.5-19.0 m (Bidlingmayer and Hem 1980). Laboratory experiments demonstrate a general preference for darker objects whether the mosquito is host-seeking, searching for a resting site, or seeking an oviposition site (Brett 1938, Frost 1954, Gilbert and Gouck, Hecht and Hernandez-Corzo 1963, Browne and Bennett 1981, Allan *et al.* 1987, Bentley and Day 1989). In the laboratory, ovipositing females of many mosquito species prefer oviposition sites (termed ovipots) painted black or brown over lighter hues. *Aedes aegypti* prefer black over clear ovipots (Fay and Perry 1965) and black over grey or white ovipots (O'Gower 1963) in two-way choice experiments.

Although different in experimental design, many other studies reach similar conclusions with *Ae. aegypti* and other "tree-hole" mosquitoes, including other Aedine mosquitoes and species in the genus *Toxorhynchites*. Gubler (1971) found a distinct preference for glass ovipots resting on black surface in his tests with *Ae. albopictus* and *Aedes polynesiensis*. Hilburn *et al.* (1983), using a two-way choice design, demonstrated that *Toxorhynchites rutilus rutilus* prefer dark-colored ovipots. Contrast between the ovipot and cage floor was investigated, with the conclusion that darker ovipots were equally preferred regardless of the degree of contrast with the surrounding environment. Contrast between target and background plays a prominent role in the orientation of many

mosquitoes, but appears to impact host-seeking behavior more than pre-oviposition behavior (Sipple and Brown 1953, McCrae 1984).

The influence of substrate color on the orientation of *Ae. aegypti* to oviposition sites also was studied. Filter paper was dyed with ink and placed on soaked cotton in petri dishes. The dark ink-dyed filter paper accumulated many more eggs than the undyed (control) counterpart (Beckel 1955). The chemical differences between dyed and undyed paper were not examined, however, and so the attractive nature of the dark-papered oviposition site may not solely be due to visual cues. By dyeing blotter paper, towelling and paper bag material, Fay and Perry (1965) recorded a higher percentage of eggs on brown rather than green or white colored substrates of the same material. With Tintex dye, O'Gower (1963) collected 73% of *Ae. aegypti* eggs on the darker filter paper (black compared to grey) in two-choice tests. As the same dye was used to make the black and grey color combinations, it may be concluded that the chemical nature of the dye did not affect female oviposition site choice. However, the grey paper was colored with less dye than the black paper (via dilution). It is possible that the amount of dye used could affect the degree of attraction to ovipositing females.

Another prominent visual signal emitted by oviposition sites is shape. Although not specifically testing *Ae. aegypti*, Browne and Bennett (1981) discovered that host-seeking females orient to the projecting ends of three-dimensional rectangular objects. In a direct comparison, most field caught species (in New Brunswick) were attracted to cuboid rather than pyramidal targets. Chadee *et al.* (1995) presented evidence that *Ae.*

aegypti female gravids do not exhibit a preference (indicated by total egg count) between round and rectangular ovipots.

Other visual indicators of an oviposition site include water color and water reflectance. The use of dyes demonstrated a distinct preference for darker-colored water by *Cx. quinquefasciatus*. The attraction to darker waters was due to the increased optical density conferred by the india ink used. Gas chromotography and evaporation/reconstitution techniques were used to isolate and remove the volatile components of the ink-test solution. The resultant non-volatile india ink solution received as many eggs as the the volatile india ink solution. No comparative data exists for *Aedes* mosquitoes.

Water reflectance is a property of both water depth and incident light. With constant ovipot volume, *Ae. aegypti* gravids did not select any particular oviposition site with water volume (and thus water depth) varying between 25 and 200 ml in a 600 ml beaker (Fay and Perry 1965). A site containing damp substrate and lacking water was not tested. O'Gower (1963) found the presence of water to act as a visual attractant to females. Ovipots containing water were preferred over ovipots with only dampened substrates under conditions of normal illumination. In the dark, however, gravid *Ae. aegypti* laid more eggs on the dampened surface ovipots. The substrates were of different material (termed as rough and porous for the moistened ovipots and smooth for the water-containing ovipots), which would allow for tactile selection by mosquitoes ovipositing in darkness. As these preferences were consistent across both 80% and saturated

humidities, O'Gower (1963) concluded that the attraction to water-containing ovipots that only occured in daylight must be due to the reflectance properties of the water.

The effects of incident light on *Ae. aegypti* oviposition have not been directly tested. Strickman (1982) found *Aedes vexans* preferred shaded areas over those exposed to direct light. Kittyapong and Strickman (1993) attempted to minimize *Ae. aegypti* breeding in Thai water storage pots by covering such pots with metal lids. They found larvae in more covered pots than they did in uncovered pots. Though indirect, this evidence indicates that *Ae. aegypti* females preferentially select shaded oviposition sites. Evidence exists that other tree-hole mosquitoes prefer oviposition sites in the shade compared to those exposed to direct sunlight (Jordan and Hubbard 1991).

Olfactory Cues

The presence of water at an oviposition site may be determined by hygroreceptors which measure the concentration of water vapor in air (Kennedy 1942). Thomson (1938) attempted to localize hygroreceptors in *Cx. quinquefasciatus*, but was unsuccessful. An experiment demonstrated the presence of hygroreceptors on the antennae of *Ae. aegypti* by use of an olfactometer with differential humidities passing through the two ports (Roth and Willis 1952). Antennal segment ablation and ultrastructure studies found correlations between the humidity response and the distribution of thin-walled trichoid or basiconic sensilla on the antennae of females (McIver 1970a). Antennal ablation was later found to be a general inhibitor of mosquito behavior, however, so Bar-Zeev (1960) performed similar experiments with mosquitoes whose antennae were either ablated,

covered with wax, or left intact. The results correlated with those of Roth and Willis (1952), and further demonstrated that *Ae. aegypti* palps do not possess hygroreceptors, but may house moisture-receptors sensitive to contact with liquid water (Bar-Zeev 1960).

Kellogg (1970) studied microelectrode recordings of *Ae. aegypti* antennal responses to water vapor. He found that one particular type of sensilla basiconica (termed A3 grooved-peg sensilla) was sensitive to changes of 2% in the relative humidity (RH). More detailed ultrastructure work showed that the absolute number of sensilla basiconica differed among mosquito species and sexes (McIver 1970a, McIver 1970b). Later studies determined that the A3 grooved-peg sensilla were in fact humidity-dependent lactic acid receptors (Davis and Sokolove 1976, Davis and Bowen 1994). They noted that these receptors would fail under conditions of low humidity. There has been no subsequent determination of the presence or absence of hygroreceptors in *Ae. aegypti*, although Davis and Bowen (1994) remarked that all mosquito oviposition-related attractants found on mosquito antennae have so far been mapped to the A2 short, sharp or short, blunt sensilla.

The differential response of mosquitoes to humidity initially measured by Roth and Willis (1952) has been confirmed for numerous species, including the tree-hole mosquitoes *Ae. aegypti* and *Toxorhynchites amboinensis*, by the use of humidity-gradients (O'Gower 1963, Jordan 1992). Under conditions of 100% RH, *Ae. aegypti* were unable to distinguish between wet and moist sites, although they chose sites containing water when the RH was lowered to 80% (O'Gower 1963). To eliminate the interaction of palpal moisture receptors, Jordan (1992) used screening to prevent test subjects from contacting water surface. Under conditions of 65% RH ovipositing *Tx.*

amboinensis females chose dull, non-reflective sites containing water over reflective sites with no water, while at 100% RH the gravids did not distinguish between the two sites: this indicated an ability to detect air moisture content. It should be noted that mosquitoes of the genus *Toxorhynchites* typically launch their eggs into an oviposition site from the air. There is some debate as to whether their elliptical flight patterns result in contact with the water. As the water was screened in these experiments, *Tx. amboinensis* likely selected an oviposition site based upon differences in local RH (when the ambient RH was not 100%). Extrapolation of these results to *Ae. aegypti* is difficult, as *Aedes* mosquitoes must land to oviposit. They may not touch the water, but chemo-tactile and moisture-related cues from the substrate may confound any implication of humidity-detection.

Chemo-tactile Cues

Kennedy (1942) observed contact between ovipositing *Ae. aegypti* females and the water surface. The volatilization of inorganic salts from water is minimal. Ikeshoji (1966) proposed the detection of water-borne oviposition stimulants are detected by *Cx. quinquefasciatus* labellar receptors. Other *Culex* mosquitoes are known to drink from the oviposition waters after landing. As they did so prior to oviposition, Weber and Tipping (1990, 1993) suggested gravid *Culex pipiens* and *Culex restuans* were "tasting" the water to determine the suitability of a site for their progeny. *Culex* mosquitoes deposit all their mature eggs in a single raft at a single site. Thus, the observation that drinking continued in the middle of oviposition indicated that determination of site-suitability was not the

only possible function of ingesting the water (Weber and Tipping 1993). Therefore assessments of site-suitability may be mediated by other contact mechanisms unrelated to drinking.

Water salinity affects the survival of both mosquito eggs and larvae, and salinity tolerances vary depending on the species involved (Macan 1961). The salt concentration in brackish-water habitats such as mangrove swamps changes with the incoming tides. In general, the mosquitoes that oviposit in these waters have higher salinity tolerance than those that do not (Petersen and Rees 1967, Petersen 1969). Freshwater habitats are not immune to changes in salinity, however. The increasing concentration of salts due to evaporation of water may indicate to mosquitoes that utilize temporary water sources that the pool or tree-hole is drying up (Pappas and Pappas 1983). Salt concentration determination by ovipositing females may determine the survival of their progeny.

Thus researchers note that the ability of inorganic salts to act as oviposition attractants or stimulants at any concentration is less important than their ability to act as repellents or deterrents at high concentrations (Petersen 1969). This was reflected in the oviposition responses of *Aedes sollicitans*, *Aedes taeniorhynchus* and *Psorophora confinnis* to varying concentrations of seven inorganic salts in the laboratory. These are salt-marsh mosquitoes, although *Ps. confinnis* is rarely found in the marshes themselves. Its populations appear confined to the marginal areas of salt-marshes that tend to have lower salinity. Regardless of the salt tested (including bicarbonates, carbonates, chlorides and sulfates), less than 20% of all oviposited eggs for any species were found in salt solutions of normality above 0.35 (Petersen 1969).

Pappas and Pappas (1983) discovered differential tolerance to sodium choloride (NaCl) among the eggs and larvae of *Culiseta inornata*. Females oviposited in waters with NaCl concentrations in which eggs remained viable: eggs were not oviposited in sites above 0.1 M NaCl, and eggs reared in higher concentrations did not hatch. Larval survival to pupation was 50% at NaCl concentrations above 0.01 M, however, indicating that gravid *Cs. inornata* select oviposition sites based on egg rather than larval survival (Pappas and Pappas 1983).

This is unlikely to be as important in the life history of *Ae. aegypti* mosquitoes, as their eggs require a dessication period prior to hatch (and females lay their eggs above the water line) (Clements 1992). The salinity tolerance of *Ae. aegypti* is lower than that of saltmarsh mosquitoes: females laid significantly fewer eggs in waters containing 5 g or more NaCl per 1 L water (Woodhill 1941, Hudson 1956). Similarly, O'Gower (1963) found *Ae. aegypti* oviposition preference for sites containing distilled water over those holding 0.5% (by volume) NaCl solutions.

Since *Ae. aegypti* oviposit above the water line, substrate conditions including texture, temperature, and moisture content may affect female oviposition site choice. Although not technically defined, researchers note a general preference for rough over smooth substrates in every experimental design used (O'Gower 1963, Fay and Perry 1965, Chadee *et al.* 1995). The number of oviposition landings on ovipots containing rough substrate was the same as on ovipots containing smooth substrate (O'Gower 1963). However 60% of collected eggs were deposited on rough substrate regardless of illumination or humidity (O'Gower 1963).

Beakers lined with blotting paper or towelling received more eggs than beakers lined with manila, bond, or filter paper (Fay and Perry 1965). The authors concluded that both substrate roughness and water content could stimulate or deter oviposition by *Ae. aegypti*; they were unable to separate these two factors. Chadee *et al.* (1995) compared the proportion of eggs laid on a hardboard "paddle" with those laid on either the water surface or the surface of the ovipot (plastic or glass). In all field tests, greater than 80% of collected eggs were found on the paddle regardless of ovipot shape or material.

Russo (1978) quanitified the degree of roughness preferred by ovipositing *Ae. vexans*. Sand was autoclaved and separated into 4 particle size groups using mesh sieves. These 4 groups were saturated with water and presented to gravid females in four-way choice tests. Most eggs were deposited in containers with 1.5 mm or 0.8 mm sand diameter. This corresponded to a significant preference for interstitial space in the range of 0.33 to 0.62 mm. Extrapolation to *Ae. aegypti* is difficult, since the yellow fever mosquito does not normally oviposit on soil. Russo (1978) noted the preferred interstitial space range allowed deposition of a cluster of 6 to 8 eggs, which is the typical cluster size of *Ae. vexans*. He suggested that this may confer some protection from the environment. Perhaps *Ae. aegypti* utilize rough substrates in an effort to protect their eggs from environmental concerns, such as the effects of dessication or direct sunlight.

Arctic mosquitoes must take into account the temperature conditions at the oviposition site. A field study with *Aedes impiger* and *Aedes nigripes* demonstrated a significant preference for sites that are moist and sheltered from the wind (Corbet and Danks 1975). These researchers also found that eggs are concentrated on rough-textured

slopes such that they attain maximal exposure to the sun. As exothermic animals, arctic mosquitoes and their eggs likely require a high degree of exposure to the sun for physiological development. In contrast, direct solar-exposure may be detrimental to the tropically-distributed *Ae. aegypti*. However, the mechanisms for determining microenvironmental conditions may be the same for arctic and tropical mosquitoes. That is, chemo-tactile cues are used to detect moisture content, substrate texture, and perhaps substrate temperature.

Biotic Factors that Influence Oviposition Site Choice

While the physical factors outlined above provide gravid females with the basic information needed to determine oviposition site suitability, their ultimate acceptance or rejection of a site depends upon a wide variety of biotic factors. The sensory modalities involved are the same, although different olfactory and chemo-tactile sensilla specific for organic chemicals are responsible for conveying information regarding oviposition site biota. The delineation of cues into visual, olfactory and chemo-tactile are not as well defined for gravid assessment of biotic factors. Therefore biotic factors are categorized by derivation. Phytotelmata, bacteria, parasites, predators, and conspecific and heterospecific immatures all affect oviposition site choice.

Cues from Phytotelmata

The site-derived cues include chemicals produced by the vegetation which houses the oviposition site. Such cues may affect oviposition site choice by indicating to the gravid mosquito the general organic content of the site (Frank and Lounibos 1983). Larval *Ae. aegypti* feed upon available microfauna and microflora, including fungi, bacteria and algae (Trager 1935, De Meillon *et al.* 1945). It is possible that the preferred bacteria are associated with specific vegetative matter. If the female detects chemicals indicative of this vegetative matter, then the site likely contains proper larval nutrients.

Some mosquito species, and indeed strains, prefer fluid from their natural habitat. Lounibos (1978) demonstrated *Eretmapodites subsimplices* preference for fruit husk water over tap water. The congeneric *Eretmapodites quinquevittatus* is never found in fruit husks in nature; gravid females selected tap water over water from fruit husks. Studies on the pitcher-plant mosquito *Wyeomyia ulocoma* indicated an even stronger specificity for waters from which larvae normally develop in nature (Lounibos and Machado-Allison 1993). The immatures of this species are only found in waters from bracts of *Heliconia* plants. These pitcher-plant bracts contain a plant-derived fluid that is present before the bract even opens to the environment. The preference for this fluid was not demonstrated with the generalist mosquito *Culex pleuristriatus*. Waters from specific trees affected tree-hole mosquitoes in a similar manner (Copeland and Craig 1992). The generalist *Aedes triseriatus* oviposited in sites containing water from either maple or beech tree-holes with no preference. The larvae of the congeneric *Aedes hendersoni* are usually found in maple tree-holes. In the laboratory most eggs were collected from sites

containing water from maples rather than beeches. In a comparison of laboratory and feral strains of *Ae. aegypti*, Leahy *et al.* (1978) found a dramatic increase in oviposition rate of the feral strain upon presentation of an oviposition site containing coconut-shell infusion.

Increased attraction due to breeding water infusion is a widespread phenomenon. Aedine mosquito preferences for hay and grass infusions were demonstrated with *Ae. aegypti*, *Ae. albopictus*, *Aedes nigromaculis*, *Ae. taeniorhynchus* and *Ae. triseriatus* (O'Gower 1963, Hazard *et al.* 1967, Wilton 1968, Ikeshoji and Mulla 1970, Gubler 1971, Bentley *et al.* 1976, Reiter *et al.* 1991). Varying concentrations of hay infusion were not deemed attractive to ovipositing *Ae. aegypti* in Trinidad (Chadee *et al.* 1993). This conflicts with the work of Reiter *et al.* (1991) in Puerto Rico. The authors suggest that differences may lie in the methods of preparation and the plant species from which the hay infusion was prepared (Chadee *et al.* 1993).

Some phytochemicals reduce the number of eggs found at experimental oviposition sites. Noting the small size of the *Aedes melanimon* populations around the *Hemizoniz fitchii* groves of northern California, Klocke *et al.* (1987) tested the effects of various *H. fitchii*-derived chemicals. The major monoterpenoid present, 1,8 cineole, elicited a negative oviposition response from gravid *Ae. aegypti*. Similarly, ovipositing *Ae. aegypti* avoided oviposition sites containing extracts from six aquatic-plant and algae species that were previously demonstrated toxic to larvae (Angerilli 1980).

Cues from Bacteria

Bacteria themselves may indicate site-suitability. Species produce specific organic chemicals that gravid females may detect. The amount of these chemicals present may invoke a dose-dependent response by gravid mosquitoes. Too low a concentration indicates a lack of food availability, which may be due to the presence of larval competitors. Too high a concentration may indicate organic pollution; *Ae. aegypti* larvae fair poorly under such conditions (Shannon and Putnam 1934).

Beehler *et al.* (1994) tested the oviposition response of *Cx. quinquefasciatus* to oviposition sites containing proteins isolated from organic infusions. Most eggs were collected from ovipots with a 1.0% lactalbumin hydrolysate solution. This positive response decreased upon addition of the antibiotic neomycin, indicating that the attraction or stimulation the lactalbumin solution was in part due to the presence of bacteria. The positive response of gravid *Cx. quinquefasciatus* to sites containing either hay infusions or their associated bacteria was demonstrated to be due to a long-range olfactory attraction: significantly more females placed in an olfactometer were trapped in the chamber containing the treatment ovipot (Hazard *et al.* 1967).

Maw (1970) found that n-capric acid, produced by pseudomonad bacteria, elicited oviposition by *Ae. aegypti*. Using a capric acid substrate, Ikeshoji *et al.* (1979) found the bacterium *Pseudomonas aeruginosa* produced a volatile compound (7,11-Dimethyloctadecane) attractive to ovipositing *Ae. aegypti*. Hasselschwert and Rockett (1988) isolated several bacterial species from *Ae. aegypti* larval rearing medium, and tested these bacteria for their effects on oviposition. Direct comparisons with controls

were not performed for most species, preventing the labelling of specific bacteria as mediating oviposition attraction or repellency. Comparisons among bacteria indicated that *Bacillus cereus* and *P. aeruginosa* elicited the strongest positive response from ovipositing *Ae. aegypti*. These authors concluded gravid mosquitoes may select sites based on the presence of specific bacterial species (Hasselschwert and Rockett 1988).

Cues from Eggs

The best-studied example of an organic cue in mosquito oviposition is the egg apical-droplet pheromone of *Cx. quinquefasciatus* (Laurence and Pickett 1985). Females lay gonotrophically-concordant eggs in a single batch as a floatilla on the surface of the water. Females are attracted to these egg rafts, but not to visual mimics constructed of polyethylene and cardboard (Bruno and Laurence 1979). The apical portion of these eggs is exposed to the environment, and a volatile liquid droplet is released from this end (Bruno and Laurence 1979). The apical-egg droplet pheromone attracts gravid conspecifics, such that a site containing eggs will receive more eggs than will a comparable control site containing only water.

Gas chromatography revealed this compound to be erythro-6-acetoxy-5-hexadecanolide (Laurence and Pickett 1982). The active enantiomer is the (-)-(5R,6S) form (Laurence *et al.* 1985, Hwang *et al.* 1987, Dawson *et al.* 1989). The mosquito *Cx. quinquefasciatus* egg apical-droplet pheromone attracted gravid *Culex tarsalis* and *Culex molestus* (Bruno and Laurence 1979), suggesting the pheromone is not species-specific. However, the mosquitoes *Ae. aegypti* and *Anopheles quadrimaculatus* did not respond to

the pheromone, indicating specificity confined to congenerics (Hwang *et al.* 1987). Researchers contend reception of this pheromone indicates to gravids the suitability of an oviposition site: at least one female has already examined the site and determined it suitable for her progeny. The response to erythro-6-acetoxy-5-hexadecanolide increases with increasing egg count and does not dissipate until the eggs hatch or die (Bruno and Laurence 1979). Researchers note that continued oviposition at a site containing many eggs is likely to lead to crowding of a particular site with larvae. Crowding may confer an advantage to any organism against predators: with so conspecifics as prey, the odds of all the progeny of a particular female being eaten diminish (Andrewartha and Birch 1954). However, overcrowding may adversely impact the survival and growth rates of mosquito larvae (Terzian and Stahler 1949). As already noted, a GRF produced by older instars reared under highly crowded conditions is toxic to conspecifics (Ikeshoji and Mulla 1970). Therefore, it seems disadvantageous for gravid *Culex* to continue ovipositing in sites with too many eggs. Knight and Corbet (1991) found that a single compound may elicit contrary responses from gravid *Ae. aegypti* depending upon source concentration. Thus, the *Culex* apical egg droplet pheromone may attract females at lower doses but repel them at higher concentrations. This has not been studied.

Field trials demonstrated the efficacy of the egg apical-droplet pheromone outdoors. Differential response of *Cx. quinquefasciatus* geographic strains was noted. The Sri Lankan strain was indifferent to the compound, while Japanese and East and West Kenyan strains showed strong positive orientation towards sites containing the

compound (Otieno *et al.* 1987, Otieno *et al.* 1988). Combining the pheromone with an insect growth regulator did not diminish this attraction (Otieno *et al.* 1988).

Gravid *Cx. tarsalis* also prefer sites containing conspecific egg rafts. Though not the same compound, breakdown products of the *Cx. quinquefasciatus* pheromone belong to the same fatty acid family as those from *Cx. tarsalis* (Starratt and Osgood 1972, 1973). This similarity may explain the limited attraction of *Culex* mosquitoes to congeneric egg rafts (Bentley and Day 1989).

Eggs of the mosquito *Ae. aegypti* do not exude this pheromone. In fact, *Aedes* mosquitoes exhibit a negative response to sites containing conspecific eggs. Kitron *et al.* (1989) studied the oviposition behavior of *Ae. triseriatus* in the field. Traps in which eggs were allowed to accumulate did not receive as many new eggs as traps in which the eggs were removed on a weekly basis. This indicated a preference for sites with fewer eggs present.

Other aedine mosquitoes exhibit a similar behavior with regards to the presence of eggs at an oviposition site. Females of *Aedes togoi* preferred control ovipots to those containing conspecific eggs at a density of 0.3 eggs/ml (Onyabe and Roitberg 1997). Ahmadi and McClelland (1983) removed newly-laid eggs of *Aedes sierrensis* after a 24 h soak in distilled water. This egg-holding water was presented as a choice to gravid females, along with an oviposition site containing distilled water. No statistically significant preference for either site was found, although 60.0% of deposited eggs were found in the control site. The avoidance of conspecific eggs also was demonstrated for *Ae. albopictus* and *Ae. polynesiensis* (Gubler 1971). In these experiments, females were

offered a choice of sites containing either water exposed to conspecific eggs or water exposed to heterospecific eggs. Both species chose the sites containing water exposed to heterospecific eggs. This indicated that the avoidance of eggs was based on species-specificity (Gubler 1971).

Some studies show that *Ae. aegypti* similarly avoids sites containing conspecific eggs. In choice tests Chadee *et al.* (1990) found gravids preferred sites devoid of eggs. If only offered sites with conspecific eggs already present in an equal number in each site, females avoided sites in which they had previously oviposited. Thus females preferred sites without eggs over those with conspecific eggs, but if given no choice preferred sites with conspecific eggs that were not their own (Chadee *et al.* 1990).

The use of randomly amplified polymorphic DNA markers demonstrated avoidance of oviposition sites containing closely-related eggs in a Puerto Rico field study (Apostol *et al.* 1994). These markers indicate genetic relatedness, allowing the authors to designate family-specificity of eggs. Though the number of families present at a site increased with increasing egg number, the average size of the family decreased. This indicates that a gravid *Ae. aegypti* avoids overburdening a site that already contains some of her eggs (Apostol *et al.* 1994).

Of note is the conflicting report of Allan and Kline (1998), who concluded *Ae. aegypti* preferred oviposition sites containing either conspecific eggs or the eggs *of Ae. albopictus* over control sites. These authors offer no explanation for why their results differ from previously published reports. Allan and Kline (1998) used freshly-laid eggs in their experiments, while Chadee *et al.* (1990) stored the eggs for some time prior to

experimentation. It is possible that differences in the time elapsed from oviposition to experimentation may account for these conflicting results.

Cues from Larvae

Several experiments demonstrate gravid mosquito orientation to waters that either previously held or currently hold conspecific larvae. While the onus of research indicates that aedine mosquitoes avoid sites containing conspecific eggs, laboratory experiments reveal that many gravids seem to prefer sites containing conspecific larvae and pupae and their associated waste products and bacteria. The attraction appears to be species specific and occurs across varying larval instars and densities.

This attraction seems limited to certain species in the genus *Aedes*. Conspecific larvae neither attracted nor repelled oviposition by *Culiseta longiareolata* (Blaustein and Kotler 1993). *Anopheles gambiae* females avoided sites containing conspecific larvae at densities of 1.5 per ml (McCrae 1984). When offered a choice between sites containing larvae and sites containing larval rearing water (LRW) from which the larvae had been removed, females oviposited preferentially in LRW. The tree-hole mosquito *Tx. amboinensis* is neither attracted to nor repelled by waters containing conspecific immatures (Linley 1988). Though fewer eggs were counted in sites containing larvae, observation revealed egg reduction was due to egg-cannibalism by larvae, and not a change in oviposition response by gravid females.

Onyabe and Roitberg (1997) found unfiltered LRW (hence replete with bacteria, food supplements and waste products) attractive to gravid female *Aedes togoi*. The

between presence of bacteria and number of eggs oviposited in the oviposition site. Treating larvae with kaolin and filtering their holding water through a 0.45 mm membrane reduced bacterial colony forming unit (CFU) counts significantly. Treating larvae with kaolin, filtration and antibiotics reduced bacteria CFU counts more, and the attraction to ovipositing mosquitoes was abolished. Oviposition sites holding this treatment received fewer eggs than control sites, indicating that bacteria mediate the observed attraction of gravid *Ae. aegypti* to sites containing conspecific immatures (Benzon and Apperson 1988).

Maire (1985) reared *Ae. atropalpus* larvae under axenic (in the absence of any other biota) conditions. Larvae raised in this sterile environment grew and developed normally. Gravid female *Ae. atropalpus* preferentially oviposited in sites containing axenic larvae and their rearing water. Maire (1985) concluded that larvae either secrete or excrete specific chemicals that attract gravid conspecifics.

Cues from Pupae

Mosquito pupae are motile, but they do not feed nor do they excrete or defecate (Clements 1992). From the standpoint of mosquito fitness, they represent to gravid females the suitability of a site perhaps more than any other immature stage could. They have successfully hatched and survived through all larval instars, and will soon emerge as adults. Therefore, a site with either pupae or their cast off skins (exuviae) may indicate oviposition site suitability to gravid females.

McCrae (1984) found no difference in the number of eggs deposited at a site when comparing tap water and tap water with pupae present at densities of 1.5 pupae per ml. Gravid *Tx. amboinensis* females were not selective of sites containing conspecific pupae: no significant alteration in oviposition rates were recorded (Linley 1988). Ovipots containing water in which pupae were held for 24 h neither repelled nor attracted *Ae. sierrensis* females (Ahmadi and McClelland 1983). Gubler (1971) tested the oviposition response of *Ae. albopictus* and *Ae. polynesiensis* to both waters which had held pupae and water that currently contained pupae. Neither type of oviposition site received a significantly different number of eggs than control sites.

By contrast, *Ae. atropalpus* responded positively to waters that previously held conspecific pupae (Kalpage and Brust 1973). Soman and Reuben (1970) found ovipots with pupal densities of 1 per 10 ml were preferred over control sites by gravid *Ae. aegypti*. A two-way choice test offered gravids sites containing either 4th instar larvae or pupae at the same density (1 per 10 ml). No preference for either site was observed. The preference for sites containing pupae over control sites was abolished at pupal densities of 1 per 2 ml (Roberts and Hsi 1977).

Cues from Predators and Parasites

An important element to the efficacy of biological control applications is that they do not repel or deter oviposition by the mosquito in question. Predatory fish may consume large quantities of *Ae. taeniorhynchus*; female mosquitoes should avoid sites containing predatory fish if possible to ensure maximum survival of their progeny. By

comparing oviposition in mangrove forest pools with and without fish, Ritchie and Laidlaw-Bell (1994) demonstrated a negative oviposition response by gravid *Ae. taeniorhynchus* to sites harboring fish. This response was eliminated if the fish were killed and dried. Thus the negative response is due to some factor associated with live fish.

Blaustein and Kotler (1993) examined a system in which conspecific immatures could also be predators. *Cs. longiareolata* larvae are cannibalistic if the available food supply is low. The presence of larvae at densities of 1 per 30 ml did not repel or deter gravid females. The addition of larvivorous tadpoles to half the oviposition sites did engender a negative oviposition response. This indicates that predation by non-related organisms is more a concern to the fitness of *Cs. longiareolata.*

Ae. aegypti avoided sites containing water in which conspecific larvae infected with the digenean *Plagiorchis elegans* had been reared (Lowenberger and Rau 1994). This effect was not diminished upon water boiling and filtration, or with the addition of antibiotics. Thus the negative response is mediated by some stabile chemical secreted or excreted from conspecific larvae infected with this parasite. Subsequent experiments demonstrated an association between strength of the avoidance and both infection intensity and site of infection in larvae (Zahiri *et al.* 1997b).

Synopsis and Introduction

Where the majority of studies have come to the same conclusion, a general consensus has been reached regarding that particular aspect of *Ae. aegypti* behavior under investigation. However, many of the experiments reviewed above produce contradictory results and conclusions. This is especially apparent regarding the response of gravid *Ae. aegypti* to oviposition sites containing conspecific larvae or eggs. The most apparent potential reason for conflicting results is the high degree of variation in experimental designs used. No two groups of investigators used the same experimental set-up. One major goal of this project is to follow the most robust of the published procedures and to standardize all materials in order to ensure reproducibility of the results.

One aspect of the experimental designs that varied so greatly was the size of the oviposition site used. The effects of oviposition site size on resulting egg distribution is investigated in the hopes of elucidating possible reasons for the presence of conflicting results in the literature. The effects of larval instar and density on site selection are studied with the aim of adding support to the literature indicating either a positive response, negative response, or lack of effect. The mechanisms that orient a gravid towards or away from sites holding conspecific larvae are investigated; a chemical basis for attraction is validated, and the origins of this chemical, or chemicals (endogenously produced by larvae or a contaminant of the LRW), are subject to careful examination. A similar study is performed on the effects that egg density and distribution may have on subsequent egg-laying. The cues involved in female detection of conspecific eggs are also the subject of investigation.

Another component of this project relates to describing the process of oviposition site selection. Although numerical collation and statistical analysis of trapping and wind tunnel experiments may reveal ultimate egg distribution, it is not known how mosquitoes behave in order to distribute these eggs. The potential ramifications of senescence and gonotrophic age on oviposition site selection are investigated. Many physiological and behavioral changes in insects due to increasing age are known. Under considerations of relative fitness, the degree of selectivity expressed by females of different age is studied.

CHAPTER 2

GENERAL METHODS

Maintenance of the Mosquito Colony

Aedes aegypti solony mosquitoes were originally collected in the Thai province of Chachoengsao in 1994, and subsequently maintained by continuous rearing at the Medical Entomology laboratory at the University of Massachusetts at Amherst. They were reared in walk-in bioroom at constant temperature (27 ± 2 °C) and constant relative humidity (65 ± 5 % RH) under a 14 L: 10 D photoregime. Lighting was provided by fluorescent lights housed in the chamber ceiling.

Eggs collected and stored on paper towel were placed in a vacuum flask containing 150 ml room temperature-equilibriated tap water (RTEW) and subjected to a vacuum for 2 h, at which time the hatched larvae were transferred to rearing trays (white photograph-developing pans). Two-hundred larvae were placed in 1 L of RTEW per tray, and fed 100 mg of a previously mixed 1:1 yeast hydrolysate:lactalbumin preparation on a daily basis.

Upon maturation (approximately 8-10 days post-hatch), *Ae. aegypti* pupae were removed by pasteur pipette and placed in a crystallizing dish containing 50 ml of RTEW. This dish was placed in colony cages (30x30x30 cm), constructed of formica and plexiglass held together by a wooden frame. Adults emerged within 48 h, and were allowed access to a 10% sucrose solution filtered through a cotton wick throughout their lifetime.

Blood-meals were provided by offering restrained chickens (less than 2.5 months olds) to the colony cages. Chickens were restrained by insertion into a nylon stocking and placed in cage for a maximum of 2 h. All birds were maintained at the University of Massachusetts small animal care facility in compliance with Institutional Animal Care and Use Committee (protocol # 17-03-1) guidelines.

Gravid females were offered a single oviposition site from days 3-5 post-feeding. This consisted of a glass crystallizing dish lined with paper towel and holding 50 ml of RTEW. The dish was removed on day 5 or 6 post-blood-meal. The paper towel upon which eggs had been laid was hung to dry for 24 h, and then stored in a plastic zip lock bag.

Maintenance of Experimental Mosquitoes

Experimental mosquitoes were hatched from eggs less than 2 months. They were treated in identical fashion to the colony mosquitoes except where mentioned in the specific experimental protocols and for the following: experimental *Ae. aegypti* were housed in smaller cages (12x12x30 cm) with no more than 500 mosquitoes (approximately equal numbers of each sex) per cage. In most experiments, females were offered a blood-meal from a restrained chicken on day 3 post-emergence. All fully-fed females were aspirated into a separate small cage, and provided access to a 10% sucrose solution. Unless otherwise noted, females used in experiments were collected from these cages 3-5 days after blood-feeding and aspirated into white transfer cartons. The transfer

cartons were placed in a cooler and taken to the building housing the experimental cages and wind tunnel.

General Design of Cage Experiments

Three Bioquip (CA) cages were used in the cage experiments. These cages consisted of a fine grade nylon weave (8 holes per cm^2) supported by a metal frame (Bioquip,CA); entrance was through a large zipper on one side. The cages measured 1.82 m in all directions. A white plastic background was spread under the cage to provide a uniform background for experimental mosquitoes. The three cages were placed in a row with 1 m between cages. Clear plastic drapes were hung between cages to prevent contamination of air from neighboring cages and their contents.

Fluorescent lights suspended above each cage provided a 14:10 L:D photoregime (0600-2000 h lights-on schedule). All windows were closed off with aluminum foil to eliminate outside sources of light. Temperature was maintained at 27 ± 4 °C. The RH was difficult to keep constant given the lack of available control, and thus a wide variation was encountered. Only experiments in which the RH fell within a range of 65 ± 10 % are included in this study.

Each experiment included the release of a single gravid female by mouth-aspiration into a cage. Each female had access to 8 oviposition sites (ovipots) arranged in a circular fashion around the periphery of the cage, such that the center of each ovipot was equidistant (50 cm) from the center of its two neighboring ovipots (Fig. 2.1). This design was used for the following reason. There is evidence that laboratory colonies of

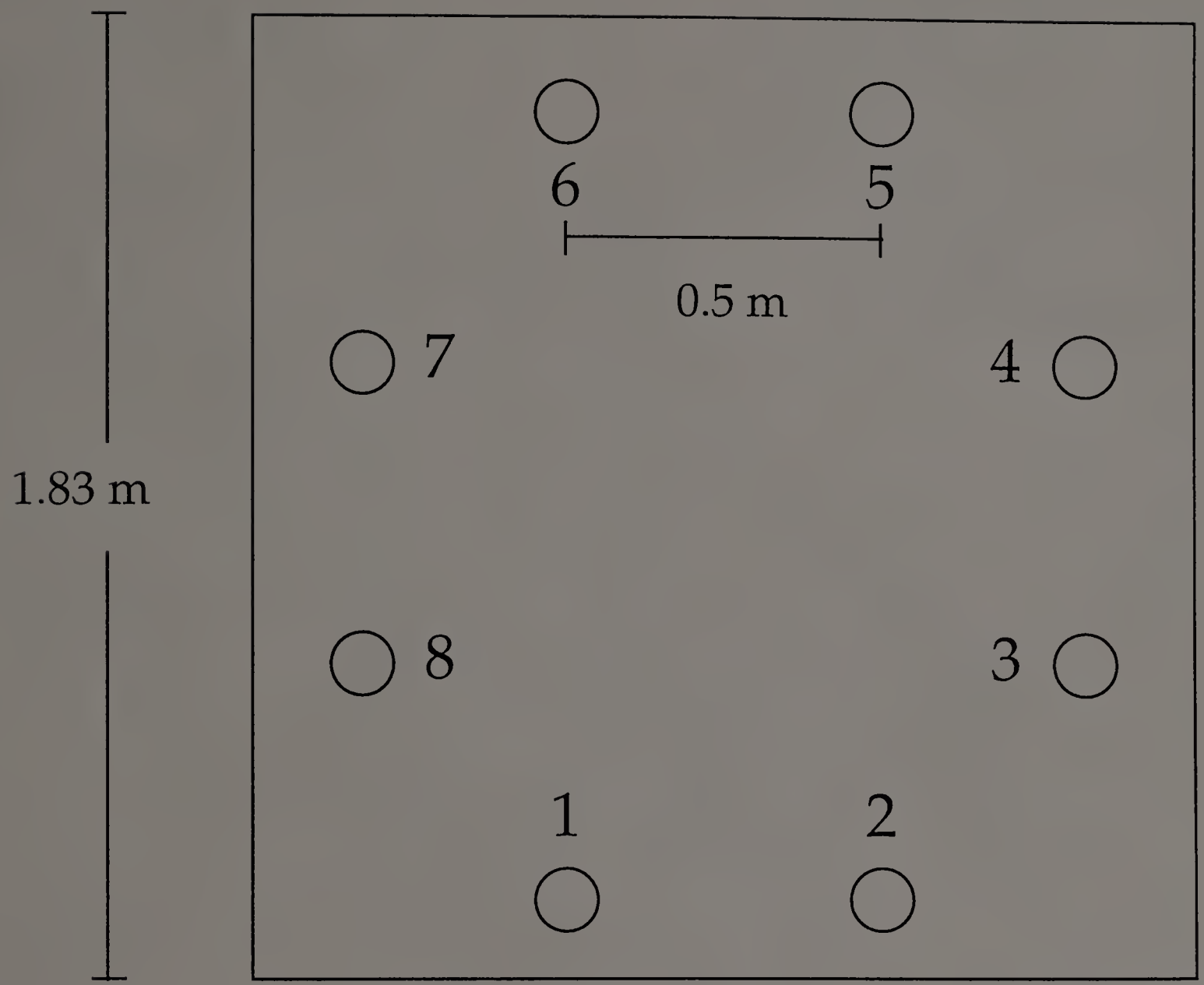

Fig 2.1. Overhead view of cage experiments incorporating 8 identical treatments. The numerical designations were also used for collection of data on experiments which required subsequent position transformation (involving 1 treatment and 7 controls).

Ae. aegypti tend to avoid laying all their eggs in one particular site (a phenomenon termed "skip oviposition"), as demonstrated by Corbet and Chadee (1993). One field experiment also found dispersal of a single female's eggs among several containers, and concluded skip oviposition may be a natural phenomenon rather than a laboratory artfiact (Apostol *et al.* 1994). With but one treatment and one control, the reliance upon counting eggs as an indication of attraction/stimulation or repellency/deterrency to the treatment may provide errant conclusions: although the treatment may be strongly attractive, the female may lay an equal or greater number of eggs in the control ovipot due to the skip nature of her oviposition. By providing more oviposition sites while maintaining only one treatment, a more accurate indication of the degree of attraction or repellency of the test oviposition site may be assessed.

The ovipots were glass jars spray-painted matte black (Testors #1032). The ovipots used in experiments were either 120, 480 or 960 ml in volume. The oviposition substrate lining the inside of jars was seed germination paper #76 (Anchor Paper, MN). Unless otherwise mentioned, all water used in experiments was distilled, passed through an organic removal-filter cartridge (Barnstead/Thermolyne, IA) and stored in a carboy. Each oviposition site was filled to 1/3 capacity with filtered-distilled water (FDW).

Experiments lasted for 23 h, allowing 1 h for removal of the females (by aspiration) and ovipots, collection of the ovipot substrate, and cleaning of the ovipots and cages prior to the next experiment. Preliminary evidence revealed peak female oviposition between 0800-1100 h daily. Assays were started and finished between 1300-1500 h to avoid interfering with peak oviposition times. Females were not given access

to sucrose during the experiments. Latex gloves were worn while handling cage contents in an attempt to prevent possible contamination by skin secretions and volatiles.

Statistical Analyses

Cage data were analyzed using a χ^2 contrast test in the program Statistix. For tests in which all eight ovipots contained the same treatment, the distribution of number of ovipots with any eggs present were compared across treatment levels. That is, the number of oviposition events (number of sites receiving any eggs across the whole sample size) was contrasted with the number of sites that didn't receive eggs. This ratio was collected for each tested factor, and then compared to assess the gravid response to different factors. See Appendix A for a sample analysis.

For experiments with one treatment (placed in a stratified random manner) and seven control oviposition sites, the data were first analyzed by comparing the number of eggs laid in the treatmnet ovipot vs. the number of eggs laid in all 7 control ovipots combined. This ratio was collected for each factor and compared across factors to determine gravid response. See Appendix B for a sample analysis.

There is a potential for a "spill-over" effect of cues in which the ovipots adjacent to the treatment ovipot received a number of eggs between the numbers collected from the treatment ovipot and the remaining control ovipots. Such an effect is evidence of a concentration gradient of chemical cues that originate from the treatment ovipot. This effect can be tested following position transformation (Fig. 2.2). In position transformation, the treatment ovipot is given the designation position 0. All ovipots to

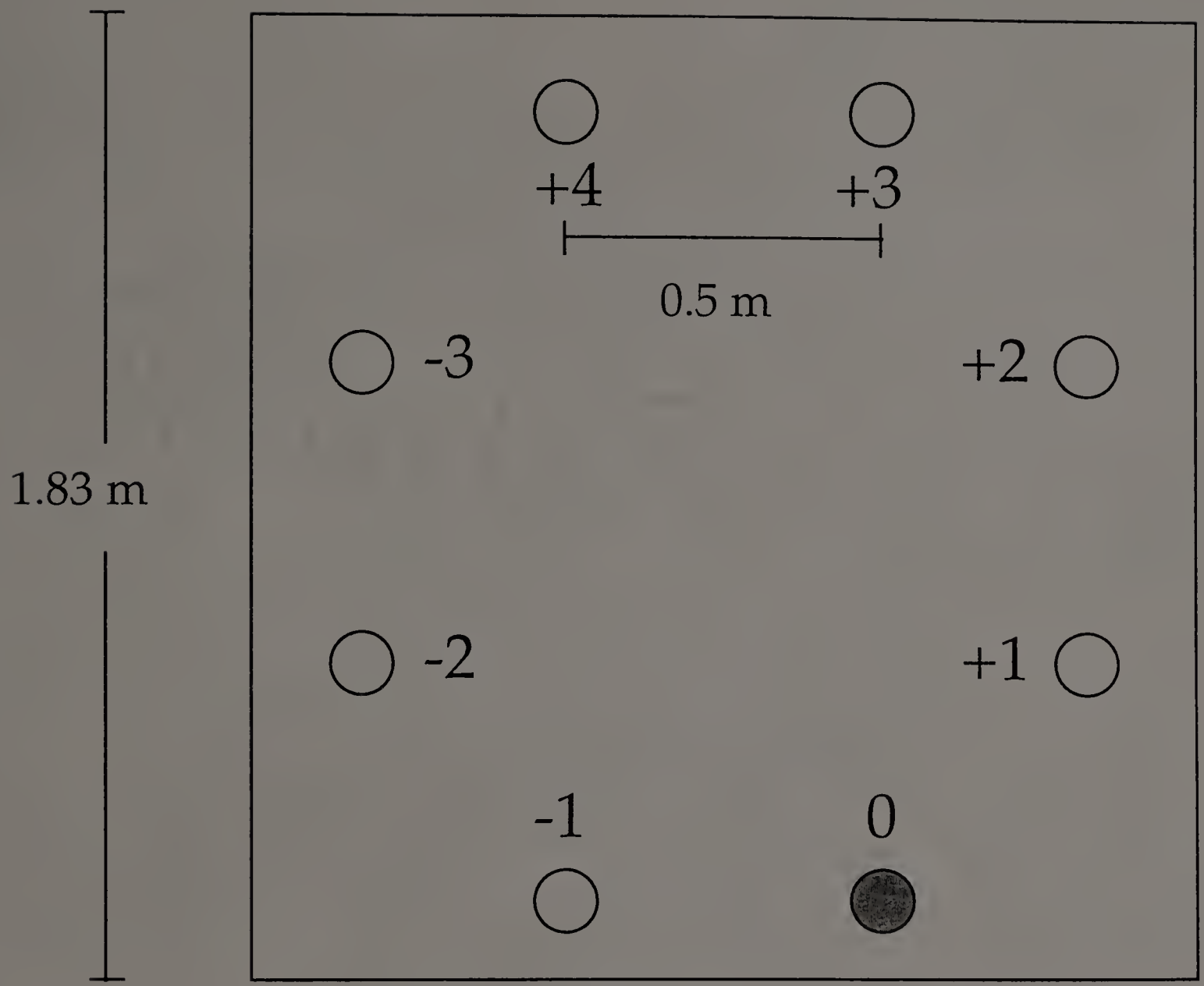

Fig 2.2. Overhead view of cage experiments incorporating 1 treatment and 7 controls, following position transformation. The treatment is marked position 0. The four ovipots further counter-clockwise are given a positive designation; the 3 ovipots further clockwise are given a negative designation.

the treatment's counter-clockwise side were given positive designations based on their position relative to the treatment ovipot. Thus, the neighboring ovipot to the counter-clockwise side was position +1; the one further counter-clockwise than that ovipot was +2. This continued until the designation of +4. The three ovipots on the clockwise side were given similar designations, except with a negative number. By comparing the number of eggs laid among the specific positions across factors (via χ^2-analysis), the relative level of spill-over effect may be evaluated. In all tested cased, no consistent spill-over was found.

As distance from the treatment ovipot is more important to the assessment of spill-over effect than the direction from the treatment, the eggs collected from ovipots the same distance from the treatment ovipot (*e.g.*, -1 and +1), were combined. These were compared with double the number of eggs found in the sites lacking complementary ovipots (position 0 and position 4). The ratio of eggs collected by position (0, 1, 2, 3 or 4) was found for each factor and compared with the same ratio collected for other factors by χ^2-analysis. Again, consistent spill-over effects were not found. See Appendix A for a sample analysis.

For all χ^2 tests, the null hypothesis was stated: there is no significant difference between the tested distributions.

CHAPTER 3

THE INFLUENCE OF CONSPECIFIC LARVAL INSTAR AND LARVAL DENSITY ON OVIPOSITION SITE CHOICE BY *AEDES AEGYPTI*

Introduction

Gravid *Ae. aegypti* select suitable oviposition sites based upon the presence or absence of specific abiotic factors, including water presence and quality, light exposure and substrate quality (Bentley and Day 1989). Suitable sites may be home to several other organisms and their waste products. In particular, a suitable site may already contain conspecific larvae (Bentley and Day 1989). These larvae may produce chemicals (including nitrogenous wastes) that indicate their presence to ovipositing females. The type and amount of these chemicals may further influence female oviposition site choice.

The cues may elicit either a positive or a negative response from gravid females. A positive response might occur because the cues a female detects signal that the site is suitable for habitation. If larvae are present, at least one other female has already laid eggs in this site, the eggs have hatched, and at least some of the larvae have survived. Females that select sites containing larvae may have a better chance of their progeny surviving than in sites in which no larvae are found. Another positive aspect of a site containing larvae is that, if predators are present, the risk to any given larva is spread around. If a female oviposits in such a site, the odds that all her progeny deposited there will be eaten are reduced.

Negative responses also are possible. A single site can only support a limited number of *Ae. aegypti* larvae at a given time, depending upon available resources such as water and food (Peters *et al.* 1969). Larvae of *Ae. aegypti* do not fare well in polluted

waters (Shannon and Putnam 1934). With increasing larval density at a site, pollution will also increase due to higher waste production. This waste is organic and provides nutrients for some biota, but the rate at which this waste is dependent upon the density of their consumers. Another negative aspect of ovipositing in a site containing larvae is that every single larva at a site represents competition for other larvae in terms of food and space requirements (Peters *et al.* 1969). Females sensing the presence of larvae at a particular site may avoid the site for being overcrowded, and not deposit eggs there.

Positive and negative cues must be weighed by each gravid female. Laboratory studies on the impact of larval presence upon aedine female oviposition site choice have been performed by numerous researchers. The species investigated include *Ae. atropalpus*, *Ae. communis*, *Ae. polynesiensis*, *Ae. togoi*, *Ae. sierrensis*, *Ae. triseriatus* and *Ae. aegypti* (Soman and Reuben 1970, Gubler 1971, Kalpage and Brust 1973, McDaniel *et al.* 1976, Ahmadi and McClelland 1983, Maire 1984, Maire and Langis 1985, Black *et al.* 1989, Corbet and Chadee 1993, Onyabe and Roitberg 1997, Zahiri *et al.* 1997a, Allan and Kline 1998) . The general consensus is that the presence of larvae is attractive to ovipositing female *Ae.aegypti* (Soman and Reuben 1970, Bentley and Day 1989, Corbet and Chadee 1993, Zahiri *et al.* 1997a). However, there are reports indicating larval presence does not elicit a positive response from this mosquito. Both Black *et al.* (1989) and Allen and Kline (1998) found no differential response between sites containing conspecific larvae and sites holding tap water. Thus, the effects of conspecific larval-presence at oviposition sites on female site choice were examined using an eight ovipot, large cage design.

The effects of larval instar were studied, based on 2 competing premises. First, a site containing later instar larvae should be attractive to ovipositing females, as it indicates a greater likelihood of her own progeny to survive to adult emergence. Fourth instars are much closer to becoming adults than are 1st instars. The second, competing, tenet is that later instars indicate a lower degree of site attraction than younger larvae, as they would offer any younger instar larvae significant competition for resources. If a female were to oviposit in a site containing 3rd instars, they may be 4th instars by the time her larvae hatch. 4th instars are much larger and consume considerably more food than do 1st instars. Perhaps the 1st instars will not be able to find enough food to survive and mature.

Another aspect investigated was the effects of larval density on oviposition site choice. Again, the literature offers conflicting reports of the effects of larval density on *Ae. aegypti* female site selection. Soman and Reuben (1970) and Roberts and Hsi (1977) concur that densities of 1 larva per 10 ml and 1 larva per 2 ml are attractive to ovipositing females. Black *et al.* (1989) disagree, finding no evidence of attraction to sites holding conspecific larvae at densities of 1 larva per 2 ml. This experiment re-examines the possible effects of density using the eight ovipot, large cage design.

Sites containing *Ae. aegypti* larvae of the same instar but different numbers were offered to individual females. There are two opposing premises upon which females may be attracted to or repelled by sites containing differing densities of larvae. Sites containing more larvae may be attractive because there is a reduced risk to any other progeny that mature there. With more larvae present, the risk that all of a particular

female's progeny at that site will be subject to predation is reduced. A competing consideration is that increasing larval density increases the likelihood of competition. Given the fixed limitations of any single site, a female may avoid sites containing higher densities of conspecific larvae to spare her progeny the effects of competition (which include reduced adult size and sometimes larval death) (Ikeshoji and Mulla 1970).

Materials and Methods

Individual females were released inside 1.82 m^3 cages containing 8 oviposition sites (Ch. 2). Females were offered 7 control sites and one treatment site. Seven of the 120 ml ovipots were lined with seed-paper substrate and filled with 40 ml of filtered-distilled water (FDW), while the remaining 120 ml ovipot contained the seed-paper substrate and the treatment. Prior to experimentation, the larvae were collected from their larval rearing trays, washed in distilled water 3 times for 1 min each wash, bathed in a fresh batch of distilled water for 2 h, and transferred into the final 40 ml of FDW used in the experiment. Including larval rearing water (LRW) in the assays (that is, collecting the proscribed number of larvae and the water in which they were reared) was considered but not used for the experiments.

Although including LRW would better approximate field conditions, the experimental design would not be as readily repeatable, as the water quality, food used, and air-borne contaminants may vary from laboratory to laboratory. It is important to note, however, that the excreted waste products from larvae, which may indeed offer cues to ovipositing females, were excluded from these experiments as much as possible. Thus

results may differ from what can be expected in natural settings or other laboratory studies that include rearing water.

To establish baseline female responses to the presence of conspecific larvae, females were offered a treatment site containing 4th instar larvae at a density of 1 larva per ml. These larvae were collected and washed in distilled water, and the placed into 40 ml of FDW (Ch. 2). Female selection of sites based upon their egg distribution was monitored.

To test the effects of instar on female oviposition site choice, 40 larvae of the stage in question were collected, washed, and placed in an ovipot containing 40 ml of FDW. Stages tested include1st-4th instar larvae and pupae. The treatment ovipot was placed in the experimental cage along with the 7 control ovipots.

Investigations of the impact of larval density on female site choice were peformed in a similar manner. Only 4th instar larvae were tested. The impacts of overcrowding on mosquito larvae are known to include the production of growth retardant factors (in certain *Culex* species) which may inhibit growth of or even kill other larvae (Ikeshoji and Mulla 1970). Although such factors have not been demonstrated in *Ae. aegypti* larval populations, the potential for their production cannot be ignored. Even in the absence of growth retardant production, competition for food and space my adversely impact *Ae. aegypti* larval growth and survival (Peters *et al.* 1969). Larvae reared under such constraints may excrete compounds (including wastes) in different quantities and qualities than those simply "placed" in a high density situation.

Therefore, all tested larvae were reared under the same densities at which they were tested; they were presented with the same amount of food (100 mg 1:1 yeast:lactalbumin) per day, regardless of density. They were washed and transferred to 40 ml of FDW as above, so the most overt extraneous debris present in the larval rearing habitat were excluded from the treatment water. Densities tested included: 1 larva per 4 ml, 1 per 2 ml, 1 per 1 ml, 2 per ml and 4 per ml. The high-density end of the range was chosen due to the research on *Cx. quinquefasciatus* performed by Ikeshoji and Mulla (1970), in which growth retardant factor production was maximized at a density of 4-6 larvae per ml. If any such factors are produced by *Ae. aegypti*, which typically inhabit cleaner waters (and at lower densities) than *Cx. quinquefasciatus*, it is reasonable that they would be produced at the low end of this 4-6 larvae per ml range. The lowest density was picked based on numerical reciprocity. As all ovipots held 40 ml water, the absolute numbers of larvae ranged from 10 to 160 larvae per treatment ovipot.

Excepting the experiments in which larval density was tested, larvae were reared under the standard feeding regime (Ch. 2). All tested females were between the ages of 6-8 days post-emergence. They were maintained on a 10% sucrose solution and offered a blood-meal 3 d post-emergence. Each trial lasted for 23 h, at the end of which females were collected, ovipots were removed, and the presence and number of eggs deposited in each oviposition site were recorded. A χ^2 contrast test was performed on the collated data.

Results

Null Hypothesis: *Females oviposit neither more nor fewer eggs in sites containing conspecific 4th larvae.*

The treatment site received more eggs (24.8%) than any other site (Table 3.1). According to an expected control distribution, the treatment site should have received 12.5% of the total eggs deposited. In 25 tests, almost twice as many eggs were oviposited in sites containing 4th instars at a density of 1 larva per ml as the expected distribution. The difference is statistically significant ($p<0.001$).

Null Hypothesis: *Females do not prefer or avoid oviposition sites containing post-eclosion immatures of differing stages present at densities of 1 per ml of water..*

A general trend of increasing egg number in the treatment site with increasing larval instar is apparent (Table 3.2). Although 1st and 3rd and 2nd and 3rd instars were not significantly different ($p=0.401$ and 0.109, respectively), treatments including 4th instar larvae or pupae received significantly more eggs than any other larval stage treatment. Sites containing either 4th instars or pupae were not significantly different ($p=0.840$). In all cases, sites containing conspecific larvae or pupae present at a density of 1 larva per ml receive more eggs than could be expected by random distribution alone (*i.e.*, 12.5%).

Table 3.1. Comparison of larval effects on *Ae. aegypti* oviposition with expected distribution if there is no effect. Larval effect tested with 4th instars at a density 1 larva per ml. Treatments sharing same letter category are not statistically different at the p=0.05 level.

	Ovipot									
	-3	-2	-1	0	1	2	3	4	Total	Category
LARVAE										a
N	25	25	25	25	25	25	25	25	200	
Oviposition Events	8	9	3	18	16	19	18	8	99	
Eggs Deposited	115	113	31	541	462	532	321	66	2181	
Eggs/Oviposition Event	14.38	12.56	10.33	30.06	28.88	28.00	17.83	8.25	22.03	
% Total Eggs in Ovipot	5.27	5.18	1.42	24.81	21.18	24.39	14.72	3.03	100.00	
Standard Error	2.45	2.77	1.02	7.26	7.39	7.14	4.58	2.17		
% Expected	12.50	12.50	12.50	12.50	12.50	12.50	12.50	12.50	100.00	b

Table 3.2. Comparison of larval instar and pupae effects on *Ae. aegypti* oviposition. Densities are 1 immature per ml. Treatments sharing same letter category are not statistically different at the p=0.05 level.

	Ovipot									
	-3	-2	-1	0	1	2	3	4	Total	Category
1ST INSTAR										a
N	17	17	17	17	17	17	17	17	136	
Oviposition Events	8	8	2	9	11	6	9	7	60	
Eggs Deposited	131	115	36	163	148	86	159	100	938	
Eggs/Oviposition Event	16.38	14.38	18.00	18.11	13.45	14.33	17.67	14.29	15.63	
% Total Eggs in Ovipot	13.97	12.26	3.84	17.38	15.78	9.17	16.95	10.66	100.00	
Standard Error	4.18	3.93	2.69	4.56	4.13	3.4	4.97	3.11		
2ND INSTAR										b
N	22	22	22	22	22	22	22	22	176	
Oviposition Events	4	10	8	11	9	9	6	12	69	
Eggs Deposited	23	142	126	223	138	117	91	196	1056	
Eggs/Oviposition Event	5.75	14.20	15.75	20.27	15.33	13.00	15.17	16.33	15.30	
% Total Eggs in Ovipot	2.18	13.45	11.93	21.12	13.07	11.08	8.62	18.56	100.00	
Standard Error	1.56	3.89	3.58	6.12	3.94	3.51	2.78	5.64		
3RD INSTAR										a,b
N	24	24	24	24	24	24	24	24	192	
Oviposition Events	11	7	7	13	10	10	11	9	78	
Eggs Deposited	203	62	84	263	215	199	187	201	1414	
Eggs/Oviposition Event	18.45	8.86	12.00	20.23	21.50	19.90	17.00	22.33	18.13	
% Total Eggs in Ovipot	14.36	4.38	5.94	18.60	15.21	14.07	13.22	14.21	100.00	
Standard Error	3.75	3.01	3.95	4.89	4.10	3.72	0.00	3.84		

Continued next page

Table 3.2. Continued.

	Ovipot									
	-3	-2	-1	0	1	2	3	4	Total	Category
4TH INSTAR										c
N	25	25	25	25	25	25	25	25	200	
Oviposition Events	8	9	3	18	16	19	18	8	99	
Eggs Deposited	115	113	31	541	462	532	321	66	2181	
Eggs/Oviposition Event	14.38	12.56	10.33	30.06	28.88	28.00	17.83	8.25	22.03	
% Total Eggs in Ovipot	5.27	5.18	1.42	24.81	21.18	24.39	14.72	3.03	100.00	
Standard Error	2.45	2.77	1.02	7.26	7.39	7.14	4.58	2.17		
PUPAE										c
N	23	23	23	23	23	23	23	23	184	
Oviposition Events	10	10	6	10	13	8	11	10	78	
Eggs Deposited	81	142	91	306	243	93	185	109	1250	
Eggs/Oviposition Event	8.10	14.20	15.17	30.60	18.69	11.63	16.82	10.90	16.03	
% Total Eggs in Ovipot	6.48	11.36	7.28	24.48	19.44	7.44	14.80	8.72	100.00	
Standard Error	2.82	3.51	2.97	7.53	6.01	3.86	4.39	5.02		

Null Hypothesis: *Females do not prefer or avoid sites containing 4th instar larvae at differing densities.*

Increasing the density of 4th instar larvae appears to increase the degree of attraction an oviposition site holds for ovipositing females (Table. 3.3). Conspecifics present at densities of 1 or 2 larvae per ml elicited the greatest egg deposition from females (24.8% and 23.1% respectively). The difference in the proportion of eggs received among the 1 and 2 larva per ml treatments is not significant ($p=0.215$). The trend of increasing attraction with increasing density is lost at densities higher than 2 larva per ml. At 4 larva per ml, the response of females becomes significantly negative (9.1% eggs laid in treatment ovipot, $p<0.05$).

Discussion

Sites containing conspecific larvae received more eggs than sites containing only FDW (Table 3.1). Discrepancies between these results and those reported by Black *et al.* (1989) are likely based upon differences in experimental design. First, providing only one treatment and seven control ovipots may produce results different from the designs of these researchers, who presented 6 ovipots to females, including 2 controls and 4 distinct treatments. The treatments contained larvae of either *Ae. aegypti* or *Ae. albopictus*: the confounding effects of introducing sites containing interspecific larvae at different densities may well have eliminated any tendency to favor sites containing conspecific larvae. Female selection of sites may have been based upon mixing cues, rendering

Table 3.3. Comparison of 4th instar density effects on *Ae. aegypti* oviposition. Treatments sharing same letter category are not statistically different at the p=0.05 level.

	Ovipot									
	-3	-2	-1	0	1	2	3	4	Total	Category
1 LARVA/ 4 ML										a,c
N	18	18	18	18	18	18	18	18	144	
Oviposition Events	7	6	10	10	5	7	7	9	61	
Eggs Deposited	84	15	187	203	55	104	114	194	956	
Eggs/Oviposition Event	12.00	2.50	18.70	20.30	11.00	14.86	16.29	21.56	15.67	
% Total Eggs in Ovipot	8.79	1.57	19.56	21.23	5.75	10.88	11.92	20.29	100.00	
Standard Error	3.48	1.16	6.03	6.46	2.21	3.57	3.42	6.05		
1 LARVA/ 2 ML										a
N	16	16	16	16	16	16	16	16	128	
Oviposition Events	9	3	5	9	8	6	10	8	58	
Eggs Deposited	142	64	66	201	136	18	253	150	1030	
Eggs/Oviposition Event	15.78	21.33	13.20	22.33	17.00	3.00	25.30	18.75	17.76	
% Total Eggs in Ovipot	13.79	6.21	6.41	19.51	13.20	1.75	24.56	14.56	100.00	
Standard Error	4.02	2.42	1.97	5.72	3.99	1.14	7.26	4.13		
1 LARVA/ 1 ML										b,c
N	25	25	25	25	25	25	25	25	200	
Oviposition Events	8	9	3	18	16	19	18	8	99	
Eggs Deposited	115	113	31	541	462	532	321	66	2181	
Eggs/Oviposition Event	14.38	12.56	10.33	30.06	28.88	28.00	17.83	8.25	22.03	
% Total Eggs in Ovipot	5.27	5.18	1.42	24.81	21.18	24.39	14.72	3.03	100.00	
Standard Error	2.45	2.77	1.02	7.26	7.39	7.14	4.58	2.17		

Continued next page

Table 3.3. Continued.

	Ovipot									
	-3	-2	-1	0	1	2	3	4	Total	Category
2 LARVA/ML										c
N	19	19	19	19	19	19	19	19	152	
Oviposition Events	8	9	10	12	8	8	8	9	72	
Eggs Deposited	61	138	227	283	109	81	141	186	1226	
Eggs/Oviposition Event	7.63	15.33	22.70	23.58	13.63	10.13	17.63	20.67	17.03	
% Total Eggs in Ovipot	4.98	11.26	18.52	23.08	8.89	6.61	11.50	15.17	100.00	
Standard Error	2.04	3.51	5.49	6.87	2.68	2.37	3.22	4.46		
4 LARVA/ML										d
N	21	21	21	21	21	21	21	21	168	
Oviposition Events	11	5	10	9	12	11	9	7	74	
Eggs Deposited	241	77	128	113	216	200	173	91	1239	
Eggs/Oviposition Event	21.91	15.40	12.80	12.56	18.00	18.18	19.22	13.00	16.74	
% Total Eggs in Ovipot	19.45	6.21	10.33	9.12	17.43	16.14	13.96	7.34	100.00	
Standard Error	5.61	2.14	3.06	3.01	5.26	4.58	3.97	2.43		

discrimination difficult. Using the current eight ovipot (one treatment, seven controls) experimental design, this potential confounding effect was eliminated.

Second, the large cage design for these experiments provided space between the ovipots and allowed greater discrimination between sites. In the design of Allan and Kline (1998), the ovipots were placed closer together. Although the exact cues used in determining the presence of larvae are not known, it is possible that such a short distance between ovipots causes a mixing of cues between treatment and control ovipots. With greater space between sites, the ability of cues to mix is likely reduced, offering females greater chances for discriminating among oviposition site contents.

Positive responses of females toward sites containing conspecific larvae indicates an evolutionary adaptation for female site selection based upon the acceptance of other females. In laboratory colonies, females are given specific windows of access to hosts and oviposition sites. Egg batches are typically collected all at once, and females are not exposed to sites containing larvae. By contrast, natural populations of *Ae.aegypti* females are in near-continuous proximity to hosts and ovipots (Edman, personal communication), and females readily encounter sites containing conspecific larvae. Larval presence signals the survival to hatch of any eggs previously deposited in the site. Larval presence could indicate to females that any eggs laid here will be at a greater advantage than if deposited in sites containing water but no larvae.

Positive response to sites containing larvae increased with increasing larval age (Table 3.2). This offers support for the tenet that older instars indicate a greater degree of survival in a particular site than do younger instars. At the same time, this argues against

the premise that younger larvae may not be able to compete with older larvae. It should be noted, however, that 3rd and 4th instars and pupae will have emerged by the time a newly-laid egg batch hatches, and thus will not actually offer any direct competition. In this sense sites containing eggs and 1st and 2nd instars are more likely to offer direct competition to newly-laid eggs that may hatch within a few days. If this is the case, females may actually avoid sites containing younger instar larvae. Sites containing 1st and 2nd instar larvae are less attractive than sites containing 4th instars or pupae, but are still more attractive than control sites (with an expected distribution of 12.5% of the total eggs received by any ovipot) (Table 3.2). Therefore, the effect of *potential* larval competition at low (1 larva per ml) density does not impact on female site choice.

Higher densities of larvae had marked effects on oviposition site selection. Sites with densities between 1 larva per 4 ml and 2 larvae per ml, all receive a greater proportion of eggs than expected by a control distribution. Gravids exhibited a negative response to oviposition sites containing larvae at densities of 4 per ml (Table 3.3); sites received but 9.1% of the total eggs distributed per female. Again, from the considerations of potential larval competition, most of these 4th instars would have emerged by the time a new egg batch hatched, and would not directly compete with these young larvae. Since females tend to avoid these sites, it is evident that females cannot measure this potential competition. Instead, they directly assess the present quantity of larvae present, likely by monitoring either larval excreta or the levels of contaminating organisms. Some of the contaminating organsims are larval food items. They are likely to be low at high

mosquito density. Other organisms may utilize larval metabolites as food. Their populations are likely to increase with increasing mosquito density.

Important to note are the lack of consistent spill-over effects. By combining the number of eggs counted in positions -1 and +1, and comparing those with neighboring ovipots, no clear relationship between distance from the treatment ovipot and proportion of eggs received was evident. However, in all such tests the treatment ovipot received significantly more eggs than any other grouping (except for the comparison of 4 larvae per ml ovipots, in which the treatment received significantly fewer eggs). This supports the results based on comparison of the treatment with all control ovipots: conspecific larvae and pupae at low densities are attractive. Analysis of oviposition incidence revealed a lack of discrimination. This indicates females likely made their decision of how many eggs to lay after they had reached the site, and perhaps after they had already begun to oviposit.

The postulate that females may select sites containing conspecific larvae at high densities due to reduced risk of predation for their own progeny (another way to spread relative risk) was not supported by my data. It should be noted that no predators were present in this study. Direct assessment of predators by female *Ae. taeniorhynchus* was established by Ritchie and Laidlaw-Bell (1994). It is possible that, not sensing any predators, spreading of risk by ovipositing in already crowded sites was not an issue with these females. Instead, the impact of larval competition appears to have been the major force in decisions to avoid sites containing 4th instars at high density.

CHAPTER 4

THE SOURCE OF ATTRACTION TO SITES HOLDING *AEDES AEGYPTI* LARVAE, AND THE MECHANISMS BY WHICH CONSPECIFIC FEMALES ASSESS THESE SITES

Introduction

The positive response gravid *Ae. aegypti* females show towards oviposition sites containing conspecific larvae at certain densities is not necessarily due to the presence of larvae. The greater attraction to later stage instars in particular attests to the required presence of available nutrients. These nutrients may take the form of soluble metabolites and/or microorganisms such as bacteria. The presence of specific organisms has been reported to augment or mitigate the mosquito response to oviposition sites. These organisms include other mosquitoes (Zahiri *et al.* 1997a, Allan and Kline 1998), bacteria (Hasselschwert and Rockett (1988), plants (Lounibos 1978), larval parasites (Lowenberger and Rau 1994) and predators (Ritchie and Laidlaw-Bell 1994) The ability to monitor contaminating organisms in sites containing conspecific larvae may provide a great survival advantage to females. Any such advantage should be readily testable under the appropriate laboratory conditions.

Barring the presence of other macroorganisms such as interspecific mosquito larvae and larval predators, gravids still must contend with the presence of contaminating bacteria. Specific bacterial species (or the specific cues they release) may provide some information important in female assessment of a site (Hasselschwert and Rockett 1988). With increasing larval instar, the numbers of prey bacteria might be relatively low due to increased larval size: larger larvae eat more than their younger counterparts (Clements

1992). At the same time, the populations of bacteria that thrive on mosquito waste products will be present in relatively high quantities. Detection of either population set may in effect substitute for direct monitoring of larval populations by gravids. Similarly, bacterial populations fluctuate with changing larval density (Hasselschwert and Rockett 1988).

The supposition that bacteria mediate the positive response females direct towards sites containing conspecific larvae has received some attention in work on *Ae. atropalpus* (Maire 1985) and *Ae. aegypti* (Benzon and Apperson 1988). In the former work, mosquitoes were reared under axenic conditions, that is, in the absence of any contaminating fauna. Maire (1985) found gravid female attraction to sites containing axenic larvae. This response was not as significant as that recorded when sites contained larvae reared under non-axenic conditions. He interpreted this to mean that both larvae and bacteria are responsible for the production of specific compounds that ovipositing females monitor. This experimental design has not been tested with other mosquitoes, probably because it is difficult to replicate due to the high cost and it is difficult to obtain the essential nutrients required.

By treating *Ae. aegypti* larvae with antibiotics, Benzon and Apperson (1988) found they could minimize bacterial growth. Testing female response to sites containing either larvae and bacteria, antibiotic-treated water containing larvae, and water treated with the equivalent amount of antibiotics, the authors found elimination of the positive response when bacterial populations were absent or severely reduced. They concluded

that bacteria, not conspecific larvae, influence female choice at sites that contain conspecific larvae (Benzon and Apperson 1988).

The most obvious distinction between these experiments is the difference in mosquito species used. Both are container breeders, although *Ae. atropalpus* is termed a rock-pool mosquito species. It has a much more temperate distribution than does *Ae. aegypti* (Maire 1985). It is not clear how this difference could impact the importance bacteria have on gravid site choice, other than the rock-pool breeding season is shorter, and the period between fall and spring oviposition is longer given their more northerly distribution. Perhaps bacterial populations do not grow as rapidly at more northerly latitudes, and are thus not suitable to entirely replace the presence of larvae as an indicator of site quality. Another important difference between the two experimental designs is that bacteria were not killed in the axenic-rearing design. Although Benzon and Apperson (1988) found no negative effects on mosquito oviposition due to the presence of antibiotics alone, they did not separately measure the response to killed bacteria. Although mosquito larvae kill bacteria, they process them and release metabolites inherently distinct from what they ate. Antibiotics prevent bacterial cell wall deposition, and render the asexually reproducing bacteria subject to cell lysis. Waters containing ruptured bacteria may offer different chemical cues than water holding axenically-reared larvae.

Another argument against the conclusions reached by Benzon and Apperson (1988) was provided in Ch. 3. Females are able to distinguish between sites containing larvae of differing instars and at differing densities. If the entire response to sites

containing larvae was in fact a response to bacteria, then the bacterial composition must change differentially in sites that hold instars of different age and larvae at different density. There is no evidence that *Ae. aegypti* larval development should alter the local bacterial species composition in any manner distinct from the way in which increasing larval density should change species composition.

Following development of a simplified axenic rearing protocol I evaluated the effects of bacteria on gravid *Ae. aegypti* oviposition site selection. Experiments were designed to indicate whether the response of gravids to sites containing conspecific 4th instar larvae at a a density of 1 larva per ml are due either entirely to larvae, entirely to bacteria, or a combination of the two.

The mechanisms by which gravid mosquitoes assess site suitablity have been tested under a variety of conditions. Visual cues such as substrate color, olfactory cues including relative humidity, and chemo-tactile cues such as water salinity have been investigated (O'Gower 1963). The mechanisms involved in the specific response to conspecific larvae has received some attention, with Soman and Reuben (1970) concluding that there was a greater reliance upon olfactory than visual cues in a two-choice experimental design.

The two-choice design does not account for the potential confounding effect of skip oviposition (relative risk reduction) reported for *Ae. aegypti*, and thus the discriminatory power of such a design is lower than that of the eight ovipot design used in the current experiments. Knowing that the presence of larvae elicits a positive response

to ovipositing females, the mechanisms behind this attraction can be studied using the large cage and eight ovipot design.

Therefore, the cues involved in gravid attraction to oviposition sites containing conspecific larvae were re-examined in the following manner. All oviposition solutions contained 4th instar larvae at a density of 1 larva per ml. One test involved the response to sites containing larvae hidden from view under a black plastic petri dish. The dish sides did not contact the inner sides of the ovipot, such that females could use both olfactory and chemo-tactile cues to assess the presence of larvae. Another test examined the response of females to sites containing larvae in a middle vial sealed off with a plastic cap. Under these conditions, females could see but could not use olfactory or chemo-tactile cues to assess larval presence. A similar experiment was performed, but with a mesh cap instead of a plastic cap. Females could see larvae and detect olfactory cues, but they could not contact them or the water that housed them. By comparing female response under these and NORMAL (in which all cues were available to females) conditions, the specific importance of each cue to female site selection was evaluated.

Materials and Methods

Axenic Rearing

The axenice rearing protocol closely follows that outlined by Maire (1985). Eggs were collected from colony egg papers between 24-48 h after deposition and surface sterilized. The egg paper was taken to a sterile containment hood (previously irradiated with ultraviolet (UV) light for 30 min) where the following procedures were performed.

Eggs were brushed into a 15 ml disposable conical tube containing a solution of sterile phosphate buffer (pH 6.5). They were agitated to remove loose-clinging debris and then transferred to a 500 µm metal screen. The screen was dipped in 70% ethanol for 30 sec and rinsed with distilled water. The ethanol bath and rinse was repeated. The screen was then bathed in a 2% sodium hypochlorite solution for 3 min; a distilled water-rinse followed. Using a sterile brush, the eggs were transferred to a 15 ml disposable conical tube containing 10 ml 0.1% benzalkonium chloride. The tube was capped and vortexed for 3 min.

Three sterile phosphate buffer washes followed. All took place in sterile conical tubes, and a new sterile pasteur pipet was used for each transfer. Each wash including gentle agitation and lasted 2 min. At this point Maire (1985) stored the eggs on sterile filter paper in a sterile petri dish for 3 weeks. This procedure resulted in decaying eggs when attempted with *Ae. aegypti*. Some eggs hatched once placed on the filter paper. Perhaps *Ae. aegypti* respond to the process differently than do *Ae. atropalpus*. It is also possible that the original egg paper had dried sufficiently that the stimulation provided by several washes prompted a hatch earlier than expected.

The process was therefore continued immediately, with no storage of eggs. Following the third phosphate buffer wash, eggs were placed in a 15 ml disposable tube containing 10 ml 0.1% benzalkonium chloride. The tube was capped and vortexed for 2 min. Eggs were sequentially washed in three sterile phosphate buffer baths (a new sterile pasteur pipet was used for each transfer); each wash was agitated for 30 sec. The eggs

and hatching larvae were then transferred to the sterile 1 l flask containing rearing medium.

Rearing medium was composed of enough autoclaved larval food (1:1 yeast:lactalbumin) to support the growth of 200 *Ae. aegypti* larvae to the pupal stage. This took approximately 8-10 days. Based on calculations used to rear colony mosquitoes, the total amount of yeast:lactalbumin added was 1 g. Fifty mg of liver powder was added to the medium. Distilled water was used to support the suspension. The 1 l flask was filled to the 200 ml mark with distilled water, and the yeast:lactalbumin and the liver powder were added. The flask screw cap was placed on top of the flask such that some gaseous exchange with the outside environment could take place, and then the flask was placed in an autoclave (the solution was autoclaved for 20 min). As soon as the temperature inside the autoclave dropped to safe levels, the door was opened and the flask cap was tightened immediately. Upon transfer of the larvae to the rearing medium in the sterile containment hood, the flask screw cap was tightened, and the flask was stored in a biochamber at (27 ± 2 °C) and constant relative humidity (65 ± 5 % RH) under a 14 L: 10 D lighting regime.

Sterility Tests

Two such flasks were made at the same time. One remained sealed until the beginning of experimentation. The other was opened every third day in the sterile hood to check for contamination. If contamination of this flask was found, the other flask was not used in experiments. The tests performed include the Tryptic Soy Agar test and the

Mycophilic Agar test. Test mixtures were made in 500 ml flasks and stored at 4 ± 2°C prior to usage. Mixtures were heated until completely liquid and then brought into a sterile containment hood for the following procedures. A sterile petri dish was opened for each test to be run, and enough agar was poured to cover approximately 70% of the petri dish bottom. The dish was swirled until a thin coating of agar covered the entire dish bottom, and the agar was allowed to dry and solidify (approximately 10 min).

The axenic rearing flask to be tested was then opened in the hood (after 30 min UV light-irradiation), and a 40 µl sample was added to each plate. Each sample formed drops on the surface of the agar, and was spread into a thin layer by the use of an ethanol and flame-treated glass stirring rod. The petri dish was then covered and sealed with parafilm, and stored upside-down in a biochamber at (27 ± 2 °C) and constant relative humidity (65 ± 5 % RH) under a 14 L: 10 D lighting regime. Each plate was checked for contamination after 24 and 48 h of storage.

Testing Response to Axenic Larvae

Once larvae had become fourth instars, the flask was opened and 40 larvae and 40 ml of rearing medium were removed and placed in the treatment ovipot. A water sample was taken and plated on both agar types. Only those waters testing free of bacterial contaminants up to 48 h after plating were considered in the results. Individual females were released in 1.82 m^3 cages containing 8 oviposition sites (Ch.2). Females were offered 7 control sites and one treatment site. Seven of the 120 ml ovipots were lined with seed paper substrate and filled with 40 ml FDW, while the remaining 120 ml ovipot

contained the seed paper substrate and the treatment. Females were 6-8 d post-emergence, and had been blood-fed 3 d prior to experimentation. Each trial lasted 23 h. At the end of the experiment, a water sample was taken and plated on both Tryptic Soy Agar and Mycophilic Agar. Only those waters testing free of bacterial contaminants up to 48 h after plating were considered in the results. Collected eggs were counted and subjected to χ^2 analysis.

Examination of Mechanisms: Conditions Tested

To test the effects of vision on female oviposition in sites containing conspecific immatures, a petri dish spray-painted matte black was affixed to a rubber stopper. The edges of the dish had previously been burned off to form a disc that fit easily into the 120 ml ovipots. Preliminary tests indicated that females neither favored nor avoided sites containing this visual blocker, when all ovipots contained identical control solutions ($p=0.874$). For the experiment, the treatment ovipot was set up as previously mentioned, with 40 fourth instars added to 40 ml of FDW. The visual blocker was then carefully placed in the ovipot. The water surface covered the upper face of the blocker, however larvae remained under the underside of the disc (Fig. 4.1). This treatment condition was termed Blind (*i.e.*, visual cues from larvae were excluded). Individual females were released and allowed to oviposit for 23 h, at which time the experiment was ended. Eggs were collected and subjected to χ^2 analysis.

Preliminary tests indicated that females do not respond any differently to sites containing a glass shell vial with a screened top than they do to sites lacking this potential

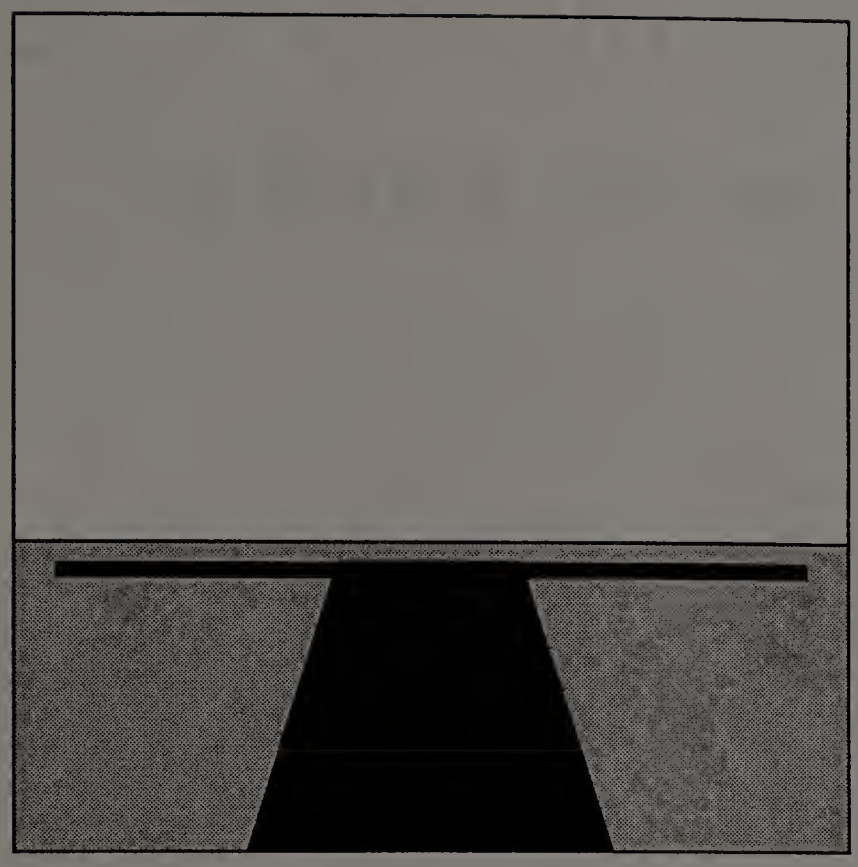

Fig. 4.1. Side view of the BLIND condition setup. A spray-painted black petri-dish with the sides cut was glued to a black rubber stopper. Larvae were placed in 40 ml of water. The stopper-dish unit was placed in the ovipot. Water flowed over the top of the dish, but larvae were trapped underneath.

obstruction (p=0.531). Ten 4th instar larvae were added to the shell vial, and 10 ml FDW was added. The vial was capped with a screen and placed in the center of the treatment ovipot (Fig. 4.2). Individual females were released and allowed to oviposit for 23 h, at which time the experiment was ended. Eggs were collected and subjected to χ^2 analysis. This experimental condition was termed SCREENED (*i.e.*, contact with larvae and their holding water was excluded).

Again, preliminary tests did not suggest differential female oviposition based upon the presence of a glass shell vial with a plastic cap in one ovipot and no such obstruction in any other ovipot (p=0.945). Ten 4th instar larvae were added to the shell vial, and 10 ml FDW was added. The vial was covered with a plastic cap and placed in the middle of the treatment ovipot (Fig. 4.2). Individual females were released and allowed to oviposit for 23 h, at which time the experiment was ended. Eggs were collected and subjected to χ^2 analysis. This experimental condition was termed CAPPED (*i.e.*, larval contact and airborne odors were excluded).

Results

Null Hypothesis: *The absence of bacteria does not render a site containing conspecific 4th instars at a density of 1 larva per ml less or more attractive than sites containing larvae and bacteria.*

The null hypothesis is proven to be false (Table 4.1): females laid more eggs in sites containing larvae and bacteria than in sites containing larvae alone. The difference is significant at $p<0.001$ by χ^2 contrast test. Sites containing larvae and contaminating

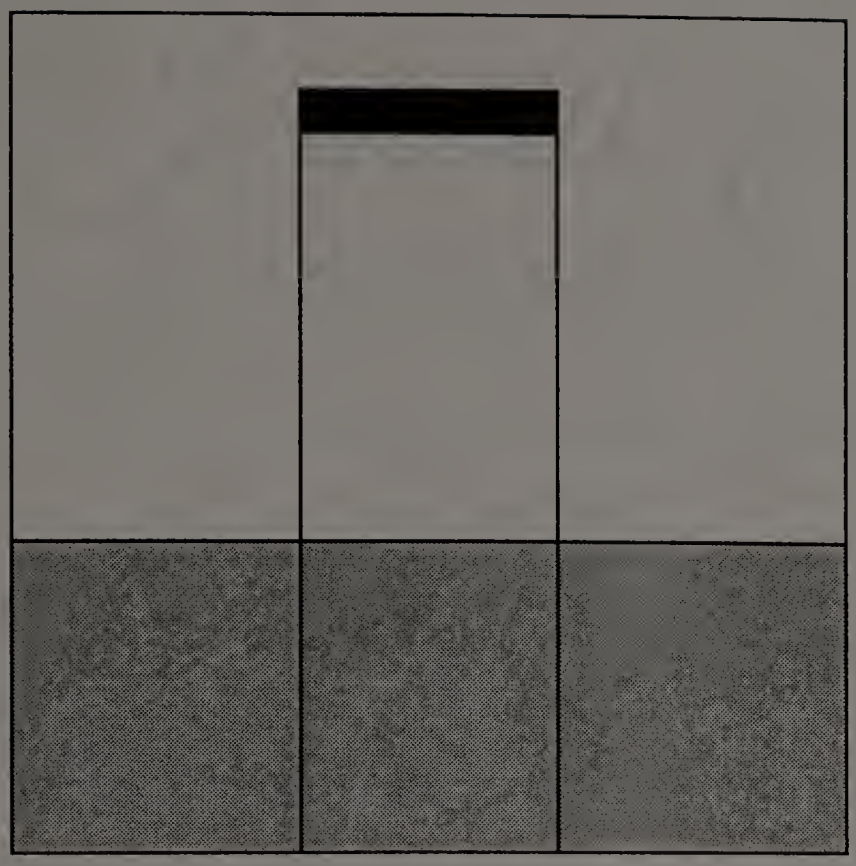

Fig. 4.2. Side view of the SCREENED and CAPPED condition setups. A shell vial containing larvae was placed in the middle of an ovipot with 40 ml of water. For the Screened experiments, a plastic cap with the center cut out and replaced by mesh was fixed to the top of the vial. For the Capped experiments, the plastic cap put on top was entire.

bacteria received almost a quarter of all deposited eggs per female; by contrast 20.0% of all eggs were laid in sites containing only larvae (Table 4.1). Female selection of sites containing conspecific larvae is based in part on the presence of larval-associated bacteria.

Null Hypothesis: *Females do not respond differently to oviposition sites containing larvae hidden from view and sites in which larvae are fully visible.*

Females oviposited 24.8% of all their eggs in the treatment ovipot when all faculties are available (NORMAL). This proportion fell to 23.2% in the absence of visual cues (BLIND) (Table 4.2), but this difference is not statistically significant (p=0.239). The null hypothesis was not disproven.

Null Hypothesis: *Females do not respond differently to sites containing larvae physically separated and screened than they do to sites in which they have full access to larvae.*

Sites containing larvae separated from the oviposition waters by the glass wall of a shell vial capped with a mesh lid (SCREENED) received a lower proportion of eggs than did sites in which females have full access to the larvae (NORMAL) (Table 4.3). The difference, 21.9% to 24.8%, respectively, is significant: p=0.026. This indicates a role of chemo-tactile cues in the assessment of sites containing conspecific 4th instars at a density of 1 larva per ml. The null hypothesis is false.

Table 4.1. Comparison of oviposition response to 4th instar larvae reared under normal and axenic conditions. Densities are 1 larva per ml. Treatments sharing same letter category are not statistically different at the p=0.05 level.

	Ovipot									
	-3	-2	-1	0	1	2	3	4	Total	Category
NORMAL										a
N	25	25	25	25	25	25	25	25	200	
Oviposition Events	8	9	3	18	16	19	18	8	99	
Eggs Deposited	115	113	31	541	462	532	321	66	2181	
Eggs/Oviposition Event	14.38	12.56	10.33	30.06	28.88	28.00	17.83	8.25	22.03	
% Total Eggs in Ovipot	5.27	5.18	1.42	24.81	21.18	24.39	14.72	3.03	100.00	
Standard Error	2.45	2.77	1.02	7.26	7.39	7.14	4.58	2.17		
AXENIC										b
N	24	24	24	24	24	24	24	24	192	
Oviposition Events	7	11	12	11	8	12	11	10	82	
Eggs Deposited	136	198	234	372	201	301	268	152	1862	
Eggs/Oviposition Event	19.43	18.00	19.50	33.82	25.13	25.08	24.36	15.20	22.71	
% Total Eggs in Ovipot	7.30	10.63	12.57	19.98	10.79	16.17	14.39	8.16	100.00	
Standard Error	2.67	3.00	3.46	5.72	2.84	4.60	4.01	2.24		

Table 4.2. Comparison of oviposition response to 4th instar larvae either normally accessible to ovipositing females or hidden from view. Densities are 1 larva per ml. Treatments sharing same letter category are not statistically different at the p=0.05 level.

	Ovipot									
	-3	-2	-1	0	1	2	3	4	Total	Category
NORMAL										a
N	25	25	25	25	25	25	25	25	200	
Oviposition Events	8	9	3	18	16	19	18	8	99	
Eggs Deposited	115	113	31	541	462	532	321	66	2181	
Eggs/Oviposition Event	14.38	12.56	10.33	30.06	28.88	28.00	17.83	8.25	22.03	
% Total Eggs in Ovipot	5.27	5.18	1.42	24.81	21.18	24.39	14.72	3.03	100.00	
Standard Error	2.45	2.77	1.02	7.26	7.39	7.14	4.58	2.17		
BLIND										a
N	24	24	24	24	24	24	24	24	192	
Oviposition Events	5	12	9	14	13	8	10	11	82	
Eggs Deposited	64	273	113	403	351	76	208	249	1737	
Eggs/Oviposition Event	12.80	22.75	12.56	28.79	27.00	9.50	20.80	22.64	21.18	
% Total Eggs in Ovipot	3.68	15.72	6.51	23.20	20.21	4.38	11.97	14.34	100.00	
Standard Error	2.21	4.27	2.02	6.53	5.32	1.54	3.22	3.54		

Table 4.3. Comparison of oviposition in the presence of 4th instar larvae either normally accessible to ovipositing females, separated by a screened vial, or segregated by a capped vial. Densities are 1 larva per ml. Treatments sharing same letter category are not statistically different at the p=0.05 level.

	Ovipot									
	-3	-2	-1	0	1	2	3	4	Total	Category
NORMAL										a
N	25	25	25	25	25	25	25	25	200	
Oviposition Events	8	9	3	18	16	19	18	8	99	
Eggs Deposited	115	113	31	541	462	532	321	66	2181	
Eggs/Oviposition Event	14.38	12.56	10.33	30.06	28.88	28.00	17.83	8.25	22.03	
% Total Eggs in Ovipot	5.27	5.18	1.42	24.81	21.18	24.39	14.72	3.03	100.00	
Standard Error	2.45	2.77	1.02	7.26	7.39	7.14	4.58	2.17		
SCREENED										b
N	19	19	19	19	19	19	19	19	152	
Oviposition Events	9	9	11	13	8	8	7	9	74	
Eggs Deposited	152	183	326	329	72	129	111	203	1505	
Eggs/Oviposition Event	16.89	20.33	29.64	25.31	9.00	16.13	15.86	22.56	20.34	
% Total Eggs in Ovipot	10.10	12.16	21.66	21.86	4.78	8.57	7.38	13.49	100.00	
Standard Error	3.56	3.25	5.79	5.86	1.31	2.43	2.02	3.57		
CAPPED										c
N	21	21	21	21	21	21	21	21	168	
Oviposition Events	12	8	11	7	9	10	9	13	79	
Eggs Deposited	249	93	257	127	185	76	217	339	1543	
Eggs/Oviposition Event	20.75	11.63	23.36	18.14	20.56	7.60	24.11	26.08	19.53	
% Total Eggs in Ovipot	16.14	6.03	16.66	8.23	11.99	4.93	14.06	21.97	100.00	
Standard Error	5.26	1.74	4.42	2.29	3.25	1.27	3.75	5.97		

Null Hypothesis: *Females do not respond differently to sites containing larvae in which both chemo-tactile and olfactory cues are abrogated than they do to sites in which they have full access to larvae.*

Females exposed to larvae under the CAPPED condition can use neither chemo-tactile nor contact cues in their assessment of larval presence. With only visual cues remaining, females laid but 8.2% of total eggs in sites containing conspecific larvae (Table 4.3). This contrasts significantly ($p<0.001$) with the NORMAL condition, in which 24.8% of all eggs were laid in the treatment ovipot, and also contrasts with the SCREENED condition ($p<0.001$), the treatment ovipot of which received 21.9% of total deposited eggs (Table 4.3). The null hypothesis is false.

Discussion

The reduced accumulation of eggs in sites containing axenically reared *Ae. aegypti* larvae suggests that bacteria are important in the determination of site suitability (Table 4.1). The evolutionary adaptations that predispose gravid females to oviposit in sites containing conspecific larvae likely include a response to appreciable levels of specific contaminating bacteria and/or their by-products. The specifics of the bacterial species involved are not known; nor is their role as either prey items for mosquito larvae or as feeders on mosquito waste products. Bacterial populations are likely to fluctuate with changes in the local environment brought about by larvae maturation and increasing larval density due to increased oviposition. Sensing the levels of these bacteria may assist females in determining site suitability. It is possible that bacterial populations indicate

larval presence (high bacterial populations at low larval density if they are prey items, high bacterial populations at high larval density if they feed on larval wastes), but it is also possible that these bacteria indicate other site-related factors entirely independent of larvae. Both scenarios are plausible, but more specific investigation of the direct effects of bacteria on female site selection will require the identification of specific bacterial species and the isolated testing of each species in the presence and the absence of *Ae. aegypti* larvae.

In either case, females will select sites containing larvae based on larval presence alone (Table 4.1). Such sites receive significantly fewer eggs than do similar sites containing both larvae and bacteria, however the net response from females is positive: 20.0% of all eggs were collected in the ovipot containing axenically-reared larvae. This is well above the expected 12.5% distribution if bacteria mediated the entire effect.

This is consistent with the results presented in Ch. 3, showing that females are able to distinguish between sites containing larvae of different age and density. If bacterial presence were the only indication of larval presence, then sites containing larvae of different age classes and different densities must contain bacterial species that differ in either quality or quantity in response to changing larval maturation and density patterns. There is no evidence that increases in larval age or density change bacterial species composition in differing manners. The simplest explanation is that larval presence can be directly detected by ovipositing females. Bacteria may assist in the determinations of site suitability either as redundant indicators of larval presence, or as indicators of site suitability in a manner that larvae do not convey. Given the increased response that

females direct towards sites containing bacteria and larvae, it is likely that bacteria convey information that larvae cannot.

These conclusions differ from those presented by Benzon and Apperson (1988), who suggested that bacteria mediate the entire attraction to sites containing conspecific larvae. Possible explanations for this discrepancy include differences in the experimental choice assay: eight ovipots with but one as treatment in the current design; Benzon and Apperson (1988) used two. Other differences include the possible negative effects of antibiotic-killed bacteria in their experiments.

Experiments on the mechanisms used by females in selecting sites containing larvae illustrate that olfactory cues are the most important, while chemo-tactile cues play some role (Table 4.3). This is shown by a lack of attraction to sites in which larvae are separated from the oviposition waters by a glass tube with a plastic cap. By comparison, glass separation with a mesh cap, allowing olfactory cues, induced a positive response from ovipositing gravids (Table 4.3). Visual cues apparently play no role in determining the presence of conspecific larvae (Table 4.2). It is concluded that larvae and their associated fauna produce compounds of varying volatility that are detectable by females. Some of these compounds are readily detectable by olfactory means, while some are only measurable under conditions in which the females may contact the oviposition waters.

These results are consitent with the previously published work of Soman and Reuben (1970). Any confounding effect due to the female tendency to skip oviposit did not affect their results. The results of the current experiment therefore validate the previous work under the conditions of a different experimental design.

The lack of importance of visual cues in assessing larval presence may be explained by the general nature of suitable *Ae. aegypti* oviposition sites. They are typically darker in color and located in the shade. Larvae of *Ae. aegypti* are browsers, and only come to the surface for respiration. Visual detection of the water contents, specifically larvae, under conditions of low light and contrast are likely difficult, and perhaps beyond the capabilities of mosquito compound eyes. The influence of chemo-tactile cues may explain a common oviposition behavior pattern witnessed during the course of this project: females often walk down to the water surface and touch it with one of their hind legs. Some mosquitoes walked across the water surface. The "drinking" of oviposition water originally observed with *Culex* mosquitoes by Weber and Tipping (1990) was not observed. They concluded in a subsequent paper that such drinking was required to assist egg expulsion (Weber and Tipping 1993). Since *Ae. aegypti* ogten lay eggs without coming into contact with water, it is not surprising that drinking behavior was not seen. It should be noted that chemo-tactile cues from the oviposition water also may be relayed through the porous substrate.

Olfactory cues are the most important elements in determining the presence of larvae. These cues may act at either short or long range (or both) and, depending on wind flow, may assist in directing mosquitoes to oviposition sites. Thus they may act as oviposition attractants. Chemical cues of low volatility will not disperse from the oviposition site. Some of these cues may act as oviposition stimulants. Further studies on the importance of olfactory cues would be assisted by the identification of specific

volatile compounds that render sites attractive. They could then be described as either oviposition attractants or oviposition stimulants (Dethier *et al.* 1960).

There are no consistent spill-over effects. By combining the number of eggs counted in positions -1 and +1, and comparing those with neighboring ovipots, no clear relationship between distance from the treatment ovipot and proportion of eggs received was evident. All such tests confirmed the data presented in the comparison of the treatment with all control ovipots. Analysis of oviposition incidence revealed a lack of discrimination. With no apparent spill-over effect and no differences in oviposition incidence, it appears that gravid females cannot detect the attractive compounds at distances spanning neighboring ovipots. This indicates that the larval-associated cues are of low volatility, and more likely act as close range attractants or oviposition stimulants.

CHAPTER 5

EFFECT OF EGG DENSITY AND DISTRIBUTION ON OVIPOSITION SITE SELECTION BY *AEDES AEGYPTI*

Introduction

Gravid *Ae. aegypti* assess oviposition sites based on both abiotic and biotic factors. Conspecific immatures are one these possible factors. As demonstrated previously (Ch. 3), females lay significantly more eggs in a treatment ovipot containing conspecific larvae than they do in ovipots containing filtered, distilled water. This relationship is more evident with increasing instar and increasing density up to two larvae per ml; the response becomes negative at high densities (*i.e.*, four larvae per ml). This suggests that larvae act as indicators of potential success, and may act as indicators of competition at high densities.

Gravids also may encounter conspecific eggs at an oviposition site. A genus-specific oviposition pheromone has been isolated and characterized from the eggs of *Cx. quinquefasciatus* (Laurence and Pickett 1985). This apical-droplet pheromone attracts gravid females, and sites containing such eggs or the chemical compound receive significantly more eggs than control sites in either laboratory or field tests (Bruno and Laurence 1979, Otieno *et al.* 1988). It is believed to act as an attractant rather than a stimulant (Bentley and Day 1989).

Yellow fever mosquito oviposition behavior is not affected by this pheromone (Bentley and Day 1989). *Aedes aegypti* are not usually found in sites containing *Culex* larvae, as the latter prefer nutrient-rich waters (Beehler *et al.* 1994). These habitats are typically too polluted for *Ae. aegypti* larvae; gravids usually oviposit in much cleaner

waters (Shannon and Putnam 1934). This may be an indication that *Ae. aegypti* larvae do not fare as well as *Culex* larvae under crowded conditions. Gravid *Ae. aegypti* preferentially oviposit in sites containing lower densities of conspecific larvae, suggesting females assess site suitability based on both indications of success and cues of overcrowding. I questioned whether gravids detect similar cues from sites containing conspecific eggs.

Eggs are another immature stage that produce that are more indicative of future competition for the offspring of ovipositing females. Previously published reports generally conclude that this is the case (Bentley and Day 1989). A variety of Aedine mosquitoes, including *Ae. aegypti*, *Ae. polynesiensis*, *Ae. sierrensis*, *Ae. togoi* and *Ae. triseriatus*, have been tested for their response to sites containing eggs under a variety of laboratory and field experimental designs (Gubler 1971, Kitron *et al.* 1989, Chadee *et al.* 1990, Ahmadi and McClelland 1993, Apostol *et al.* 1994, Onyabe and Roitberg 1997, Allan and Kline 1998). Most of these studies demonstrated female avoidance of sites containing conspecific eggs. However, the most recent of these reports concludes that gravid *Ae. aegypti* show neither preference nor avoidance of sites in which conspecific eggs are found (Allan and Kline 1998). This apparent spurious conclusion may be due to differences in experimental design; re-examination of gravid response to sites containing conspecific eggs is warranted. I examined the effects of conspecific egg-presence at oviposition sites on female site choice using the eight ovipot, large cage design.

First, female responses to the presence of eggs was investigated, and results assessed under the implications of two competing paradigms. The first is that eggs

indicate potential success of a site; this would hold true if females preferentially oviposit in sites containing eggs. This might occur in the same manner by which conspecific larvae are attractive to ovipositing females. The opposing postulate is that eggs are a sign of future larval competition. Gravids should then avoid these sites and select ovipots free of egg-contamination.

A possible density-dependent response to sites containing eggs also was investigated. Such a response was found with regards to female assessment of sites containing 4th instar conspecifics: low larval densities were attractive to ovipositing females, while high larval densities elicited an avoidance response (Ch. 4). A density-dependent relationship based on presence of eggs has not been investigated with *Ae. aegypti*. Because most researchers found eggs to be repellent/deterrent to gravid oviposition, the degree of the this negative response was not a major concern.

Another experiment involves the response to eggs placed in different physical distributions on the paper substrate. As opposed to larvae, eggs are non-motile, so the cues they emit are more fixed than those from larvae, which may spread throughout the aquatic medium. Although chemical cues may be volatile and spread through the air, any chemicals of low volatility, and certainly contact-related cues, are fixed to the immediate area of the egg. The concentration gradients of these cues may therefore differ under differing egg distributions. If placed in a line, females could not avoid coming into contact with some eggs as they approached the water line. If eggs were distributed in a patch, females were more likely to not come into contact with any eggs, but if they did contact any, they were likely to contact a high concentration of eggs. The response of

females to sites containing eggs at the same density but different distributions (LINE or CLUMP) was investigated.

Materials and Methods

Individual females were released in 1.82 m^3 cages containing 8 oviposition sites (Ch. 2). Females were offered 7 control sites and one treatment site. Seven of the eight 120 ml ovipots lined with seed-paper substrate were filled with 40 ml of filtered distilled water (FDW), while the remaining ovipot contained seed-paper substrate and was treated with eggs. Eggs were brushed off colony egg papers onto a dampened seed-paper substrate by means of a fine paintbrush. All eggs used in experiments were of 1-2 wks of age.

To establish the baseline female response to the presence of conspecific eggs, females were offered a treatment site containing eggs at a density of 1 egg per ml of ovipot water. Eggs were not washed as they had already undergone their development period (*i.e.*, had dried and tanned): it was possible they might hatch if wetted. Female selection of sites based upon her deposition of new eggs was monitored.

To test the effects of egg density on female oviposition site choice, eggs were collected and placed on a paper substrate such that if all eggs hatched, the corresponding larval density would be either 1 per 4 ml water, 1 per 2 ml water, 1 per 1 ml water or 4 per 1 ml water. The water volume remained constant at 40 ml for all experiments. Water was added after the seed-paper was placed in the glass container. This treatment ovipot was placed in the experimental cage along with the 7 control ovipots.

The high-density end of the range was chosen due to the research on *Cx. quinquefasciatus* performed by Ikeshoji and Mulla (1970), in which growth retardant factor production was maximized at a density of 4-6 larvae per ml. If any such factors are produced by *Ae. aegypti*, which typically inhabit cleaner waters (and at lower densities) than do *Cx. quinquefasciatus*, it seems plausible that they might be produced at the low end of this 4-6 larvae per ml range. Again, this is based on the presumption that all eggs will hatch. The lowest density was picked based on numerical reciprocity. As all ovipots held 40 ml water, the absolute numbers of eggs ranged from 10 to 160 eggs per treatment ovipot.

There is some evidence that population regulation may occur in Aedine mosquitoes at the time of egg hatch. Edgerly *et al.* (1998) found installment hatching in *Ae. triseriatus*, in which only some of the eggs present in a site hatched at any given time. Gillet *et al.* (1977) put forth the hypothesis that *Ae. aegypti* larvae from eggs that hatch first browse over the other unhatched eggs on their way into the water. In so doing they eat bacteria. He suggested these bacteria provided the hatching stimulus for eggs based on the amount of oxygen they consumed. At high bacterial densities, the local oxygen tension was reduced; this provided the ultimate hatching stimulus for *Ae. aegypti* eggs. With newly-hatched larvae reducing these bacterial populations, the oxygen tension would rise and other eggs would not hatch.

The specific factors which ultimately lead to particular eggs hatching or not hatching are not known, so installment hatching could not be accounted for in this experiment. The hypothesis of Gillet *et al.* (1977) remains unverified, and it is apparent

that this idea falls apart under conditions of site flooding in which eggs are submerged. Larvae could not browse over other eggs and would swim away immediately after hatching.

The effects of egg distribution were monitored by a similar design. Gravid females typically walk around the seed-paper substrate after they have landed on it. Preliminary observations indicated they often walk down to the water edge (though they do not land or walk on the water). Given the possible importance of contact with eggs, two different egg distributions were tested for gravid-response. Both involved eggs present at a density of 1 per ml of water. In each distribution, eggs were placed 4-5 mm apart from their nearest neighbor. The LINE distribution involved placing 40 eggs in a ring around the substrate such that the eggs were horizontal to the water surface and approximately 1 cm above it. The CLUMP distribution involved placing 40 eggs 4-5 cm apart in a 2 cm by 2 cm square. The bottom of this square was about 1 cm above the water surface.

All tested females were between the ages of 6-8 days post-emergence. They were maintained on a 10% sucrose solution and offered a blood-meal 3 d post-emergence. Individual females were released into a large cage containing the seven controls and one treatment ovipot. The position of the treatment was decided by a stratified random assignment. Each trial lasted for 23 h. At the end of the trial females were collected, ovipots were removed, and the presence and number of eggs present in each oviposition site were recorded. The starting egg count in the treatment site was subtracted from the

total egg count at the end of the experiment to obtain the number of newly-deposited eggs. A χ^2 contrast test was performed on the collated data.

Results

Null Hypothesis: *Females oviposit neither more nor fewer eggs in sites containing conspecific eggs.*

The treatment site received fewer eggs (1.74%) than any other site (Table 5.1). According to an expected control distribution, the treatment site should have received 12.5% of the total eggs deposited. The difference from the expected distribution was statistically significant ($p<0.001$).

Null Hypothesis: *Females do not exhibit any preference or avoidance for sites containing eggs at differing densities.*

Increasing density of eggs appears to increase the negative oviposition response from ovipositing females, up to a density of 1 egg per ml (Table 5.2). Eggs present at densities of 1 per 4 ml water elicited the greatest egg deposition from females (8.1%). The greatest negative response occurs with eggs present at a density of 1 per 1 ml (1.74%). This is significantly lower than the proportion of eggs laid due to any other treatment, including eggs present at higher densities. The response is significantly different from an expected (12.5%) distribution regardless of the egg density tested.

Table 5.1. Comparison of egg effects on *Ae. aegypti* oviposition with expected distribution if there is no effect. Eggs tested at density 1 per ml. Treatments sharing same letter category are not statistically different at the p=0.05 level.

	Ovipot									
	-3	-2	-1	0	1	2	3	4	Total	Category
EGGS										a
N	25	25	25	25	25	25	25	25	200	
Oviposition Events	8	7	8	1	6	14	7	6	57	
Eggs Deposited	201	178	153	20	103	356	56	84	1151	
Eggs/Oviposition Event	25.13	25.43	19.13	20.00	17.17	25.43	8.00	14.00	20.19	
% Total Eggs in Ovipot	17.46	15.46	13.29	1.74	8.95	30.93	4.87	7.30	100.00	
Standard Error	5.35	4.82	3.99	1.05	2.43	9.38	1.73	2.21		
% Expected	12.50	12.50	12.50	12.50	12.50	12.50	12.50	12.50	100.00	b

Table 5.2. Comparison of egg density effects on *Ae. aegypti* oviposition. Treatments sharing same letter category are not statistically different at the p=0.05 level.

	Ovipot									
	-3	-2	-1	0	1	2	3	4	Total	Category
1 EGG/ 4 ML										a
N	19	19	19	19	19	19	19	19	152	
Oviposition Events	8	10	9	8	11	10	7	13	76	
Eggs Deposited	127	230	207	97	226	53	106	158	1204	
Eggs/Oviposition Event	15.88	23.00	23.00	12.13	20.55	5.30	15.14	12.15	15.84	
% Total Eggs in Ovipot	10.55	19.10	17.19	8.06	18.77	4.40	8.80	13.12	100.00	
Standard Error	3.25	5.87	6.01	2.78	5.97	2.12	2.63	3.77		
1 EGG/ 2 ML										b
N	21	21	21	21	21	21	21	21	168	
Oviposition Events	11	8	12	5	18	6	9	10	79	
Eggs Deposited	233	124	315	77	335	81	152	79	1396	
Eggs/Oviposition Event	21.18	15.50	26.25	15.40	18.61	13.50	16.89	7.90	17.67	
% Total Eggs in Ovipot	16.69	8.88	22.56	5.52	24.00	5.80	10.89	5.66	100.00	
Standard Error	5.06	2.12	6.79	1.88	7.11	1.65	3.22	1.81		

Continued next page

Table 5.2. Continued.

	Ovipot									
	-3	-2	-1	0	1	2	3	4	Total	Category
1 EGG/ ML										c
N	25	25	25	25	25	25	25	25	200	
Oviposition Events	8	7	8	1	6	14	7	6	57	
Eggs Deposited	201	178	153	20	103	356	56	84	1151	
Eggs/Oviposition Event	25.13	25.43	19.13	20.00	17.17	25.43	8.00	14.00	20.19	
% Total Eggs in Ovipot	17.46	15.46	13.29	1.74	8.95	30.93	4.87	7.30	100.00	
Standard Error	5.35	4.82	3.99	1.05	2.43	9.38	1.73	2.21		
4 EGGS/ ML										b
N	18	18	18	18	18	18	18	18	144	
Oviposition Events	12	8	11	4	8	9	10	10	72	
Eggs Deposited	269	112	250	60	76	138	204	245	1354	
Eggs/Oviposition Event	22.42	14.00	22.73	15.00	9.50	15.33	20.40	24.50	18.81	
% Total Eggs in Ovipot	19.87	8.27	18.46	4.43	5.61	10.19	15.07	18.09	100.00	
Standard Error	6.54	2.28	5.37	1.57	1.72	3.12	4.78	5.34		

Null Hypothesis: *Females do not exhibit any preference or avoidance for oviposition sites containing eggs present at differing distributions.*

Sites containing eggs placed at equal densities but under different distributions receive similar numbers of eggs ($p>0.75$) (Table 5.3). Both treatment sites receive significantly fewer eggs (6.7% and 6.5% respectively) than expected if the eggs were laid in a random manner (12. 5%).

Discussion

My results agree with the general consensus in the literature that gravid *Ae. aegypti* avoid sites in which conspecific eggs are already present. This differs from the recent conclusions of Allan and Kline (1998), likely due to differences in experimental design. The use of 8 ovipots, with but one as treatment, likely reduces any potential confounding effects due to "skip oviposition." This phenomenon is the tendency of females to avoid laying all their eggs in one site (Corbet and Chadee 1993). While not demonstrated conclusively under field conditions, there is evidence that indicates skip oviposition is a factor in laboratory experiments (Corbet and Chadee 1993). The work of Allan and Kline (1998) did not account for skip oviposition, and it is possible that the lack of effect they witnessed was due to this effect. Although their treatment may have contained a repellent/deterrent to oviposting females, this could have been masked by their tendency to not lay all their eggs in the lone control site.

Sites containing eggs present at a density of 1 per ml water receive significantly fewer eggs than should be expected by random oviposition (Table 5.1). This contrasts

Table 5.3. Comparison of egg distribution (LINE or CLUMP) effects on *Ae. aegypti* oviposition. Both distributions tested with density of 1 egg per ml. Treatments sharing same letter category are not statistically different at the p=0.05 level.

	Ovipot									
	-3	-2	-1	0	1	2	3	4	Total	Category
LINE										a
N	23	23	23	23	23	23	23	23	184	
Oviposition Events	11	9	12	7	8	7	10	10	74	
Eggs Deposited	315	124	253	81	73	137	82	153	1218	
Eggs/Oviposition Event	28.64	13.78	21.08	11.57	9.13	19.57	8.20	15.30	16.46	
% Total Eggs in Ovipot	25.86	10.18	20.77	6.65	5.99	11.25	6.73	12.56	100.00	
Standard Error	7.21	2.67	6.58	2.01	1.99	4.01	2.61	3.63		
CLUMP										a
N	24	24	24	24	24	24	24	24	192	
Oviposition Events	14	10	7	8	8	12	13	12	84	
Eggs Deposited	431	153	62	101	127	195	261	219	1549	
Eggs/Oviposition Event	30.79	15.30	8.86	12.63	15.88	16.25	20.08	18.25	18.44	
% Total Eggs in Ovipot	27.82	9.88	4.00	6.52	8.20	12.59	16.85	14.14	100.00	
Standard Error	8.22	3.10	1.64	1.89	2.41	3.76	4.86	4.11		

with the response to sites containing larvae, which received significantly more eggs than should be expected by random oviposition (Ch. 3). Larvae and pupae at a site are indicative of potential reproductive success. Combined with the information that increasing instar brings increased oviposition response (Ch. 3), it appears that larvae and puape are more indicative of success than competition, except at very high larval densities. The reason eggs do not elicit similar responses likely lies in the hatching biology of *Ae. aegypti*. The eggs are laid above the water surface, and require a period of conditioning prior to hatch. Any larvae present in a site are likely to have emerged as adults by the time any newly-laid eggs hatch (*i.e.*, upon the next flooding of the oviposition habitat). Thus, larvae present at a site do not compete directly with the progeny of the female assessing the site. In contrast any eggs already present at a site will likely hatch at the same time as those of the female assessing the site for oviposition. Over the evolutionary history of *Ae. aegypti*, females have likely come to respond to oviposition cues from larvae and eggs in the opposite manner because they provide different information about risk and success.

The negative response to eggs intensified with increasing egg density (Table 5.2) up to a density of 1 egg per ml. Surprisingly, an egg density of 4 per ml received significantly more eggs than the 1 per ml treatement. It is not clear why a higher density, indicating more competition, would elicit a negative response from gravids but one that is not as strong as a response to a lower density of eggs. It is possible that a threshold is reached at 1 per ml so that greater densities do not have any enhanced effect on ovipositing females. That the 4 per ml egg density is not as significantly

repellent/deterrent as the 1 per ml egg density indicates experimental limitiations due to small sample size.

Females do not discriminate between ovipots containing an equal density of eggs in either LINE or CLUMP distributions (Table 5.3). Both received proportions of eggs significantly fewer than expected by random oviposition. This emphasizes the relatively low importance of contacting eggs in oviposition-site selection of females. The LINE distribution prevents females from walking to the water surface without touching an egg. If an egg is contacted, the females can move up the substrate and not touch another egg. If sites with eggs distributed in a line received fewer eggs than sites containing clumped eggs, it might be an indication that contacting but a single egg is of importance in site-selection. The CLUMP distribution allows females to walk over most of the substrate without contacting any eggs. If an egg is contacted the female is likely to contact more eggs by walking in any direction other than the one she came by. If sites containing a clumped distribution of eggs received significantly fewer eggs than sites containing a line of eggs, it would likely indicate that the number of eggs a female contacts is relevant to oviposition site-selection.

That females respond in an identical manner to either distribution demonstrates that eggs present at equal density do not present a different set of cues to gravids. This suggests the presence of some egg-related chemicals that spread throughout the ovipot region. They may be olfactory in nature, in which case the local air would be saturated, or they may be chemo-tactile in nature, spreading through the damp, porous substrate. It is possible that the cues used may be visual, but given the fact that the CLUMP

distribution of eggs gives a larger, cohesive optical "target" than does the LINE distribution, and that no differences were found in response to the two distributions, it seems unlikely that female response is due only to visual cues.

Again, there are no consistent spill-over effects. By combining the number of eggs counted in positions -1 and +1, and comparing those with neighboring ovipots, no clear relationship between distance from the treatment ovipot and proportion of eggs received was evident. All such tests confirmed that the presence of eggs elicits a negative response from females: the treatment ovipot received significantly fewer eggs than did control ovipot groups. Analysis of oviposition incidence revealed a lack of discrimination. The cues emitted from eggs do not appear to repel or deter oviposition in control ovipots neighboring the treatment ovipot, nor do they cause the female to visit the treatment ovipot at a reduced frequency. This indicates that gravid females detect egg-associated cues only at close range.

With the geographic overlap of *Ae. aegypti* and *Aedes albopictus* populations in Asia and recently in North America, competitive displacement of species due to competition has been given much consideration. These species may oviposit in similar containers and their hatching biology is the same. There is one report on the *Ae. aegypti* oviposition response to sites containing *Ae. albopictus* eggs (Allan and Kline 1998). They concluded *Ae. aegypti* preferred oviposition sites containing either conspecific eggs or the eggs of *Ae. albopictus* over control sites. My results contradict their findings regarding conspecific eggs, likely due to differences in experimental design. Their conclusions based on *Ae. aegypti* response to sites containing *Ae. albopictus* are based on

the same experimental design; therefore the positive response might be unfounded. Since eggs are a sign of future competition, and *Ae. albopictus* larvae compete with *Ae. aegypti* larvae in the same habitats, females of either species should avoid sites containing heterospecific eggs. Re-examination of gravid *Ae. aegypti* response to heterospecific eggs using an 8 ovipot design is warranted.

CHAPTER 6

THE MECHANISMS OF GRAVID *AEDES AEGYPTI* REPELLENCY OR DETERRENCY TO OVIPOSITION SITES CONTAINING CONSPECIFIC EGGS

Introduction

Gravid *Ae. aegypti* tend to avoid ovipositing in sites containing conspecific eggs (Bentley and Day 1989, Ch. 5). This avoidance response appears to be density-dependent and independent of egg distribution. The mechanisms by which females assess sites containing eggs has not been studied.

Eggs may present an entirely different set of cues than do larvae. Given the opposite response of gravids to sites containing eggs and larvae, at least one cue must be specific for either eggs or larvae. Some general *Ae. aegypti*-restricted cue may be found in both eggs and larvae, but the presence or absence of some stage-specific cues are required for discrimination.

In comparing the density-dependent response by which females favored sites containing larvae, it was demonstrated that both olfactory and chemo-tactile cues impart discriminatory information to females (Ch. 4). Visual cues were found to be of no importance in female site-selection. This is not surprising since oviposition sites are typically dark and in the shade and larvae are in the water and generally browse on the sides and bottom of their habitat rather than near the surface. It appears unlikely that gravids could come to rely on visual cues for assessing larval presence under these circumstances.

Although the oviposition sites would be the same (dark and in the shade), eggs are fixed and present on the surface of the oviposition substrate. Females have a greater

chance of visually recognizing or touching eggs than they do larvae. Thus, visual cues could play a role in selection of sites containing eggs. Chemo-tactile cues also may be of greater importance in detection of eggs than of larvae, for similar reasons.

With a lack of discrimination between sites containing an equal density of eggs distributed in a line or a clump, it appears that eggs of equal density present the same cues regardless of position on the substrate (Ch. 5). This indicates females probably rely more on chemical rather than visual or contact-related cues. If vision were important, then some differences would have been expected since the patchy distribution provides a larger visual target. If contact were important females should have responded in a more negative fashion to eggs placed in a clumped distribution. The density-dependent response (more negative with increasing density) indicated that cues females receive from eggs are probably olfactory in origin. If chemical cues are disbursed through the seed-paper substrate, then chemo-tactile cues may also play a role in gravid appraisal of oviposition sites containing eggs.

The relative importance of chemo-tactile, olfactory and visual cues related to egg presence were tested in a direct manner. The effects of visual were tested using visual mimics, specifically, pencil-marked ovals of egg size and shape. The role of chemo-tactile cues were assessed by placing eggs in a screened shell vial in the center of the ovipot. Olfactory-derived cues were tested by bleaching eggs. Eggs bleached for a short period of time retain their shape and therefore present the same set of contact cues as non-bleached eggs, but they no longer present any egg-derived chemical cues. The

importance of olfactory cues are demonstrated by comparing gravid response to eggs fully exposed, bleached eggs, and eggs separated by a screened-shell vial.

Materials and Methods

All experiments involved the testing of eggs, real or surrogates, at a density of 1 per ml of water. To test the effects of vision on female oviposition in sites containing conspecific eggs, a black pencil was used to draw egg mimics on the seed-paper substrate. Each mimic was shaped as an oval, length 1.5 mm by diameter 0.5 mm. This treatment condition was termed DRAWN. Since no differences in female response to eggs of different distribution are known (Ch. 5), the 40 drawn eggs were placed in a line around the ovipot, with the egg length along the horizontal axis. Once the substrate was placed in the ovipot, 40 ml FDW was added. This treatment ovipot was placed in a large cage with seven control ovipots in the described manner (Ch. 2). Individual females were released and allowed to oviposit for 23 h, at which time the experiment was ended. Eggs were counted and subjected to χ^2 analysis.

Preliminary tests indicated that females do not respond differently to sites containing a glass shell vial with a screened top than they do to sites lacking this potential obstruction ($p=0.531$). Ten eggs were collected off colony rearing papers and placed on a damp seed-paper. This paper was cut to the shell vial dimensions, and 10 ml of filtered-distilled water (FDW) was added. Egg papers were placed such that eggs faced the interior of the vial. With the egg paper lining the vial, the eggs could not be seen. The vial was capped with cloth screening and placed in the center of the treatment ovipot (Fig

6.1). The treatment ovipot was placed in a stratified random manner in a large cage with 7 control ovipots. Individual females were released and allowed to oviposit for 23 h, at which time the experiment was ended. Eggs were collected and subjected to χ^2 analysis. This experimental condition was termed SCREENED.

The final treatment involved the placement of bleached eggs in a line (length of egg along the horizontal axis) around the ovipot waterline. Colony egg papers were dipped in a crystallizing dish containing bleach for 15 sec. Treatments of shorter periods resulted in non-bleached eggs, while longer washes with bleach resulted in egg collapse or even destruction. After the bleach bath, the egg paper was soaked in distilled water for 30 mins. The eggs turned white within the first few minutes of this wash, and most floated off the paper during the course of the wash. At the end of this bath, eggs were collected by means of a fine paintbrush and placed in a new dish of distilled water. They were left to soak for 2 h. A final soak of 2 h in a fresh batch of distilled water followed. The eggs were then examined for integrity under a dissection microscope. Intact bleached eggs had the same porous appearance as non-bleached counterparts. These eggs were brushed onto filter paper and allowed to dry for 15 min, at which time they were brushed onto a dampened seed-paper to be used in the treatment ovipot. Forty ml of FDW was added to this ovipot, which was then placed in a large cage with 7 control ovipots. Individual females were released and allowed to oviposit for 23 h, at which time the experiment was ended. Eggs were counted and subjected to χ^2 analysis. This experimental condition was termed BLEACHED.

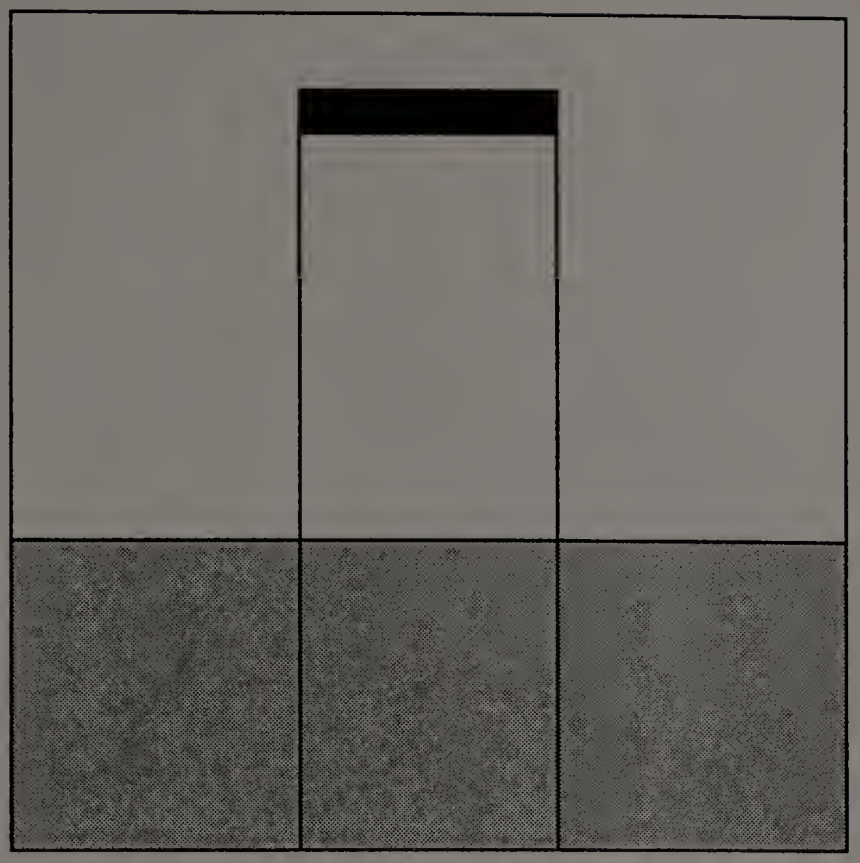

Fig. 6.1. Side view of the SCREENED condition setups. A shell vial containing seed-paper substrate and 40 eggs was placed in the middle of an ovipot with 40 ml of water. A plastic cap with the center cut out and replaced by mesh was fixed to the top of the vial.

Results

Null Hypothesis: *Females do not respond differently to sites containing visual egg mimics and sites containing an equal density of eggs.*

This hypothesis appears to be false (Table 6.1). Females laid a significantly smaller proportion of their eggs in the treatment site containing actual eggs (NORMAL). The DRAWN treatment received 13.0% of female eggs which was not different from the expected random distribution of 12.5%. Female selection of sites containing conspecific eggs does not appear to be due to visual cues (at least not to the cues presented by these egg surrogates). Thus, abrogation of the response must be due to missing olfactory and/or chemo-tactile cues.

Null Hypothesis: *Females do not respond differently to sites containing eggs physically separated and screened than they do to sites in which they have full access to eggs.*

Sites containing eggs separated from the oviposition waters by the glass wall of a shell vial capped with a mesh lid (SCREENED) received a greater proportion of eggs than sites in which females had full access to eggs (NORMAL) (Table 6.1). However, the difference between SCREENED and the expected distribution was significant ($p<0.05$), indicating that some egg-associated olfactory cues were present. The difference in the proportion of eggs received by either SCREENED or NORMAL treatment ovipots containing (9.2% to 1.7%, respectively) was significant ($p<0.001$). This indicates that olfaction is not the only means, indeed is not the most significant mechanism, by which females assess the presence of eggs. The null hypothesis proved false.

Table 6.1. Comparison of egg mimics (DRAWN) and eggs either normally accessible to ovipositing females, separated by a screened vial or pretreated with bleach. Densities are 1 egg per ml water. Treatments sharing same letter category are not statistically different at the p=0.05 level.

	Ovipot									
	-3	-2	-1	0	1	2	3	4	Total	Category
SCREENED										a
N	18	18	18	18	18	18	18	18	144	
Oviposition Events	8	10	13	9	13	10	5	8	76	
Eggs Deposited	26	259	228	113	316	134	53	106	1235	
Eggs/Oviposition Event	3.25	25.90	17.54	12.56	24.31	13.40	10.60	13.25	16.25	
% Total Eggs in Ovipot	2.11	20.97	18.46	9.15	25.59	10.85	4.29	8.58	100.00	
Standard Error	1.21	6.54	5.27	2.68	7.23	3.31	1.42	2.64		
BLEACHED										b
N	16	16	16	16	16	16	16	16	128	
Oviposition Events	9	4	11	8	12	13	7	10	74	
Eggs Deposited	231	61	228	206	232	306	82	127	1473	
Eggs/Oviposition Event	25.67	15.25	20.73	25.75	19.33	23.54	11.71	12.70	19.91	
% Total Eggs in Ovipot	15.68	4.14	15.48	13.99	15.75	20.77	5.57	8.62	100.00	
Standard Error	4.86	1.35	4.75	3.91	4.39	6.22	1.70	2.13		

Continued next page

Table 6.1. Continued.

	Ovipot									
	-3	-2	-1	0	1	2	3	4	Total	Category
DRAWN										b
N	24	24	24	24	24	24	24	24	192	
Oviposition Events	13	11	8	12	13	7	7	11	82	
Eggs Deposited	286	217	132	184	167	119	62	254	1421	
Eggs/Oviposition Event	22.00	19.73	16.50	15.33	12.85	17.00	8.86	23.09	17.33	
% Total Eggs in Ovipot	20.13	15.27	9.29	12.95	11.75	8.37	4.36	17.87	100.00	
Standard Error	5.93	4.56	2.35	3.81	3.91	2.43	1.02	5.48		
NORMAL										c
N	25	25	25	25	25	25	25	25	200	
Oviposition Events	8	7	8	1	6	14	7	6	57	
Eggs Deposited	201	178	153	20	103	356	56	84	1151	
Eggs/Oviposition Event	25.13	25.43	19.13	20.00	17.17	25.43	8.00	14.00	20.19	
% Total Eggs in Ovipot	17.46	15.46	13.29	1.74	8.95	30.93	4.87	7.30	100.00	
Standard Error	5.35	4.82	3.99	1.05	2.43	9.38	1.73	2.21		

Null Hypothesis: *Females do not respond differently to sites containing eggs in which both chemo-tactile and olfactory cues are abrogated as they do to sites in which they have full access to eggs.*

Females exposed to BLEACHED eggs can use neither chemo-tactile nor olfactory cues in their assessment of larval presence. It is important to reiterate that chemo-tactile comprises both physical and chemical aspects. Chemical aspects are obliterated with treatment by bleach, while the contact aspects of chemo-tactile should remain intact. With only altered visual cues (*i.e.*, color) and contact cues presumably remaining, females oviposited 14.0% of eggs in the treatment ovipot (Table 6.1). This is not significantly different from an expected random distribution, and is significantly different from the proportion of eggs received in the NORMAL treatment ($p<0.001$). By comparison, in the SCREENED condition, sites containing bleached eggs received a significantly greater proportion of new eggs than did sites containing eggs separated and screened ($p<0.001$). These results reflect the importance of chemical cues in the appraisal by gravid females of ovipots containing eggs. Thus, the null hypothesis proved false.

Discussion

By comparing the full range of treatment conditions it is apparent that females rely on chemicals associated with eggs to determine the presence of eggs at a site. Visual mimics either do not approximate the visual profile of actual eggs, or there is no response to eggs based on visual cues. As mentioned with the lack of visual response to larvae (Ch. 4), these sites are typically dark and in the shade. Mosquitoes likely use vision to

detect differences in color and contrast (Sipple and Brown 1953, McCrae 1984). Under conditions of low light it may be impossible for gravids to detect eggs by visual means. This evidence is supported by the findings that a patchy egg distribution (a large visual target) does not elicit a response different from eggs distributed in a line (several small visual targets) (Ch. 5).

Olfactory cues do play some role in female detection of sites containing eggs (Table 6.1). With all egg-associated chemical cues nullified (BLEACHED), females oviposit in treatment sites as if there were no eggs present. Under the SCREENED condition, in which only air-borne cues may be detected by gravids, there is some negative response. That this response is significantly mitigated compared to the NORMAL condition indicates an additional role of chemo-tactile cues. The degree of this mitigation (1.7% eggs laid in NORMAL treatment ovipot compared to 9.2% eggs recovered in the SCREENED treatment ovipot) suggests that chemo-tactile cues play a greater role in detection of eggs than do olfactory cues.

Physical contact cues are presumably present under conditions of treatment with bleach. That the negative response to eggs is no longer apparent with BLEACHED eggs indicates that physical contact with eggs does not assist the female in-site assessment (Table 6.1). The term "chemo-tactile" cue is insufficient to explain the nature of female response to sites containing eggs. The response is entirely based upon chemical cues. These cues may be highly volatile, and thus olfactory in nature, or may be fairly inert, and thus chemo-tactile in nature. Chemo-tactile cue detection does not require female contact with the eggs themselves since these cues may be dispersed through the porous, damp

paper substrate in a limited fashion. The way such cues my travel through the substrate are not known, and their degree of spread cannot be speculated on at this time. The way in which chemicals spread differently through the air and the much more viscous medium of water is understood, however, at least in a general sense. Chemicals that may spread more readily through a damp paper than air are likely large molecules (and thus more inert) and hydrophilic in nature (Zumdahl 1992). By contrast, those compounds that disperse readily in air are probably smaller molecules and not as likely to be polar (and thus do not bind to water molecules with the affinity of hydrophilic compounds).

By combining the number of eggs counted in positions -1 and +1, and comparing those with neighboring ovipots, no clear relationship between distance from the treatment ovipot and proportion of eggs received was evident. Thus no spill-over effects were found. Analysis of oviposition incidence revealed a lack of discrimination. Gravid females must likely come very close to a site containing eggs to assess its suitability. The chemical cues that volatilize do not spread very far. This further emphasizes the importance of close-range chemical assessment via olfactory and chemo-tactile means.

CHAPTER 7

OVIPOSITION INCIDENCE AND PROPORTION OF EGGS LAID IN OVIPOSITION HABITATS OF DIFFERENT SIZE

Introduction

Gravid *Ae. aegypti* oviposit in a wide variety of natural and artificial containers, including tree-holes, refuse plastic and metal containers, water storage jars and discarded tires (Bentley and Day 1989). This wide variety of container types reflects a high degree of flexibility in the oviposition behavior of this mosquito species. With sufficient resources such as nutrients and water, and the appropriate degree of shelter from direct sunlight, the basic requirements for successful oviposition are fairly minimal. The fact that humans inadvertently provide many suitable oviposition habitats so close to their homes is likely a major contributing factor in the urbanization of *Ae. aegypti* populations throughout the world (Gratz 1993).

Investigations of *Ae. aegypti* oviposition behavior, both laboratory and field-based, have used a wide variety of ovipots to test hypotheses. These ovipots differ in color, substrate material, nutrient concentration and physical dimension. The ramifications of several of these factors have been studied, and a general picture of *Ae. aegypti* preference for dark ovipots, rough substrate, and clean water has emerged (Bentley and Day 1989, Ch. 1). However, the effect of oviposition site size on deposition of eggs has not been studied.

Oviposition habitat size may contribute to a gravid female's assessment of site suitability, particularly in relation to available resources for her progeny. Although evaluation of a site is indeed dependent upon several other abiotic and biotic factors, size

may give females an initial indication of how many resources are present and how many eggs to lay. Given the fixed space limitations of a site, females must consider many larvae may be supported by the resources present.

During tests on mechanisms of attraction and repellency, it became apparent that density-dependent responses may play a significant role in the response of gravids (Ch. 3, 5). To assess density of conspecific immatures, ovipositing females likely need to be able to measure some physical aspects of the oviposition site. If so there should be a fixed physical factor by which some basis of density can be determined. Density-dependent responses were found in experiments using ovipots of constant size (Ch. 3, 5). The substrate surface area (SSA) to water volume (WV) ratio was kept constant. The physical attributes that contribute to determinations of the density-dependent response are likely one of the following three: (1) the total size of the ovipot is a possible indicator of the total resources a habitat can contain; (2) the number of eggs that a site receives might be limited by SSA; while (3) assessment of larval presence may reflect upon WV. These considerations were examined.

Investigations of density-dependent response involved a standard 120 ml glass ovipot fitted with substrate paper and 40 ml of filtered-distilled water (FDW). In this experiment, gravids were provided different size classes of ovipots. The substrate surface area: water volume ratio was kept constant by adding one-third the total ovipot volume in water regardless of ovipot size. The oviposition response was measured in terms of oviposition incidence.

To test these effects in the presence of an attractive immature stage (and thus monitor a density-dependent response), 4th instar conspecifics were placed in one treatment ovipot at a density of 1 per ml of FDW; the other seven ovipots were controls containing only FDW. Again, the substrate surface area: water volume ratio was kept constant by maintaining the water volume at one-third that of the total ovipot volume. Female response was evaluated by comparing the proportion of eggs received in the treatment ovipots of different size.

To test the effects of SSA and WV, ovipots of different height but constant diameter were tested with combinations of SSA and WV. The treatments included high and low SSA amd high and low WV. The oviposition incidence in response to these parameters was recorded.

Materials and Methods

The first experiment examines the oviposition incidence of females exposed to 8 identical ovipots in a large cage. All ovipot size classes were tested independently of each other. The size classes include: SMALL (120 ml), MEDIUM (480 ml) and LARGE (960 ml) glass jars painted matte black. The specific physical dimensions of the ovipots follow. SMALL measured 6.5 x 6.0 cm (height x diameter). MEDIUM ovipots were 9.0 x 8.5 cm, while LARGE ovipots were 18.0 x 8.5 cm. The seed-paper substrate was cut to line the entire interior of each ovipot type, and one-third the total ovipot volume of FDW was added. The 8 ovipots were placed in a circle around the perimeter of a large cage (Ch. 2). Individual gravids were released and allowed to oviposit for 23 h, at which time

the experiment was ended. Sites receiving eggs were noted and egg numbers recorded; data were subjected to χ^2 analysis.

The reaction of females to ovipot size in the presence of an attractive treatment (larvae) was tested in the following fashion. All size classes were tested independently. A single treatment ovipot, consisting of 4th instars at a density of 1 per ml of water, was placed in the cage with 7 control ovipots (Ch. 2). The 4th instars were reared under conditions of 1 larva per ml, collected and washed in distilled water, and finally collected into the appropriate volume (40 ml, 160 ml or 320 ml, by increasing size class) FDW and placed in the treatment ovipot. Individual gravids were released and allowed to oviposit for 23 h, at which time the experiment was ended. The proportion of eggs received in the treatment ovipot was counted, and χ^2 analysis was performed comparing the number of eggs in the treatment ovipot within each of the three different size classes.

The final analysis involved comparing the effects of SSA and WV on female oviposition site choice. The MEDIUM and LARGE size class ovipots were used for this experiment. By keeping the SSA:WV ratio constant throughout all previous experiments, any possible confounding effects could not be accounted for. By testing low (20 cm^2) and high (200 cm^2) substrate surface areas with combinations of low (40 ml) and high (400 ml) water volumes, these effects could be tested directly (Fig. 7.1). The combinations of low SSA, high WV and high SSA, low WV were tested in the MEDIUM size ovipots. The combinations of low SSA, low WV and high SSA, high WV were examined in the LARGE class of ovipots. Note that the low SSA, low WV combination left much of the interior of the large ovipots exposed (*i.e.*, they were not covered by seed-paper). All

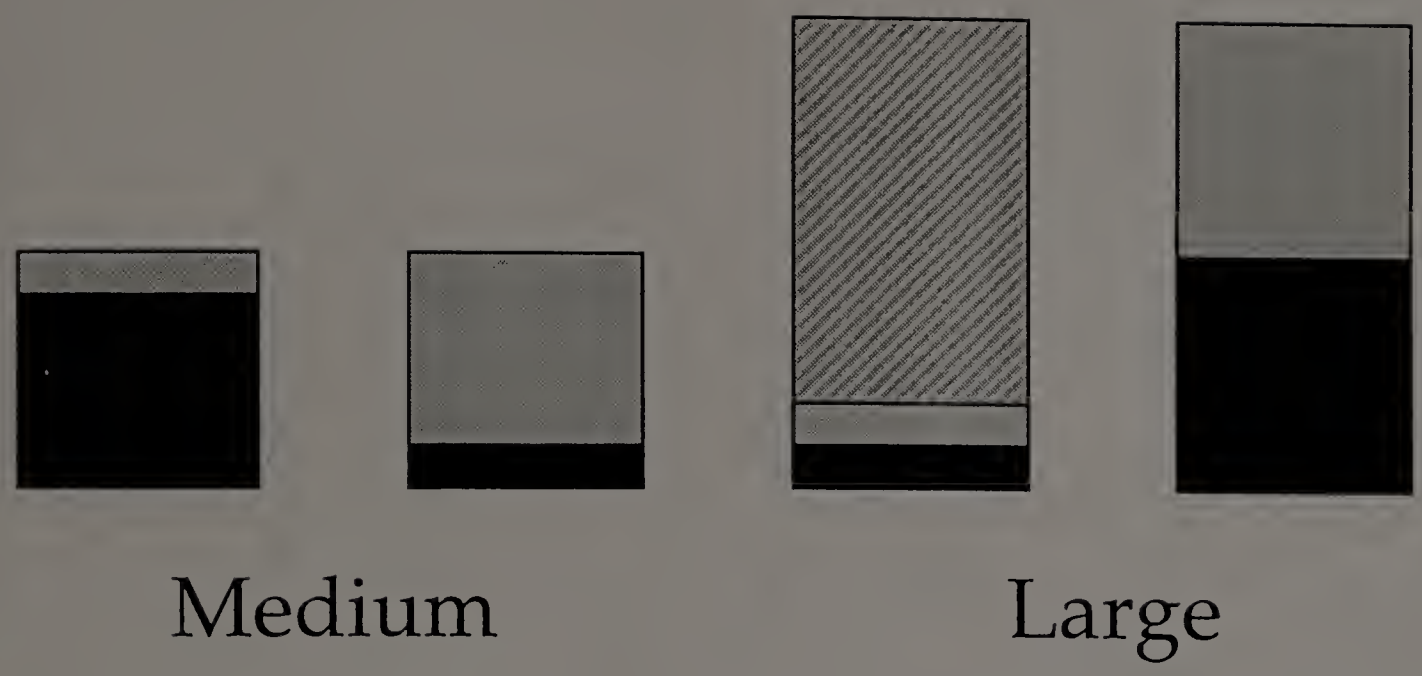

Fig. 7.1. Design for comparing *Ae. aegpypti* response to combinations of substrate surface area (light blocks) and water volume (dark blocks) in ovipots of constant diameter. The dashed markings represent the inner wall of the glass ovipot not covered by either substrate or water in the Low SSA: Low WV treatment combination.

experiments involved placing 8 identical ovipots in a large cage. Individual gravids were released and allowed to oviposit for 23 h, at which time the experiment was ended. The number of eggs at each site was counted, and data were subjected to χ^2 analysis.

Results

Null Hypothesis: *Oviposition site size does not affect gravid* Ae. aegypti *oviposition incidence.*

When only SMALL size class jars were provided, 60.4% received eggs (from the 240 possible oviposition events) (Table 7.1). This is significantly different from the 44.0% of potential oviposition sites used when all were of MEDIUM size and 37.5% when all were LARGE. The average number of ovipots visited was not significantly different between MEDIUM and LARGE ovipots ($p>0.15$). This indicates that females visit SMALL sites more frequently than they do larger sites. The null hypothesis proved to be false.

Null Hypothesis: *Oviposition site size does not affect the proportion of eggs laid in a treatment ovipot containing 4th instars at a density of 1 per ml.*

When presented with only SMALL ovipots, one containing a treatment of one 4th instar per ml, gravids oviposited 24.8% of their eggs in the treatment site (Table 7.2). This is significantly lower than the proportion of eggs received by either MEDIUM or LARGE treatment ovipots (34.3% or 32.2%, respectively). The number of eggs laid in the treatment ovipot was not significantly different between MEDIUM and LARGE size

Table 7.1. Comparison of oviposition incidence among different size class ovipots. Treatments given the same letter designation are not signficantly different from each other at the p=0.05 level.

	Ovipot									
	1	2	3	4	5	6	7	8	Total	Category
SMALL										a
N	30	30	30	30	30	30	30	30	240	
Oviposition Events	15	19	16	14	21	16	24	20	145	
Eggs Deposited	551	218	168	127	266	217	479	136	2162	
Eggs/Oviposition Event	36.73	11.47	10.50	9.07	12.67	13.56	19.96	6.80	14.91	
Oviposition Events/N	0.500	0.633	0.533	0.467	0.700	0.533	0.800	0.667	0.604	
MEDIUM										b
N	29	29	29	29	29	29	29	29	232	
Oviposition Events	12	13	10	12	13	15	13	14	102	
Eggs Deposited	355	432	201	313	319	353	341	370	2684	
Eggs/Oviposition Event	29.58	33.23	20.10	26.08	24.54	23.53	26.23	26.43	26.31	
Oviposition Events/N	0.414	0.448	0.345	0.414	0.448	0.517	0.448	0.483	0.440	
LARGE										b
N	28	28	28	28	28	28	28	28	224	
Oviposition Events	14	11	10	9	8	4	14	14	84	
Eggs Deposited	501	98	284	315	133	75	398	319	2123	
Eggs/Oviposition Event	35.79	8.91	28.40	35.00	16.63	18.75	28.43	22.79	25.27	
Oviposition Events/N	0.500	0.393	0.357	0.321	0.286	0.143	0.500	0.500	0.375	

Table 7.2. Proportion of eggs deposited in a treatment site containing 4th instar conspecifics at a density of 1 larva per ml among different size class ovipots. Treatments sharing same letter category are not statistically different at the p=0.05 level.

	Ovipot									
	-3	-2	-1	0	1	2	3	4	Total	Category
SMALL										a
N	25	25	25	25	25	25	25	25	200	
Oviposition Events	8	9	3	18	16	19	18	8	99	
Eggs Deposited	115	113	31	541	462	532	321	66	2181	
Eggs/Oviposition Event	14.38	12.56	10.33	30.06	28.88	28.00	17.83	8.25	22.03	
% Total Eggs in Ovipot	5.27	5.18	1.42	24.81	21.18	24.39	14.72	3.03	100.00	
Standard Error	2.45	2.77	1.02	7.26	7.39	7.14	4.58	2.17		
MEDIUM										b
N	21	21	21	21	21	21	21	21	168	
Oviposition Events	8	5	8	11	9	7	10	8	66	
Eggs Deposited	89	33	110	431	194	86	175	137	1255	
Eggs/Oviposition Event	11.13	6.60	13.75	39.18	21.56	12.29	17.50	17.13	19.02	
% Total Eggs in Ovipot	7.09	2.63	8.76	34.34	15.46	6.85	13.94	10.92	100.00	
Standard Error	2.31	1.22	2.43	10.01	4.58	2.07	4.16	3.52		
LARGE										b
N	18	18	18	18	18	18	18	18	144	
Oviposition Events	10	8	6	12	6	4	7	7	60	
Eggs Deposited	178	103	37	339	55	86	160	94	1052	
Eggs/Oviposition Event	17.80	12.88	6.17	28.25	9.17	21.50	22.86	13.43	17.53	
% Total Eggs in Ovipot	16.92	9.79	3.52	32.22	5.23	8.17	15.21	8.94	100.00	
Standard Error	4.85	2.67	1.53	9.03	1.62	2.41	4.22	2.78		

class ovipots ($p>0.2$). Since females laid more eggs in an attractive treatment site of MEDIUM or LARGE size than corresponding sites of SMALL size the null hypothesis proved to be false.

Null Hypothesis: *Substrate surface area and water volume do not affect oviposition incidence.*

The gravid female response to ovipot size did not differ when comparing MEDIUM and LARGE size ovipots. Thus treating these ovipots as a single size class was justified for testing oviposition incidence in response to differing combinations of SSA and WV. Gravids visited similar numbers of identical ovipots regardless of the combinations of physical parameters tested (Table 7.3). Faced with low SSA and either low or high WV, or faced with high SSA and low or high WV, females oviposited in similar numbers of oviposition sites ($p>0.05$). That is, regardless of the amount of substrate surface area present, nor the volume of water at a site, females visited a similar number (between 32.2% and 42.2%) of oviposition sites. This indicates that neither SSA nor WV are limiting factors that might affect oviposition site choice, so the null hypothesis stands.

Discussion

Oviposition site size does have an effect on *Ae. aegypti* oviposition behavior. Gravids visit more smaller sites than they do larger ones (Table 7.1). They oviposit fewer eggs in smaller attractive treatment sites than they do in larger attractive sites (Table 7.2).

Table 7.3. Oviposition incidence under combinations of high and low substrate surface area (SSA) and water volume (WV). Treatments sharing same letter category are not statistically different at the p=0.05 level.

	Ovipot									
	1	2	3	4	5	6	7	8	Total	Category
High SSA, Low WV										a
N	25	25	25	25	25	25	25	25	200	
Oviposition Events	8	9	10	9	11	12	8	9	76	
Eggs Deposited	168	121	231	99	248	252	131	156	1406	
Eggs/Oviposition Event	21.00	13.44	23.10	11.00	22.55	21.00	16.38	17.33	18.50	
Oviposition Events/N	0.320	0.360	0.400	0.360	0.440	0.480	0.320	0.360	0.380	
Low SSA, High WV										a
N	21	21	21	21	21	21	21	21	168	
Oviposition Events	7	8	9	7	11	9	9	11	71	
Eggs Deposited	134	84	168	99	205	145	120	261	1216	
Eggs/Oviposition Event	19.14	10.50	18.67	14.14	18.64	16.11	13.33	23.73	17.13	
Oviposition Events/N	0.333	0.381	0.429	0.333	0.524	0.429	0.429	0.524	0.423	

Continued next page

Table 7.3. Continued

	Ovipot 1	2	3	4	5	6	7	8	Total	Category
Low SSA, Low WV										a
N	23	23	23	23	23	23	23	23	184	
Oviposition Events	10	11	10	8	9	10	6	9	73	
Eggs Deposited	151	238	103	125	213	182	56	176	1244	
Eggs/Oviposition Event	15.10	21.64	10.30	15.63	23.67	18.20	9.33	19.56	17.04	
Oviposition Events/N	0.435	0.478	0.435	0.348	0.391	0.435	0.261	0.391	0.397	
High SSA, High WV										a
N	24	24	24	24	24	24	24	24	192	
Oviposition Events	7	7	8	6	10	9	8	7	62	
Eggs Deposited	117	92	78	104	212	163	125	138	1029	
Eggs/Oviposition Event	16.71	13.14	9.75	17.33	21.20	18.11	15.63	19.71	16.60	
Oviposition Events/N	0.292	0.292	0.333	0.250	0.417	0.375	0.333	0.292	0.323	

Given a fixed number of eggs to deposit in any single egg batch, a female that visits more smaller sites will not lay as many eggs in each site.

By combining the number of eggs counted in positions -1 and +1, and comparing those with neighboring ovipots, no clear relationship between distance from the treatment ovipot and proportion of eggs received was evident from the data presented in Table 7.2. Thus no spill-over effects were found. Analysis of oviposition incidence revealed a lack of discrimination. These data support the contention that larvae present at an attractive site emit close-range cues that elicit a positive response from gravid females. The degree of this response is mitigated by oviposition site size.

The trend of increasing positive response with increasing treatment ovipot size held true for ovipots of increasing diameter but not of increasing height. Similarly, the reduction in oviposition incidence occured with increasing ovipot diameter but was not seen among ovipots different only in height. SMALL ovipots were smaller than MEDIUM or LARGE ovipots in every dimension. LARGE and MEDIUM ovipots only differed in height. Thus, the lack of significant difference seen between MEDIUM and LARGE class ovipots regarding both oviposition incidence in the face of identical ovipots, and proportion of eggs laid in an attractive treatment ovipot, indicates one of two possible relationships between gravids and oviposition sites. The first is that a threshold SSA or WV is reached, beyond which gravids do not "need" or respond in any greater fashion. The second is that gravid response is directly proportional to ovipot diameter (*i.e.*, water surface area).

From Table 7.3 it is apparent that neither SSA nor WV hold any particular importance in *Ae. aegypti* oviposition. This concurs with preliminary observations, in which gravids were seen to oviposit in containers with just the slightest amounts of available substrate above the water line, or containers holding very small amounts of water.

Since increasing egg density correlates with an increasing negative oviposition response, it was presumed that SSA might impact female oviposition behavior. That it does not, indicates that the presence of eggs, rather than their exact number, is probably the biggest consideration of gravids that encounter sites in which eggs are already present. If egg presence is detected, females may well be repelled or limit their oviposition. If more eggs are detected, this response will be stronger.

The amount of SSA available should not impact female acceptance of a site when there are no eggs present. Barring the presence of conspecific eggs, SSA is unlikely to be a limiting factor due to the inert nature of the eggs a gravid is about to lay: eggs do not compete for resources, and SSA likely conveys little information about larval density. Regardless of the presence of eggs or larvae, it appears that SSA conveys too little information about the larval habitat to be particularly useful to an ovipositing mosquito.

Water volume, however, is a measure of the potential competition for resources that a gravid's progeny may face. The number of eggs already present, or the number of eggs a female will deposit at a site, may be expected to reflect water volume, as density is a measure of larvae (the competing stage) per unit water volume. That they do not, testifies to either an inability to measure the amount of water, or to an oviposition

relationship that is independent of oviposition habitat size. The latter is not true (Tables 7.1, 7.2).

As mentioned previously, female reliance on visual cues once at a site are probably limited due to conditions of low direct light. That mosquitoes may visually assess water volume at a site has been suggested, but appears unlikely for *Ae. aegypti* due to its visual environment (Fay and Perry 1965). There is no other way for females to measure water volume in a direct fashion.

The lack of difference between MEDIUM and LARGE class ovipots in all tests indicates that there is a potential ovipostion relationship with ovipot diameter. This relationship may come in the form of water surface area. This provides a two-dimensional measurement of a three-dimensional physical attribute, but this inadequacy might be overcome by the ease with which females can measure the water surface area. The visual contrast of reflected water vs. dull substrate may give visual recognition of the surface area. Alternatively, females may walk around the ovipot or fly across it. By performing the same tests with ovipots of different diameter, this relationship could be confirmed or refuted.

CHAPTER 8

STUDIES ON THE EFFECTS OF SENESCENCE AND GONOTROPHIC CYCLE OF GRAVID *AEDES AEGYPTI* ON OVIPOSITION SITE SELECTION

Introduction

The ramifications of age, both physiological (termed senescence) and gonotrophic, on mosquito oviposition behavior has not been investigated. Older females are considered more important vectors of disease: they are more likely to have acquired and be capable of transmitting an etiologic agent. Recent evidence suggests that older *Ae. aegypti* have a lower daily mortality rate than their younger counterparts (Harrington, personal communication). This further emphasizes that older mosquitoes may make a more significant contribution to disease transmission than younger mosquitoes: not only have they survived long enough to become competent vectors, but they are less likely to die. If oviposition trapping programs are to be implemented, the possibility of differential trapping of older and younger mosquitoes should be investigated.

Females may initiate oviposition within the first week of their emergence and lay subsequent egg batches throughout their lifetime. All else being equal, older females are likely to have had more chances to oviposit than younger females. Relative fitness is a measurement of the number of surviving progeny of a particular female. A core tenet of ecology is that organisms should strive to maximize their fitness (Charnov and Skinner 1985). An older female that has already oviposited on at least one occasion has a much greater level of fitness (barring the death of all her progeny) than a younger female that has yet to oviposit.

The gonotrophic cycle refers to the process of taking a blood-meal, developing oocytes with the digested proteins and other nutrients, and finally ovipositing one batch of eggs (Clements 1992). Female *Ae. aegypti* do not oviposit all their eggs in a single batch. Indeed, they do not acquire and assimilate sufficient nutrients from a single full blood-meal to develop and oviposit all of their egg follicles in a each batch (Clements 1992). A female that has taken a blood-meal but has not oviposited in her lifetime is in her first gonotrophic cycle. While older females are likely to have had more chances to oviposit than younger females, it is possible that younger and older females may be in the same gonotrophic cycle. Again referring to relative fitness, a female in a later gonotrophic cycle is likely to have a greater fitness than a female in her first gonotrophic cycle (Charnov and Skinner 1985).

Given this theoretical concept of fitness, *Ae. aegypti* females under differing fitness constraints were compared for their degree of selectivity in choosing oviposition sites. Selectivity may be manifested either by the number of ovipots in which females choose to oviposit, or by the number of eggs laid in sites containing a known factor of attraction or stimulation. When all oviposition sites contained the same treatment, females that laid eggs in significantly more oviposition sites were considered more selective than those that laid eggs in fewer oviposition sites. This is because more "fit" females are under less pressure to deposit all their eggs at the first suitable site. By contrast, females that have not oviposited have a fitness of zero, and may be under pressure to oviposit immediately (and perhaps in a single oviposition site) to enhance their relative fitness. Thus, females of advanced age or gonotrophic cycle, and

consequently higher potential fitness, were expected to lay eggs in more oviposition sites than those of younger age or earlier gonotrophic cycle.

If a treatment site of particular attractiveness is present, more selective females will lay more eggs in that site relative to all other sites. This holds because older females with potential high fitness can strive to give the best possible advantage to their subsequent progeny. Note, this assumes that the most attractive site is selected because of the advantage it offers a female's progeny. Although it is unlikely that less fit gravids will not recognize the advantages of the same site, they are under more pressure to lay their eggs in any suitable site in order to enhance their fitness. Therefore, females of advanced age or gonotrophic cycle are expected to lay proportionately more eggs in the treatment site than are females of younger age or less advanced reproductive state.

The following experiments tested whether degree of selectivity is related to post-emergence age or gonotrophic cycle. Specifically, females of the same gonotrophic cycle but different age were compared regarding the number of identical oviposition sites in which they choose to oviposit. The proportion of eggs they deposit in an attractive treatment site relative to 7 control sites was also compared. The attractive site consisted of conspecific 4th instar larvae, which were previously determined to be preferentially selected by females over unoccupied control sites. Similarly, females of the same age but different gonotrophic cycle are compared under the same experimental design.

Materials and Methods

Individual females were released in 1.82 m^3 cages containing 8 oviposition sites (Ch. 2). For assays in which all ovipots contained the same treatment, the ovipots consisted of 120 ml ovipots lined with seed-paper substrate and filled with 40 ml of filtered-distilled water (FDW). For tests in which females were offered 7 control sites and one treatment site, 7 of the 120 ml ovipots were lined with seed-paper substrate and filled with 40 ml of FDW, while the remaining ovipot contained the seed-paper substrate and fourty 4th instar conspecifics in 40 ml of FDW. Prior to experimentation, larvae were collected from their larval rearing trays, washed in distilled water 3 times for 1 min each wash, bathed in a fresh batch of distilled water for 2 h, and placed into the final 40 ml of FDW used in the experiment. Each trial lasted for 23 h, at the end of which females were collected, ovipots were removed, and the presence and number of eggs deposited in each oviposition site were recorded. A χ^2 contrast test was performed on the collated data.

To test the effects of age on oviposition site selection, two groups of females were compared. Both groups were collected 7 d after taking a blood-meal. The younger group was offered its blood-meal just 3 d post-emergence, and was thus 10 days post-emergence in age when the trial began. The older group was not given the opportunity to feed until 17 d post-emergence; its experimental age was 24 d post-emergence.

To test the possible effects of gonotrophic cycle on oviposition site choice, two groups of females of identical age but different gonotrophic cycle were compared. Females in their first gonotrophic cycle were not fed until day 17 post-emergence, and

were tested on day 20 post-emergence. Females of advanced gonotrophic cycle were in their third cycle by the time of experimentation. Specifically, a large group of females 3 d post-emergence were offered a blood-meal. Those that fully engorged were placed in a separate cage and allowed to oviposit 7 d post-emergence. They were then fed on day 9 post-emergence, and fully engorged females were removed and placed in a separate cage. These females were given access to oviposition sites on day 13 post-emergence. They were collected and given their third blood-meal on day 17 post-emergence. Those that fully engorged were tested in the large cage design on day 20 post-emergence.

Results

Null Hypothesis: *Females in the same gonotrophic cycle but separated by 2 weeks in post-emergence age do not visit idenitical oviposition sites at different frequencies.*

The numbers of oviposition sites and the number of eggs collected for females tested on day 10 post-emergence (7 d after a blood-meal) are given in Table 8.1. Females oviposited in 55.7% of the available oviposition sites, and averaged 16.7 eggs per oviposition event (oviposition event is the mean number of eggs received per ovipot that received any eggs). Females ovipositing on day 24 post-emergence (7 d after a blood-meal) oviposited in 56.0% of available oviposition sites, with a mean number of 17.3 eggs deposited per oviposition event (Table 8.1). χ^2-tests confirmed that females of different age did not oviposit in significantly different numbers of oviposition sites ($p=0.969$) so the null hypothesis stands.

Table 8.1. Comparative oviposition incidence by females separated in age by 2 weeks; no test ovipot present. Treatments sharing same letter category are not statistically different at the p=0.05 level.

	Ovipot									
	1	2	3	4	5	6	7	8	Total	Category
AGE=10 Days										a
N	24	24	24	24	24	24	24	24	192	
Oviposition Events	12	14	13	10	14	16	13	15	107	
Eggs Deposited	91	321	276	86	171	421	200	221	1787	
Eggs/Oviposition Event	7.58	22.93	21.23	8.60	12.21	26.31	15.38	14.73	16.70	
Oviposition Events/N	0.500	0.583	0.542	0.417	0.583	0.667	0.542	0.625	0.557	
AGE=24 Days										a
N	21	21	21	21	21	21	21	21	168	
Oviposition Events	9	15	11	12	13	10	10	14	94	
Eggs Deposited	67	303	181	227	298	109	164	276	1625	
Eggs/Oviposition Event	7.44	20.20	16.45	18.92	22.92	10.90	16.40	19.71	17.29	
Oviposition Events/N	0.429	0.714	0.524	0.571	0.619	0.476	0.476	0.667	0.560	

Null Hypothesis: *Egg distribution in the presence of an attractive oviposition site is not different among females in the same gonotrophic cycle but separated by 2 weeks in age.*

The treatment site (position 0) received a greater percentage of eggs than any other site when offered to females 10 days post-emergence (22.10%) (Table 8.2). Females 2 weeks older also deposited the most eggs in the treatment site (22.48%) (Table 8.2). A comparison by the χ^2-test reveals that the distribution of eggs among all ovipots did not differ significantly (p=0.778) between younger and older females, so the null hypothesis stands.

Null Hypothesis: *Females of the same age but separated by two gonotrophic cycles do not visit idenitical oviposition sites at different frequencies.*

The distribution of eggs and the average numbers of eggs deposited per oviposition event by females in their first gonotrophic cycle is illustrated in Table 8.3. Females in their first gonotrophic cycle oviposited in 53.0% of available containers, and averaged 15.75 eggs per oviposition event. By contrast, females in their third gonotrophic cycle deposited eggs in a similar number of oviposition sites (58.2%), but laid fewer eggs per oviposition event (7.73) (Table 8.3). Analysis by a χ^2-test reveals that the number of oviposition sites visited by the 2 groups of females did not differ statistically (p=0.990), so the null hypothesis was not disproven.

Table 8.2. Response of females separated in age by 2 weeks measured by proportion of eggs deposited in a treatment site containing 4th instar conspecifics at a density of 1 larva per ml. Treatments sharing same letter category are not statistically different at the p=0.05 level.

	Ovipot									
	-3	-2	-1	0	1	2	3	4	Total	Category
AGE=10 Days										a
N	22	22	22	22	22	22	22	22	176	
Oviposition Events	8	10	9	14	16	13	11	11	92	
Eggs Deposited	201	196	57	478	352	347	227	305	2163	
Eggs/Oviposition Event	25.13	19.60	6.33	34.14	22.00	26.69	20.64	27.73	23.51	
% Total Eggs in Ovipot	9.29	9.06	2.64	22.10	16.27	16.04	10.49	14.10	100.00	
Standard Error	2.83	2.53	1.36	6.75	5.06	4.52	3.08	4.18		
AGE=24 Days										a
N	18	18	18	18	18	18	18	18	144	
Oviposition Events	7	7	12	12	12	8	13	6	77	
Eggs Deposited	136	128	279	392	283	188	277	61	1744	
Eggs/Oviposition Event	19.43	18.29	23.25	32.67	23.58	23.50	21.31	10.17	22.65	
% Total Eggs in Ovipot	7.80	7.34	16.00	22.48	16.23	10.78	15.88	3.50	100.00	
Standard Error	2.41	2.09	4.83	6.55	4.97	3.07	4.72	1.36		

Table 8.3. Comparative oviposition incidence by females in 1st and 3rd gonotrophic cycles; no test ovipot present. Treatments sharing same letter category are not statistically different at the p=0.05 level.

	Ovipot									
	1	2	3	4	5	6	7	8	Total	Category
FIRST CYCLE										a
N	25	25	25	25	25	25	25	25	200	
Oviposition Events	12	13	12	16	13	14	14	12	106	
Eggs Deposited	221	225	87	346	238	141	200	211	1669	
Eggs/Oviposition Event	18.42	17.31	7.25	21.63	18.31	10.07	14.29	17.58	15.75	
Oviposition Events/N	0.480	0.520	0.480	0.640	0.520	0.560	0.560	0.480	0.530	
THIRD CYCLE										a
N	23	23	23	23	23	23	23	23	184	
Oviposition Events	14	12	14	16	15	13	10	13	107	
Eggs Deposited	149	83	71	180	142	73	36	94	828	
Eggs/Oviposition Event	10.64	6.90	5.05	11.28	9.45	5.61	3.65	7.20	7.73	
Oviposition Events/N	0.609	0.522	0.609	0.696	0.652	0.565	0.435	0.565	0.582	

Null Hypothesis: *Egg distribution in the presence of an attractive oviposition site is not different among females of the same age but separated by two gonotrophic cycles.*

Females in their first gonotrophic cycle deposited the most eggs in the treatment site; (Table 8.4) this site (ovipot 0) received an average of 18.24% of the total eggs deposited. This demonstrates a similar oviposition pattern by females in their third gonotrophic cycle; the treatment site received 20.99% of eggs. The egg distribution pattern among all ovipots did not differ significantly between females in the first and third gonotrophic cycles, according to a χ^2-test ($p=0.106$). The null hypothesis stands.

Discussion

Increasing physiological age has been demonstrated to alter the physiology and behavior of many insects (Rockstein and Miquel 1973, Stoffolano 1976). Indeed, in *Ae. aegypti* advancing age has been shown to change physiological processes (Mellink and Van Zeben 1976). As already mentioned, gonotrophic age is a distinct measurement, and may be the better one to assess the fitness of a female mosquito.

The oviposition patterns of females in the same gonotrophic cycle but 2 weeks apart in age were not significantly different. Females laid similar numbers of eggs in similar numbers of oviposition sites (Table 8.1), and responded to an attractive oviposition site in the same manner (Table 8.2), regardless of post-emergence age. This might be expected under these conditions since both groups of females had a relative fitness of zero at the onset of the experiment. Chronological age might not carry as much importance as gonotrophic age.

Table 8.4. Response of females separated in 1st and 3rd gonotrophic cycles measured by proportion of eggs deposited in a treatment site containing 4th instar conspecifics at a density of 1 larva per ml. Treatments sharing same letter category are not statistically different at the p=0.05 level.

	Ovipot									
	-3	-2	-1	0	1	2	3	4	Total	Category
FIRST CYCLE										a
N	21	21	21	21	21	21	21	21	168	
Oviposition Events	6	7	11	13	11	14	8	11	81	
Eggs Deposited	89	93	193	246	159	238	105	226	1349	
Eggs/Oviposition Event	14.83	13.29	17.55	18.92	14.45	17.00	13.13	20.55	16.65	
% Total Eggs in Ovipot	6.60	6.89	14.31	18.24	11.79	17.64	7.78	16.75	100.00	
Standard Error	1.88	2.23	4.31	5.57	3.39	5.32	2.30	5.14		
THIRD CYCLE										a
N	17	17	17	17	17	17	17	17	136	
Oviposition Events	11	9	3	14	10	6	8	6	67	
Eggs Deposited	143	87	28	186	156	94	131	61	886	
Eggs/Oviposition Event	13.00	9.67	9.33	13.29	15.60	15.67	16.38	10.17	13.22	
% Total Eggs in Ovipot	16.14	9.82	3.16	20.99	17.61	10.61	14.79	6.88	100.00	
Standard Error	4.81	2.68	1.73	6.10	5.17	3.22	4.45	2.17		

In comparing females in their first or third gonotrophic cycles, large differences in both relative fitness and physiological status (*e.g.*, the changes in levels of circulating hormones) might affect female oviposition behavior. For example, the concentration of a particular hormone may alter the sensitivity of antennal chemo-sensory receptors such that gravids that have not oviposited (nulliparous) assess oviposition site cues in a manner disctinct from gravids that have oviposited previously (parous). Gravids of differing gonotrophic age might select oviposition sites in a differential manner.

Gravids of the same post-emergence age but with one group nulliparous and the other biparous did not exhibit differences in either the number of oviposition sites receiving eggs (Table 8.3), or in the proportion of eggs deposited in a single attractive site with seven less attractive sites in proximity (Table 8.4). This suggests that females of differing parity and relative fitness assess and respond to oviposition site cues in a similar, if not identical, fashion.

These experiments indicate no changes in oviposition site selection based on differences in either senescence or gonotrophic age, at least within the parameters tested. Although the tested attractive site contained but one potential attractant, the similarity with which gravids responded to a series of identical sites suggests that the baseline response to oviposition sites does not change with increasing physiological or gonotrophic age. Mosquito programs that plan on trapping gravid *Ae. aegypti* as a control strategy need not be concerned with obtaining a population of skewed physiological or gonotrophic age.

CHAPTER 9

LIMITED RANGE ATTRACTION OF *AEDES AEGYPTI* TO OVIPOSITION SITES CONTAINING CONSPECIFIC LARVAE

Introduction

Gravid *Ae. aegypti* lay a greater proportion of eggs in oviposition sites that contain conspecific larvae than they do in corresponding control sites (Ch. 3). This positive response increases with larval instar and increasing density, up to a threshold density of 2 larvae (4th instar) per ml of water, above which the response becomes negative.

The experimental design of Corbet and Chadee (1993), upon which my large-cage protocol used to demonstrate *Ae. aegypti* preference to sites containing conspecific larvae was based, suggested using oviposition incidence as an indicator of female preference or avoidance. These authors found a direct correlation between the average proportion of eggs in sites and the average number of times those sites received eggs. The degree of attraction of a treatment could be indicated by plotting both sets of data on a bar chart. A clear, bell-shaped trend was observed for gravid oviposition when offered conspecific 3rd instar larvae as the treatment. This trend was equally apparent for both the number of eggs laid per site and the number of times a site received eggs.

This design also demonstrated a "spill-over" effect: ovipots adjacent to the treatment received a number of eggs and a number of visits intermediate between the treatment ovipot and the remaining ovipots. The authors concluded attractive olfactory compounds originating from the treatment ovipot spread through the experimental cages forming a concentration gradient. Though this gradient was highest near the treatment

oviposition site, it was sufficiently strong around the neighboring control ovipots to result in a positive directed response from gravid females.

Results from the current series of experiments found either no or very poor correlations between oviposition incidence and proportion of eggs laid. As an example, the response of gravids to a treatment site containing one 4th instar per ml shows a significantly greater proportion of eggs laid in the treatment ovipot compared to controls (Fig. 9.1). This response is not apparent when measuring oviposition incidence (Fig. 9.2). The spill-over effect noted by Corbet and Chadee (1993) is also not apparent under consideration of either set of data. The degree of discrimination seen in the work of Corbet and Chadee (1993) is not found in the current series of experiments.

The reason for such discrepancies are not readily apparent since the experimental designs are similar. The lack of spill-over effect in the current experiments suggests that the olfactory cues apparent in earlier work were not disseminating in the same fashion in the large cage design incorporated in this project. Corbet and Chadee (1993) used cages approximately 30 cm in all dimensions; this contrasts with the 1.82 m dimensions of the large cages used. The eight oviposition sites in both projects were placed equidistant from their neighbors around the periphery of the cage. In this experiment this corresponds to a distance of 50 cm between ovipots. Although the distance between ovipots in the Corbet and Chadee (1993) paper was not mentioned, if it was scaled exactly it was about 8.25 cm. Even if not scaled exactly, the distance between ovipots was quite small compared to the current experiments.

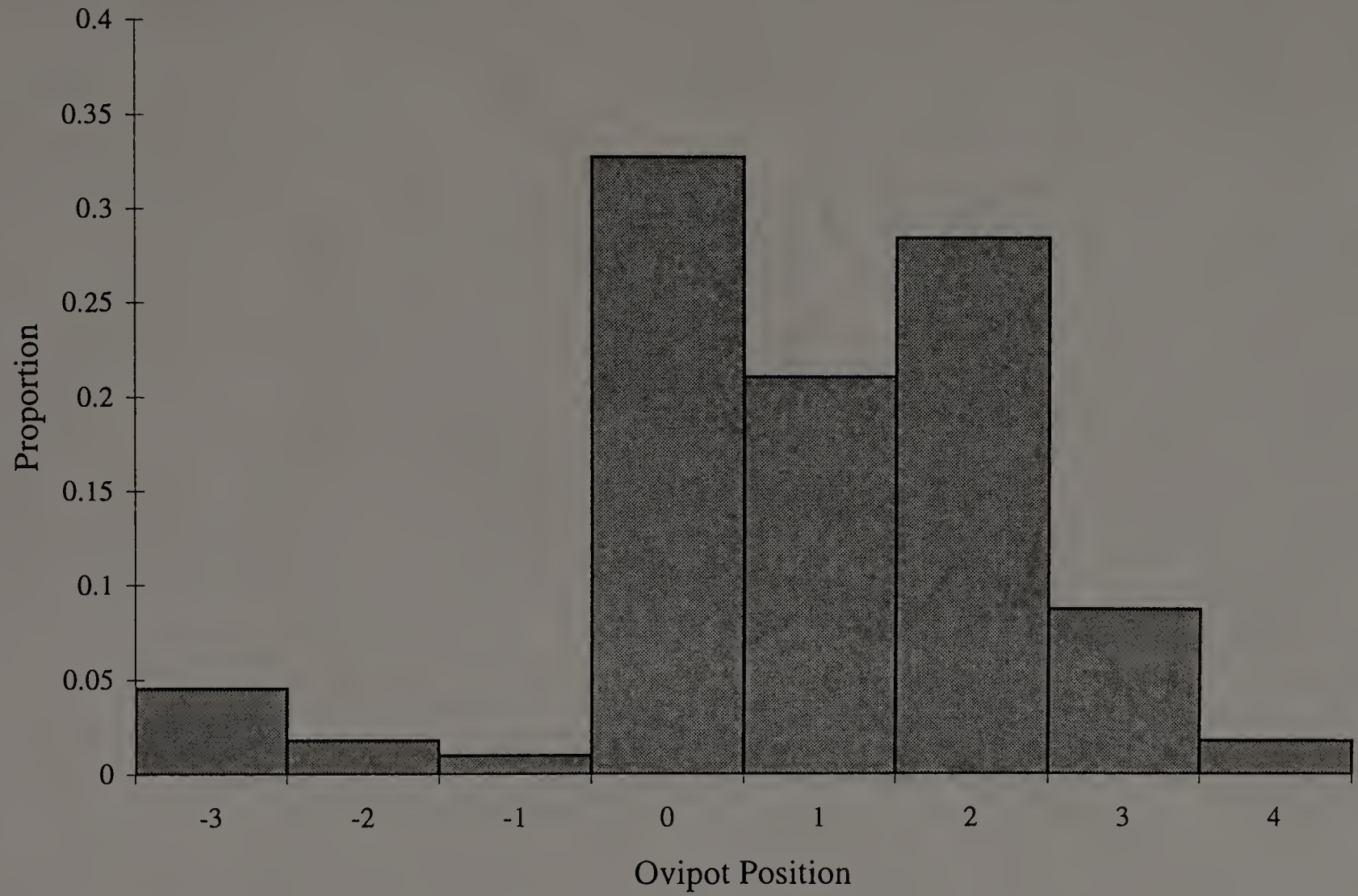

Fig. 9.1. Proportion of eggs deposited by ovipot position. The treatment ovipot (position 0) contains one 4th instar per ml water.

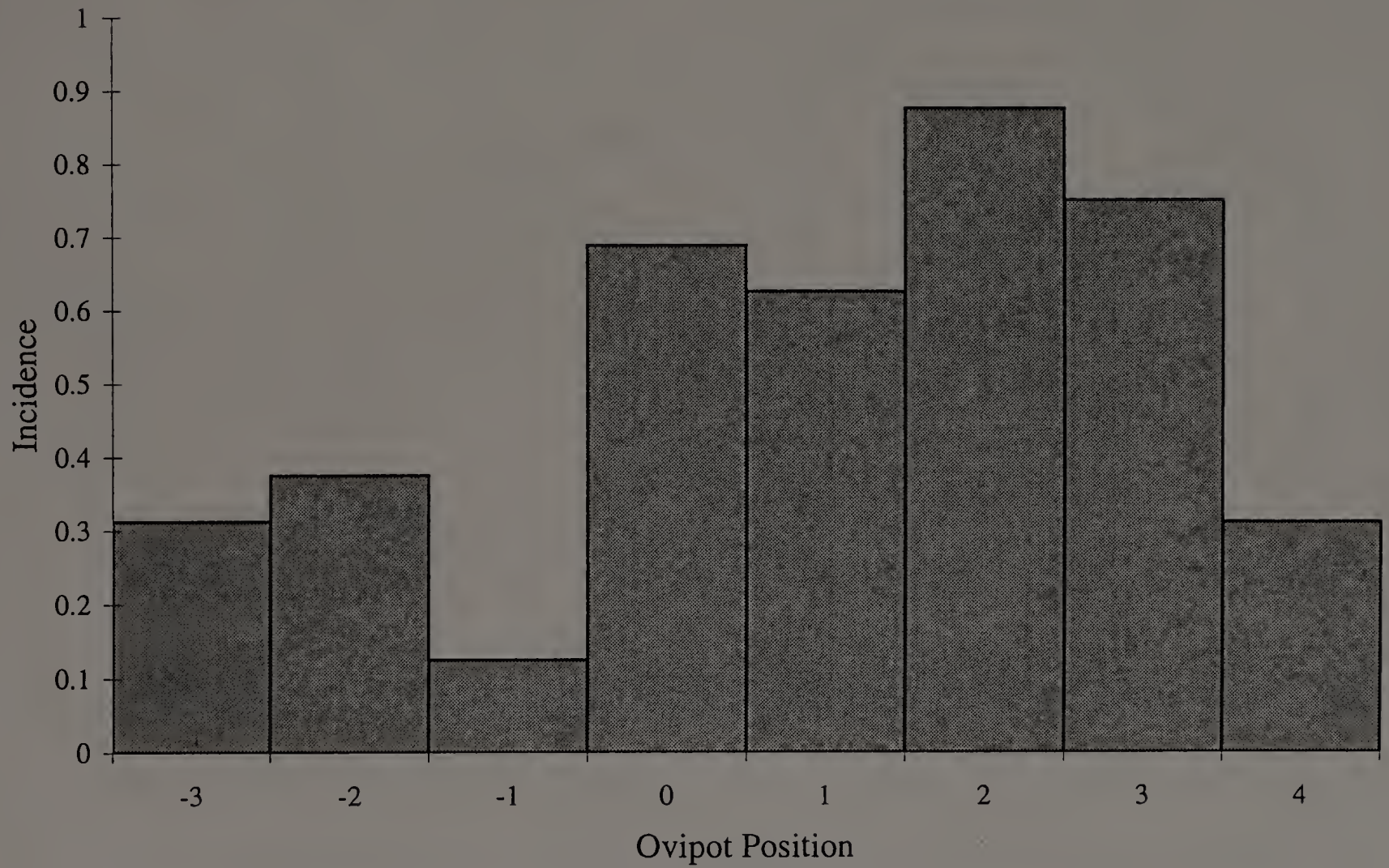

Fig. 9.2. Incidence of eggs deposited by ovipot position. The treatment ovipot (position 0) contains one 4th instar per ml water.

Olfactory cues are likely the most important cues which serve to attract gravids to sites containing conspecific immatures (Ch. 4). Olfactory cues are the only ones that might mitigate the spill-over effect observed in the work of Corbet and Chadee (1993). The distances at which thes cues operate under conditions of no or minimal wind and the way in which these cues disseminate from a point source are unknown.

The current experiments tested the range at which these olfactory cues serve to orient gravid *Ae. aegypti* to oviposition sites containing conspecific larvae at an attractive density. Several experimental designs were used to assess this orientation behavior. All took place in a wind tunnel, such that directed wind-flow and the distribution of olfactory cues are better accounted for. The first involves the trapping of gravids in one of two upwind cages (one containing a control ovipot and one containing an attractive treatment ovipot). The second experiment incorporated the observation of specific oviposition behavior in the presence of 2 oviposition sites (one control and one attractive treatment). The third experiment included trapping of gravids in one of two cages placed upwind (containing the attractive treatment ovipot) and downwind (containing the control ovipot), rather than horizontally. The distance between female release site and trapping cages were manipulated to help determine the distance at which olfactory cues operate.

Materials and Methods

Wind Tunnel Trapping

The wind tunnel (2.54x1.06 x0.70 m) was constructed of plexiglass held together by metal support beams. The total experimental volume measured 1.88 m^3. Air was

pushed through the tunnel by means of a fan connected to an air intake tube. Laminar flow was provided by passing the air through a series of two filters prior to entry into the experimental area. The first lattice-like filter was plastic and consisted of square holes measuring 1 cm across. The second filter was a fine nylon mesh (100 holes per cm^2). Air was allowed to exhaust from the cage via a rear screen (36 holes per cm^2). Air-flow in all experiments was maintained at 20 cm/sec linear windspeed.

The entire wind tunnel was housed in a separate chamber constructed of metal support beams and a double-layer of black-plastic tarp to prevent contamination from external light sources. Lighting was provided by suspended fluorescent lights, set at a 14L:10D h photoperiod regime, light phase (0600-2000 h). Temperature was maintained at 27 ± 4 °C. As with the cage experiments, RH was difficult to control, and thus experiments in which the RH fell outside the range of 65 ± 10 % are excluded from this study.

White sheets were draped over the sides of the wind tunnel to provide a uniform background for the experimental mosquitoes. Mosquitoes sense flight speed and direction by a process known as optomotor anemotaxis (Clements 1992), which involves processing cues regarding upwind flight in relation to the visual background. In an effort to provide visual landmarks which mosquitoes might use for optomotor anemotaxis, a white sheet with spray-painted matte-black (Testors #1032) lines of increasing width was placed under the plexiglass bottom of the wind tunnel. The lines increased by 1 cm in width from the rear of the cage toward the wind source. The center of each line was spaced 20 cm apart from the center of the next line.

Entrance to the wind tunnel was by means of a plexiglass door built into the side. Ten gravid females were released (between 1300-1500 h) per experiment by an external releasing mechanism. The white transfer carton containing the 10 females was placed in a styrofoam box (0.027 m^3), the front and back of which had been cut out and covered with mesh (36 holes per cm^2) to allow air flow. The carton was opened in this box, the box was placed 1.25 m downwind from the front of the trapping cages which housed the ovipots, and a wire (the other end of which was external to the tunnel) was attached to the lid of the box. The wind tunnel was sealed and the release box lid was opened by means of pulling on the wire.

For experiments involving the trapping of females, two plexiglass cages were placed at the end of the tunnel nearest the intake fan (Fig. 9.3). These cages took up the full height and width of the wind tunnel, and were 50 cm in depth. The part of the cages nearest the fan are covered with a 36 holes per cm^2 mesh. The side of the cages open to the experimental area was plexiglass with a central opening in which a mesh-covered wire frame funnel was placed. This funnel allowed mosquitoes entrance but not egress, and was porous on all surfaces to allow airflow. The opening of the funnel that entered the cage was 3.5 cm in diameter, while the tunnel-opening of the funnel measured 12 cm. The funnel-end of the cages was covered with a sheet of black cardboard, with holes cut out where the funnels were located, to provide a uniform visual backdrop for the experimental mosquitoes.

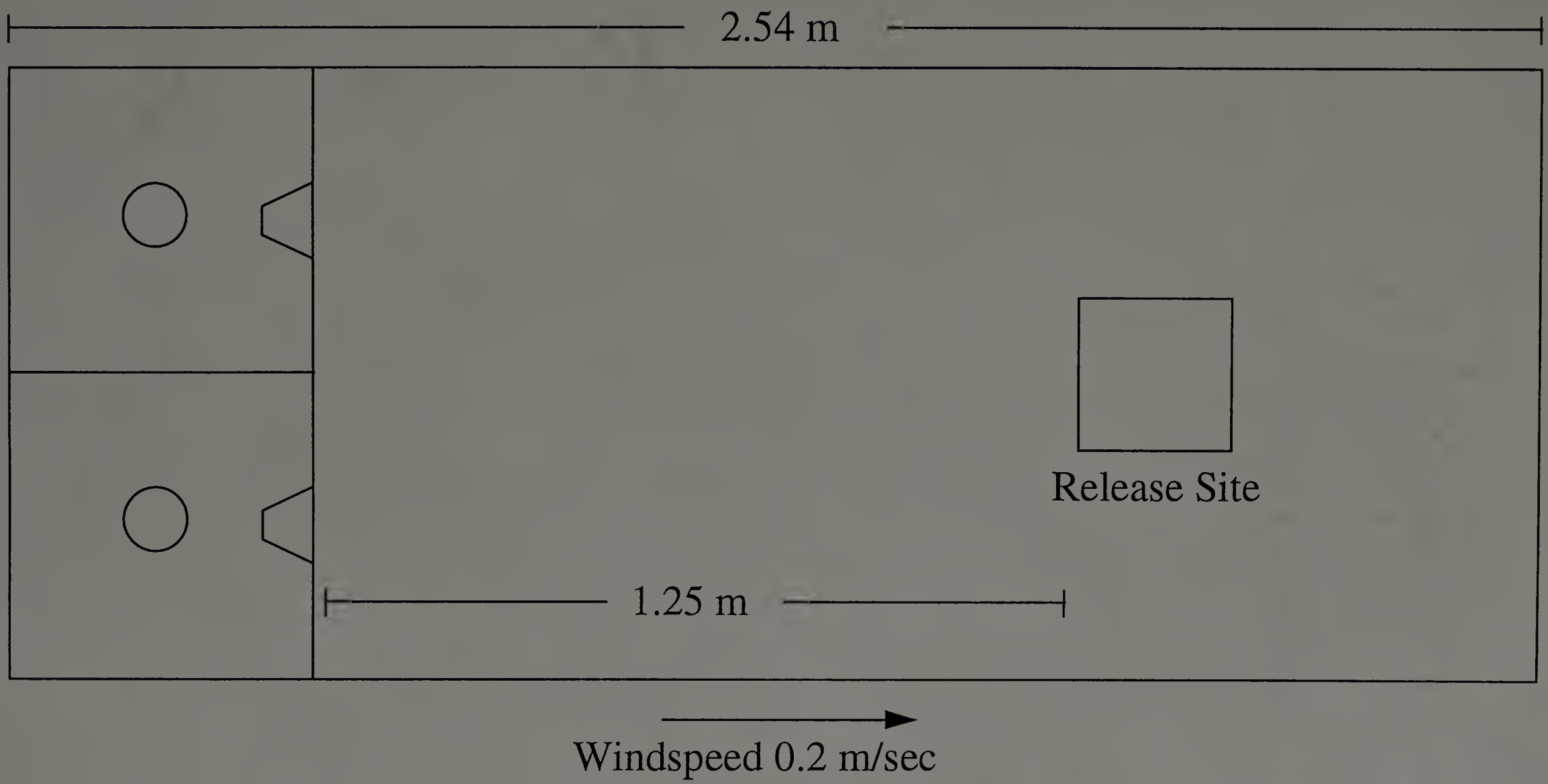

Fig. 9.3. Overhead view of the wind tunnel design. Circles represent oviposition sites located in plexiglass cages. Ovipots contained control or treatment on an alternating basis.

Inside each cage a wooden platform 24 cm high was centered, upon which the 120 ml ovipot was placed (Fig. 9.4). This placement ensured the ovipot opening was vertically level with the middle of the funnel aperture at a distance of 8 cm from the center of the ovipot to the funnel aperture (Fig. 9.4).

Preliminary experiments were performed to test the validity of this wind tunnel design. Ten females were released into the wind tunnel with the identical condition (ovipot containing 40 ml of filtered-distilled water (FDW)) in both cages. In 5 replicates, females did not select one cage over the other ($p>0.85$). This indicated that there was no position effect that might compound the analysis. To test the discriminatory power of this design a positive and negative control were offered at the same time. The positive control ovipot contained 40 ml of FDW and the negative control ovipot was dry. Females were released in batches of 10. Nearly all gravids (6 replicates) were caught in the positive control cage. The difference in numbers caught was highly significant ($p<0.01$). This showed females could discriminate between the cages based on olfactory cues alone.

The control ovipot contained the seed-paper substrate and was filled with 40 ml of FDW (one-third the volume of the 120 ml ovipot). The treatment ovipot held 4th instar *Ae. aegypti* larvae at a density of 1 per ml of FDW. Females were released in batches of 10 approximately 1.25 m downwind of the cage openings. After each 23 h trial, the mosquitoes and egg papers were collected, and the cages, ovipots and wind tunnel were cleaned in preparation for the next trial. The number of females trapped in each cage was recorded, collated and analyzed by a students t-test. Ovipots and plexiglass were handled

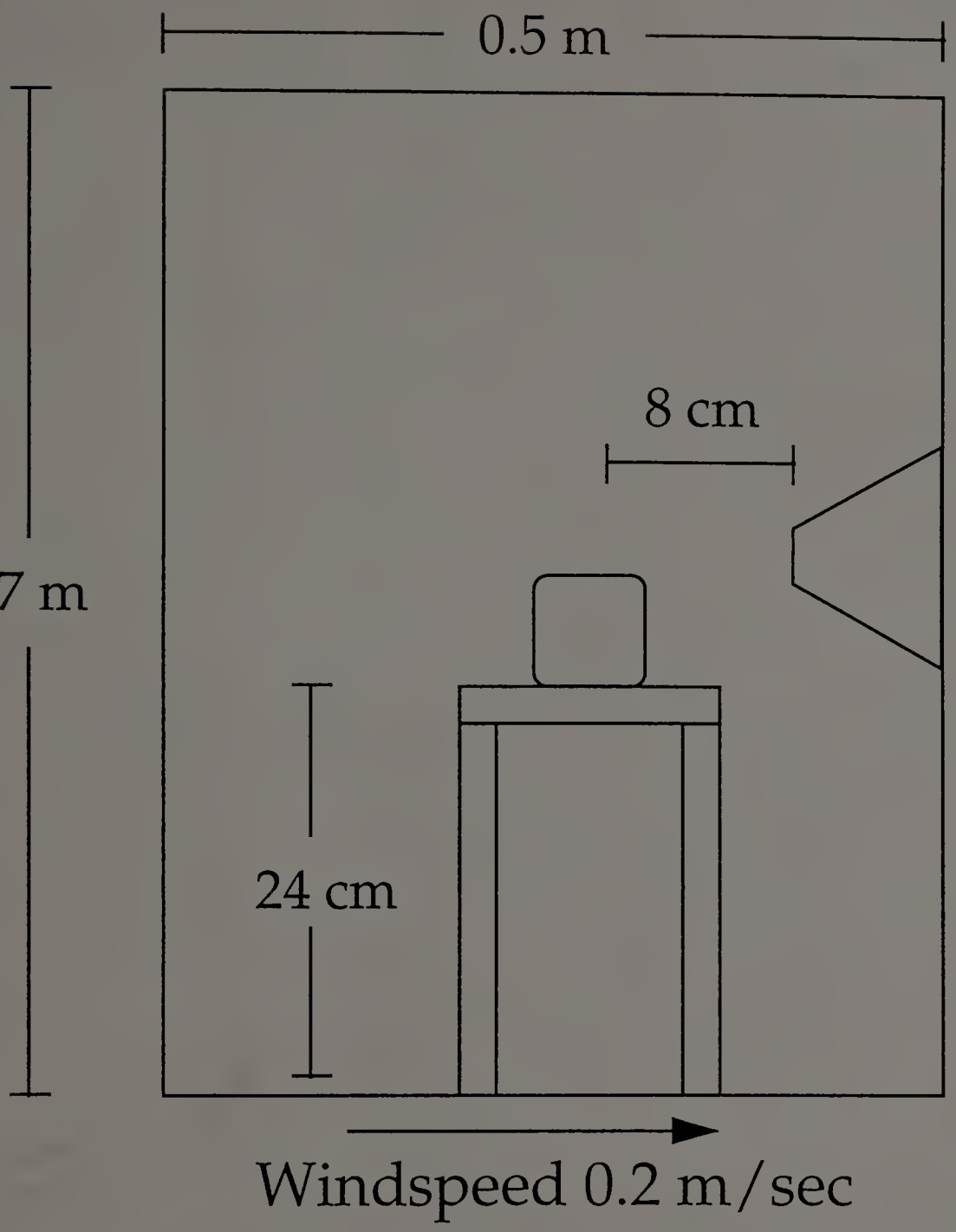

Fig. 9.4. Side view of a wind tunnel trapping cage. The glass ovipot was placed on a wooden stand such that the ovipot opening was level with the middle of the funnel, and that the middle of the ovipot was located 8 cm upwind of the nearest funnel opening.

while wearing latex gloves. Any plexiglass touched by skin was rinsed with a 70% ethanol solution, and the tunnel was "aired out" for a 10 min period.

Wind Tunnel Observations

For observational experiments, cages were removed and ovipots were placed on the floor of the wind tunnel, 1 m upwind from the release box (Fig. 9.5). Ovipots were placed 40 cm apart from each other in a horizontal line. The white sheets were removed from the sides of the wind tunnel to allow observation through the plexiglass. Each 120 ml ovipot contained seed-paper substrate and was filled with 40 ml of FDW. Experiments lasted until specific behavioral events were witnessed. Females age 6-8 d post-emergence and blood fed on day 3 post-emergence were released individually. Recordings were made of which ovipot the female first appraoched, which she first landed in, and which first received eggs. The area around an ovipot in which females had to fly before considering the flight an "approach flight" was a circle of 10 cm radius. Female selections were recorded, collated and analyzed by t-test at the $p=0.05$ level. Ovipots and plexiglass were handled while wearing latex gloves. Any plexiglass touched by skin was rinsed with a 70% ethanol solution, and the tunnel was "aired out" for a 10 min period.

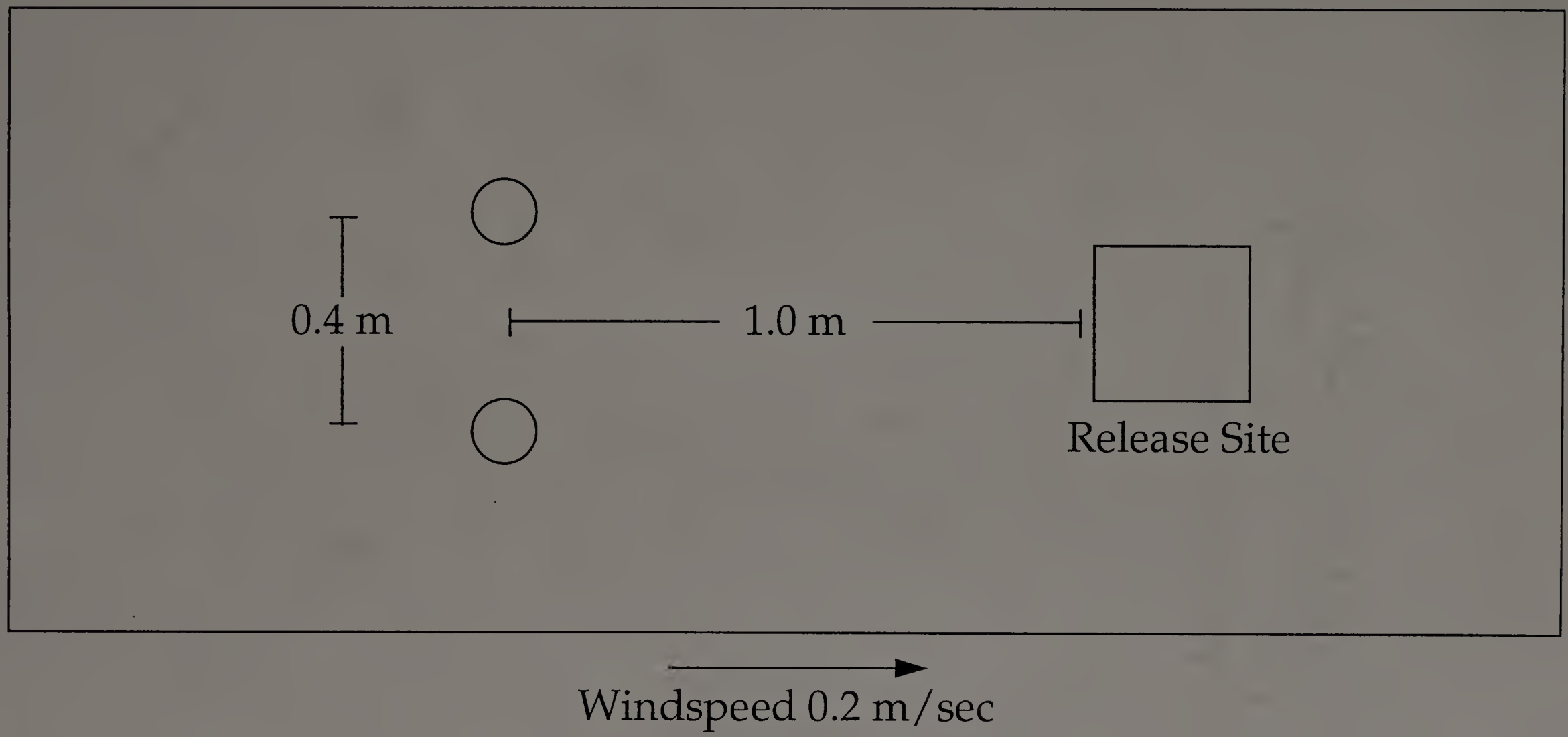

Fig. 9.5. Overhead view of wind tunnel for observation experiments. Ovipots (represented by circles) rested on the tunnel floor. Ovipots contained control or treatment on an alternating basis.

General Design of Olfactometer Experiments

The wind tunnel was used to house the olfactometer in these experiments. The olfactometer was linear in design, with treatments placed upwind and downwind of the release site. The release site was a cylindrical (9.0 cm diameter x 7.6 cm length) white ice cream carton. Individual cartons (9 cm diameter x 8.9 cm length) were placed at the ends of the release carton in varying numbers, and taped on the outside. The distal ends of these cartons were attached to either more connecting cartons or to a trapping cage (Fig. 9.6). The number of cartons placed between the release site and either the upwind or downwind cage varied between 1 and 3. All possible numerical combinations were tested, such that there could be 3 cartons between the release site and both trapping cages, or there could be 1 between the release site and the upwind cage and 2 between the realease site and the downwind cage.

Trapping cages were cylindrical in shape, with a white carton frame supporting a wire mesh (25 holes per cm^2) cone. The base of the cone was distal and the apex was proximal to the release site (Fig. 9.7). Cage lids, of identical construction, were located on the end furthest from the release site. Windflow was maintained such that the airspeed at the release site was 20 cm/sec. Entrance to the cages from the connecting cartons was via a hole cut through the wire mesh. A white cardboard disc was supported on the cage side of this hole by 3 wire stems (Fig. 9.7). This allowed mosquito entrance into a cage, but prevented mosquito egress. This was an issue in these experiments because the trapping cages were small and in preliminary trials some females flew back out. Holes

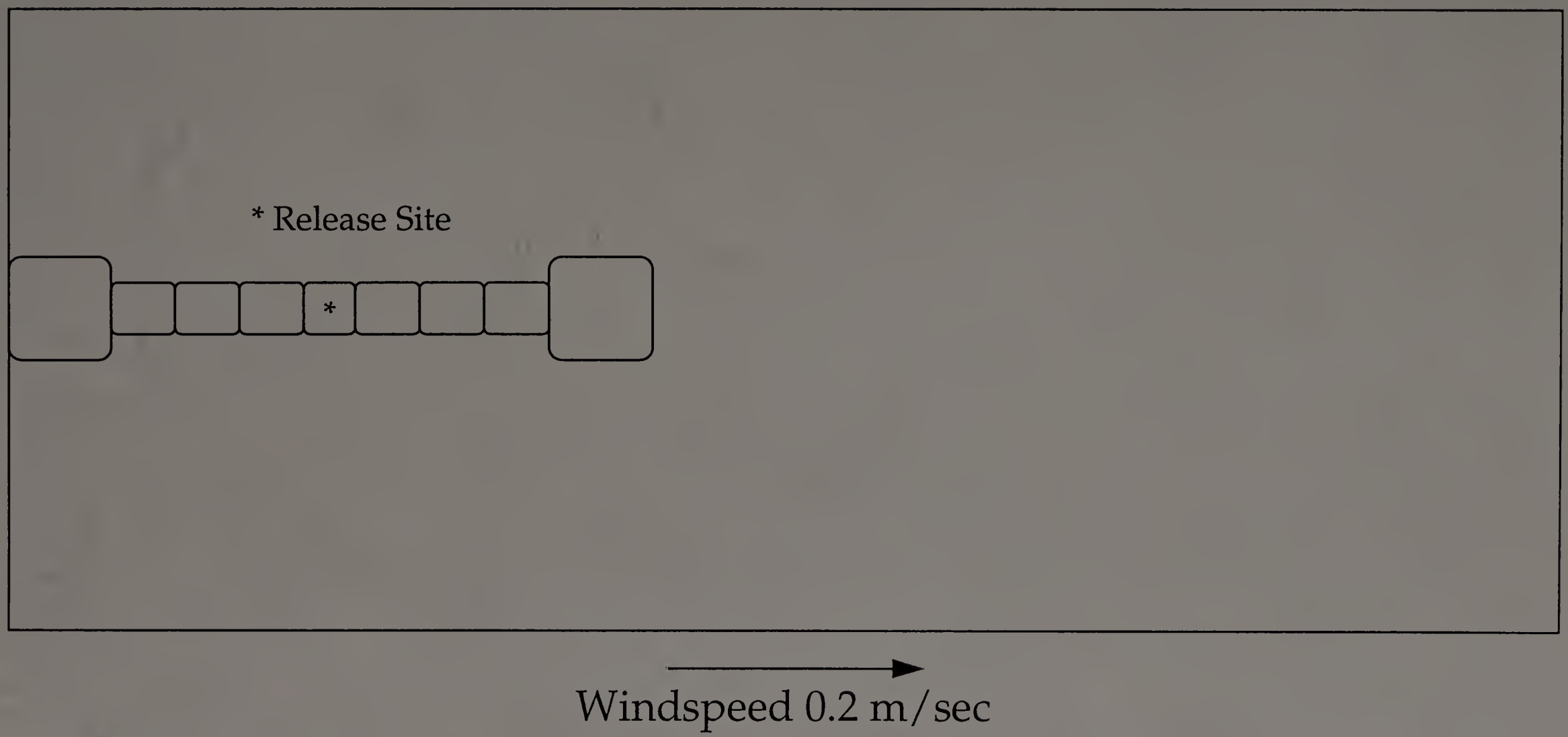

Fig. 9.6. Overhead view of olfactometer assays. Trapping cages are located at either end of the olfactometer. Between one and three cartons were placed between the release site and either cage. The entire apparatus was contained within a wind tunnel.

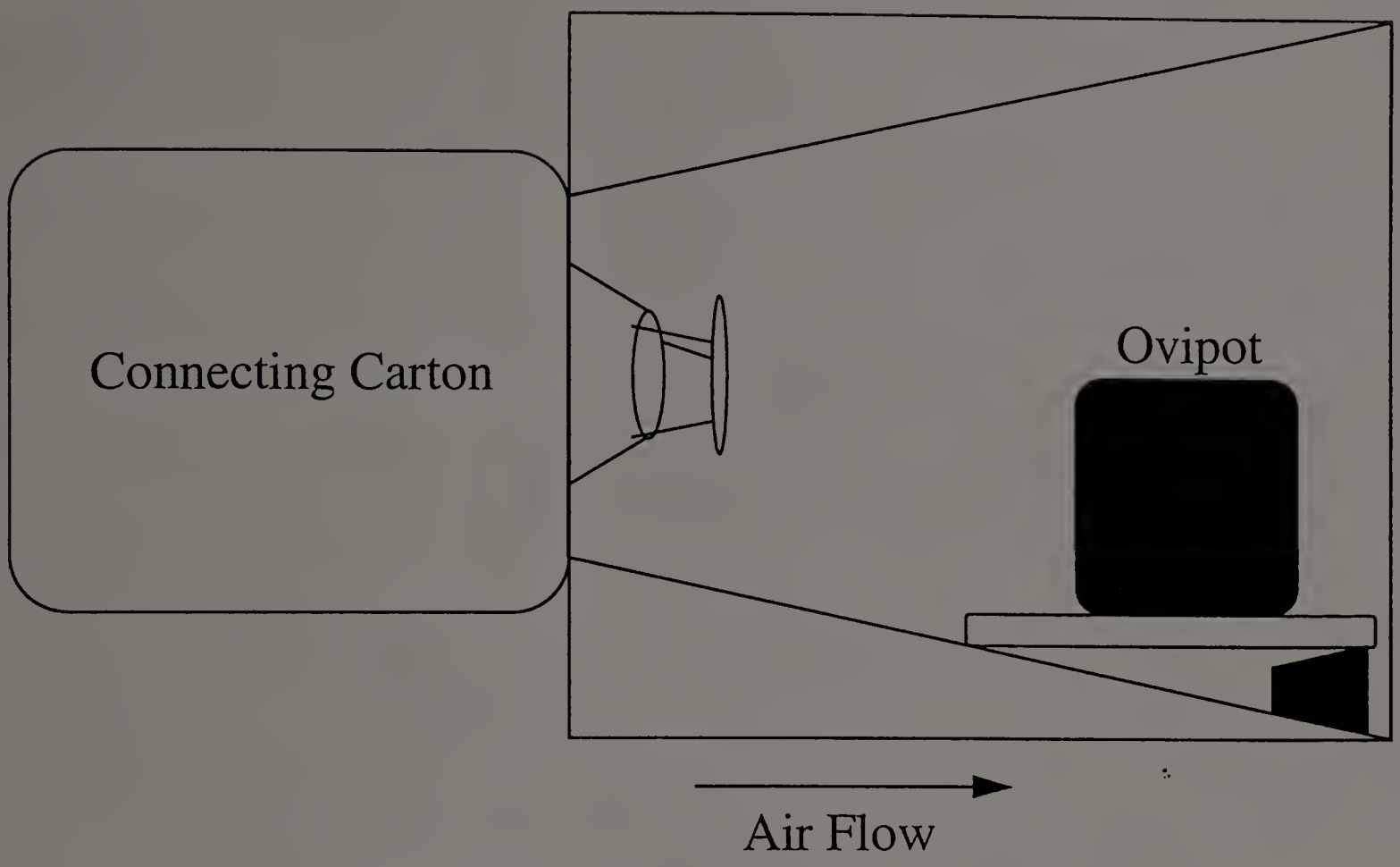

Fig. 9.7. Side view of an olfactometer trapping cage. The glass ovipot was placed on a plastic petri dish, held level with a rubber stopper. A white cardboard disc was attached to the entrance funnel to prevent mosquito egress once they had entered the cage. This cage is downwind of the release site. A cage identical in all ways except for the oviposition site treatment was located upwind of the release site.

cut into the sides of the wire mesh provided aspirator access points for collecting mosquitoes. Holes were plugged with cotton during experimentation.

At the end of each cage, distal to the release site, a black rubber stopper was glued into place, and a clear plastic petri dish was glued atop this stopper such that it formed a level surface upon which the 120 ml ovipot could be placed. Each ovipot was lined with seed-paper and 40 ml of FDW was added. This treatment ovipot was placed in the upwind cage for these experiments. Ten females age 6-8 d post-emergence and blood fed on day 3 post-emergence were placed into the central release carton, and the wind tunnel was sealed. Each trial lasted 23 h (starting and ending between 1300-1500 h), allowing time between trials for mosquito and egg paper collection, ovipot and carton cleaning, and setup of the next experiment. The number of females caught in the upwind an downwind cages were compared by a student's t-test. The number of females trapped in the upwind cage were analyzed across all distance comparisons by an analysis of variance (ANOVA).

All cage contents were handled while wearing latex gloves to prevent possible contamination.

For all experiments involving two way choices, a Student's t-test was performed on the data to be analyzed in the spreadsheet program Microsoft Excel. All such experiments were designed as two-tailed, with the null hypothesis stated as: the difference between the two treatments (measured in either number of females caught or number of eggs collected) is zero.

Results

Null Hypothesis: *Gravids do not discriminate between upwind control and more attractive treatment sites.*

Fifteen of the 100 females released did not fly into either cage: they remained in the main experimental area or the release box. Data for females entering the cages demonstrate that 54% of trapped females were caught in the treatment cage, while 46% were trapped in the control cage (Table 9.1). This difference is not significant by t-test ($p>0.55$). Preliminary experiments demonstrated that plume mixing first occured 60 cm downwind of the cage openings, and the point sources are approximately 30 cm further upwind of the cage opening. This indicates that olfactory cues derived from sites containing conspecific larvae do not orient gravids at a distances between 30 and 90 cm downwind. The null hypothesis stands.

Null Hypothesis: *On the basis of approach flight, first landing or initial oviposition, females do not discriminate between upwind control and more attractive treatment oviposition sites.*

Data in Table 9.2 demonstrate that females did not approach sites containing conspecific 4th instars at a density of 1 per ml at any greater frequency than they did to control sites containing FDW ($p>0.10$). Initial landings were distributed in a similar fashion: females first landed in either treatment or control sites with the same regularity ($p>0.75$). Initial oviposition also occured in either oviposition site with equal frequency ($p>0.40$). Gravid females do not appear to discriminate between control and treatment

Table 9.1. Comparison of the number of gravid *Ae. aegypti* caught in upwind control and attractive treatment cages in a wind tunnel. Linear wind speed is 20 cm/sec.

Parameter	4th Instar	Control
Mean	4.6	3.9
Variance	9.6	5
Observations	10	10
Pooled Variance	7.29	
df	18	
p-value (one-tail)	0.29	
p-value (two-tail)	0.57	

Table 9.2. Gravid female selection of oviposition sites based on approach flight, first landing and first oviposition. Linear wind speed is 20 cm/sec.

	Approach Flight	First Landing	First Oviposition
4th Instars	0.60	0.52	0.56
Control	0.40	0.48	0.44
Observations	25	25	25
df	48	48	48
p-value (one-tail)	0.08	0.39	0.20
p-value (two-tail)	0.16	0.78	0.41

sites based on which site they approach first, which they land in first, and which they oviposit in first). Sixty percent of females first landed in the ovipot they first approached. About 88% of females initiated oviposition in the ovipot they first landed in. This suggests that females land in a site before making the decision of whether to oviposit in that site. The null hypothesis is not disproven.

Null Hypothesis: *Females do not discriminate between an upwind treatment and a downwind control site regardless of the distance either is from the release site.*

The number of females trapped in the upwind cage separated from the release by 3 cartons and the number of females trapped in the downwind cage separated from the release site by 3 cartons was not significantly different ($p>0.65$). The number of females caught in an upwind cage containing the treatment ovipot (one 4th instar per ml) was not significanly different ($p>0.85$) across all distances tested (Fig. 9.8). Even if the treatment cage was but 9 cm upwind of the release site, and the control cage was 27 cm downwind of the release site, females flew into these cages in equal numbers. Given the distance of the ovipot from the cage opening, this corresponded to 22 cm between the upwind oviposition site and the release site, and 44 cm between the control oviposition site and the release site. The null hypothesis stands.

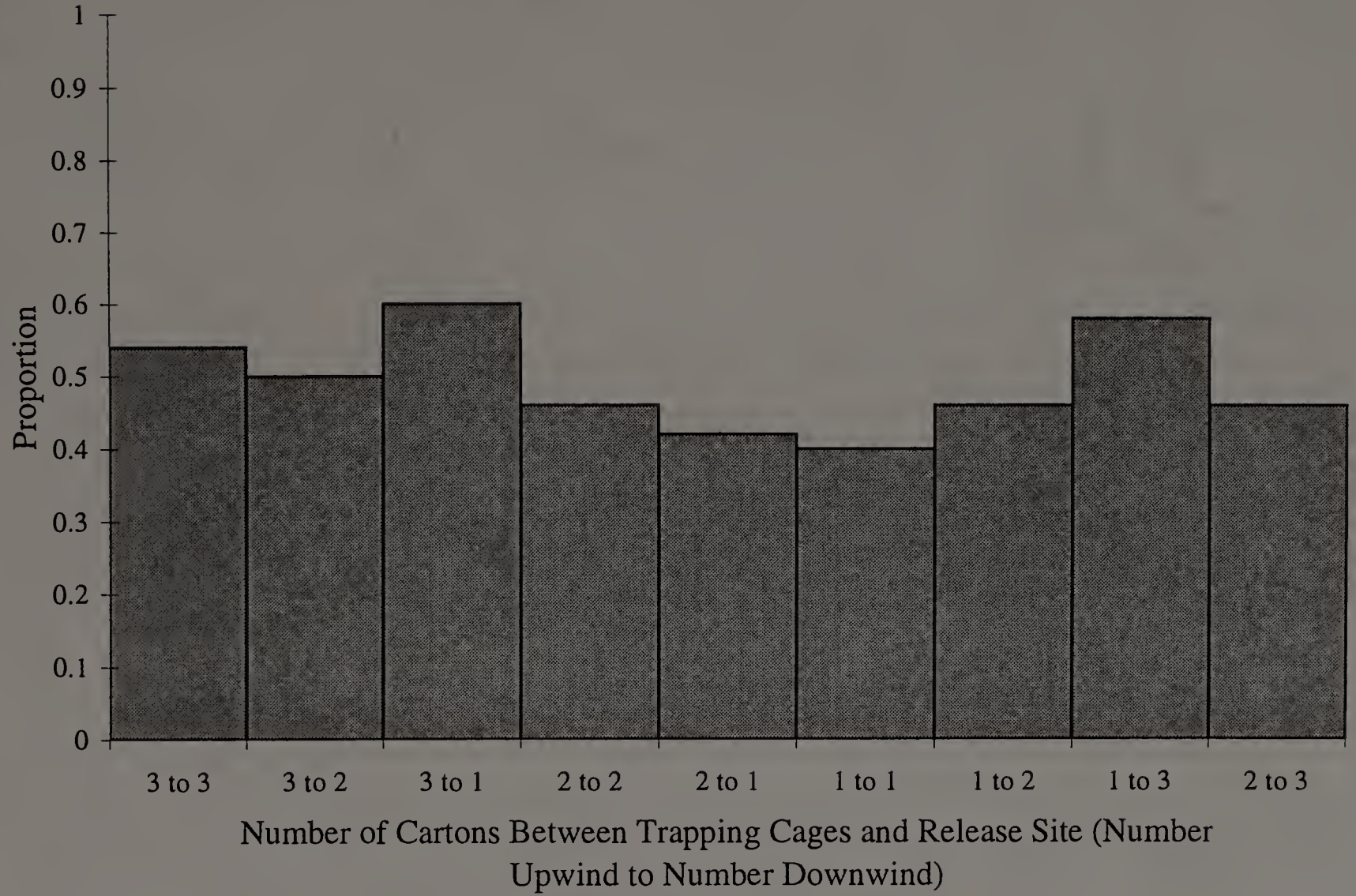

Fig. 9.8. Proportion of females trapped in the upwind cage containing ovipot with one 4th instar per ml water. Linear wind speed is 20 cm/sec.

Discussion

Olfactory and visual cues may act to orient gravid *Ae. aegypti* to oviposition sites. By providing uniform visual cues and constant windflow, the range at which gravids respond to olfactory cues from sites containing conspecific 4th instars was investigated. Based on the number of females trapped in either a treatment or a control cage, females are not able to detect conspecific larvae-associated cues at distances 1 m or greater downwind. An equal number of females were trapped in control and treatment cages upwind (Table 9.1). By isolating the oviposition sites behind uniform visual backgrounds and forcing female choice some distance away from the actual oviposition site, the only response that could be measured was long range selection of sites.

To determine female response with all cues available, the cages were removed. Only behaviors specific to initial choice were observed. Females did not distinguish between control and treatment sites upon approach flight, first landing or initial oviposition (Table 9.2). This indicates that initial choice is not based upon larval-associated cues, when the oviposition sites are 40 cm apart horizontally and 1 m upwind of the release site.

Even with an attractive treatment upwind at a shorter distance than a control site downwind, females were trapped in equal numbers in both cages. Visual cues were again blocked, so females were forced to rely entirely on their olfactory senses. This reliance did not allow for discrimination between sites. Females were not trapped in greater numbers in the upwind treatment cage with distances as short as 22 cm between the upwind source and the release site.

Taken together, these data indicate that the olfactory cues associated with conspecific 4th instars and their larval waters do not orient females to oviposition sites from a long range. The positive response females exhibited towards such sites in the large cage experiments, measured by proportion of eggs deposited, is due to female evaluation of cues at close-range. This conclusion is further supported by evidence suggesting first landing is the most likely indicator of initial oviposition.

This could explain why the spill-over effect and bell-shaped distribution evident in the work of Corbet and Chadee (1993) was not seen in the large cage experiments of this project. The ovipots of Corbet and Chadee (1993) were probably less than 10 cm from their nearest neighbors. This is well under the tested minimum range of 22 cm (at which no discrimination was found). The oviposition sites in the large cage design were placed 50 cm from neighboring ovipots. No olfactory discrimination was evident at such a range in the wind tunnel. Although olfactory cues might operate at 10 cm, allowing a spill-over or contaminatory effect of the neighboring ovipots, they would not facilitate such a spill-over in the large cage design.

Females rely on olfaction more than any other mechanism to determine the presence of conspecific larvae (Ch. 4). Yet these olfactory cues operate at a very limited range. Given that initial landing is the best indicator of first oviposition, it is possible that olfactory cues operate only within the oviposition site. If they operate in such a manner they might be better classified as oviposition stimulants rather than oviposition attractants. If stimulants, they would not be involved with female orientation to sites at

all; they would only affect female choice (to initiate oviposition or to determine how many eggs to lay) once she has reached the oviposition site.

Stimulants are generally considered chemo-tactile in nature (Dethier *et al. 1960*), and a chemo-tactile response to sites containing larvae is evident (Ch. 4). But both a chemo-tactile and an olfactory stimulant would be redundant. Discrimination of sites based largely on olfactory cues was evident in the work of Corbet and Chadee (1993); if olfactory cues were stimulants, there would be no spill-over effect into the ovipots nearest the treatment site. That some spill-over was witnessed supports the classification of larval-associated olfactory cues as close-range oviposition attractants rather than stimulants.

The limited range of attraction explains why first landing was a better indicator of initial oviposition than approach flight was. Approach flight might have been a successful indicator had the oviposition sites been placed closer together in the wind tunnel; they were 40 cm apart horizontally. Only controls containing water were offered as contrasts with the treatment of one 4th instar per ml water. Control sites were suitable for mosquito oviposition. That the majority of females that landed in either site stayed in that site indicates females select sites based primarily on suitability: each tested site me the basic needs for mosquito oviposition. Likely replacing the control with a treatment that repels females (based on olfactory cues) would have skewed the initial landings in favor of the positive treatment site.

An overall picture of gravid selection of oviposition sites containing conspecific larvae emerges. Females orient to sites based on long range visual cues (contrast or

darkness) and long range olfactory cues (presence of water). They fly towards such sites, monitoring short range olfactory cues (larval-associated) as they near the site. They land in any site deemed suitable at this point and, based on very short range olfactory cues and chemo-tactile cues, make the final determination of whether to lay eggs in that site. The number of eggs laid is likely a function of possible skip oviposition (by which females avoid laying all eggs in one site); mitigating chemo-tactile factors that might indicate how many larvae the site can support; and possibly indirect measurements of available larval resources, such as oviposition site water surface area (Ch. 7).

The differential effects of long and short range cues are demonstrated by tests of *Ae. aegypti* oviposition response to paired ovipots of differing concentrations of hay infusion (Reiter 1991). The most attractive grouping, in terms of number of eggs collected, were the 10% and 100% hay infusion pairs. Pairs of 10/10 or 100/100 infusion concentrations received fewer eggs than the 10/100 infustion pair. The 10% infusion ovipot received significantly more eggs than its 100% infusion neighbor. The combined evidence suggested that initial orientation to the pair was due to the high concentration of attractive chemicals dispersing from the 100% infusion ovipot. That the 10% infusion ovipot received more eggs indicated that the 100% infusion ovipot was somehow less attractive to the ovipositing female than the 10% infusion ovipot at close range. This supports the idea that the final assessment of site-suitability is based on close range cues, likely short-range olfactory attractants/repellents and chemo-tactile stimulants/deterrents.

APPENDIX A
SAMPLE ANALYSIS OF PROPORTION OF EGG DATA

Table A.1. Comparison of proportion of eggs laid in treatment containing 4th instars and expected distribution.

	Ovipot								
	-3	-2	-1	0	1	2	3	4	Total
LARVAE									
Eggs Deposited	115	113	31	541	462	532	321	66	2181
% Total Eggs in Ovipot	5.27	5.18	1.42	24.81	21.18	24.39	14.72	3.03	100.00
% Expected	12.50	12.50	12.50	12.50	12.50	12.50	12.50	12.50	100.00

541 eggs were laid in the treatment ovipot. This means 2181-541=1640 eggs were laid in control ovipots.

The ratio of 541:1640 is compared across treatments, in this case an expected distribution.
The ratio for the expected distribution is calculated by multiplying 2181 by 0.125
This gives us a ratio of 273:1908

Using a chi-square test thusly:

	Treatment	Not in Treatment
Larvae	541	1640
Expected	273	1908

A p-value below 0.001 results, indicating that the treatment ovipot containing larvae recevied significantly more eggs than expected.

APPENDIX B
SAMPLE ANALYSIS OF OVIPOSITION INCIDENCE WITH ALL OVIPOTS CONTROLS

Table B.1. Comparison of incidence of eggs laid among identical ovipots across gravid age.

	Ovipot								
	1	2	3	4	5	6	7	8	Total
AGE=10 Days									
N	24	24	24	24	24	24	24	24	192
Oviposition Events	12	14	13	10	14	16	13	15	107
AGE=24 Days									
N	21	21	21	21	21	21	21	21	168
Oviposition Events	9	15	11	12	13	10	10	14	94

The test statistic is the number of oviposition events to the number of non-oviposition events.

For 10 days, then, the ratio is 107:85, while for 24 days the ratio is 94:74

By chi-square analysis, the ratios of oviposition events to non-events are not significantly different across age ($p>0.05$). Thus, there are no differences among 10 and 24 day old females in terms of oviposition incidence.

APPENDIX C
SAMPLE ANALYSIS OF NEIGHBOR OVIPOT EFFECTS

Table C.1. Comparison of proportion of eggs laid in treatment containing 4th instars and spill-over effects.

	Ovipot								
	-3	-2	-1	0	1	2	3	4	Total
LARVAE									
Eggs Deposited	115	113	31	541	462	532	321	66	2181
% Total Eggs in Ovipot	5.27	5.18	1.42	24.81	21.18	24.39	14.72	3.03	100.00
% Expected	12.50	12.50	12.50	12.50	12.50	12.50	12.50	12.50	100.00

The distance from the treatment ovipot is more important in calculating the spill-over or neighbor effect than is the direction from the treatment ovipot. By combining the number of eggs deposited by distance rather than direction (thus adding eggs collected from -1 and +1) and comparing these with neighboring ovipots, any possible spill-over effects can be recognized.

As the position 0 and 4 ovipots do not have counterparts, the numbers of eggs they receive is multiplied by 2.
The ratio described in Appendix A is again calculated. This ratio is compared among neighboring ovipots. To give an overall picture of the lack of a neighboring effect, all ratios are presented here together.

	Ovipot Placement				
	Position 1	Position 2	Position 3	Position 4	Treatment
Eggs in ovipot	493	645	436	132	1082
Expected	557.6	557.6	557.6	557.6	557.6
Eggs in other ovipots	2295	2143	2352	2656	1706
Expected	2230.4	2230.4	2230.4	2230.4	2230.4

Ultimately, the treatment ovipot receives more eggs than any other position control, but the next highest amount is the combined egg total from ovipots 2 away from the treatment. Next comes position 1, than 3, than 4. There is no apparent neighbor effect.

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