

# EXPERIMENTAL TEST OF THE INFLUENCE OF AQUATIC MACROPHYTE COVER ON THE SURVIVAL OF ANOPHELES LARVAE

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**ABSTRACT.** Emergent or submergent macrophytes can enhance the survival of *Anopheles* larvae by providing favorable microhabitat and refuge from predation. The relationships among the amount of aquatic macrophyte cover, the density of *Gambusia affinis*, and survivorship of *Anopheles freeborni* larvae were tested experimentally. Larval survivorship was positively related to amount of plant cover and negatively related to *Gambusia* density. Larval survivorship was lowest in treatments with low plant cover and high fish density (0–15% survivorship) and highest in treatments with high plant cover and no fish (41–76% survivorship). Similar patterns of larval survivorship were found in enclosure experiments conducted at 3 sites, using 3 species of aquatic macrophytes (*Potamogeton pectinatus*, *Myriophyllum aquaticum* and *M. spicatum*).

## INTRODUCTION

Many species of anopheline mosquitoes prefer habitats with well-developed beds of submergent, floating-leaf or emergent aquatic macrophytes (Hess and Hall 1943, Aitken 1945, Service 1980). In North America, larvae of most *Anopheles* spp. are typically found in sites with abundant rooted aquatic vegetation, such as in rice fields and adjacent irrigation ditches (Stone 1953, Hoy et al. 1971), freshwater marshes (Collins et al. 1983, Balling and Resh 1984, Walker et al. 1988) and the vegetated margins or littoral zones of reservoirs (Hess and Hall 1943, Rozeboom and Hess 1944).

Within a given site, *Anopheles* eggs and larvae tend to be positively associated with aquatic vegetation. Early research by Hess and coworkers in the Tennessee Valley (Hess and Hall 1943, Rozeboom and Hess 1944) documented the positive relationship between densities of eggs and larvae of *An. quadrimaculatus* Say and the amount of plant cover present. In particular, they found that the amount of intersection line (i.e., "the line of intersection between three interfaces, water-air, water-plant and plant-air" Hess and Hall 1943), which they quantified as the intersection line value ("the number of meters of intersection line per square meter of water surface"), was a useful correlate of the density of immature anophelines. Recent studies have further documented the correlation between *Anopheles* larval density and plant cover or intersection line value (Balling and Resh 1984, Walker et al. 1988, Collins and Resh 1989).

Many investigators have suggested that aquatic vegetation promotes anopheline production because it provides a refuge for larvae from predators, such as *Gambusia affinis* (Bailey and Baerg 1966, Bay 1967, Collins et al. 1983). Alternative, but not mutually exclusive, hypotheses for the beneficial effects of aquatic vegetation have also been proposed, including enhanced food resources in vegetated regions

versus open water, shelter from physical disturbance, and favorable conditions for oviposition (Hess and Hall 1943, Collins et al. 1985, Orr and Resh 1987, Walker et al. 1988, Collins and Resh 1989). However, critical experimental tests of these hypotheses, especially under field conditions, are lacking.

This study has been designed: 1) to quantify experimentally the relationships among the amount of aquatic vegetation, predator (*G. affinis*) density and survivorship of *Anopheles* larvae and 2) to assess the generality of these relationships by experimentally testing the survivorship of *An. freeborni* Aitken larvae using different levels of aquatic plant cover, different *G. affinis* densities and different species of macrophytes. Our primary hypothesis ( $H_1$ ) is that larval survivorship is positively correlated with amount of plant cover and negatively correlated with predator density. Two additional hypotheses were tested. The microhabitat hypothesis ( $H_2$ ) specifies that aquatic plant cover creates favorable microhabitat conditions (shelter from adverse weather and enhanced food resources) for *Anopheles* larvae that are independent of its potential role in providing refuge from predation. A key prediction of this hypothesis is that there is a positive correlation between larval survivorship and amount of plant cover in the absence of predators. Our third hypothesis, the refuge hypothesis ( $H_3$ ), predicts a positive relationship between amount of plant cover and larval survivorship only when predators are present.

## METHODS

**Study sites:** The first study site, Coyote Hills Marsh, is a 6 ha man-made freshwater marsh located in Coyote Hills Regional Park (Fremont, Alameda Co., CA). From late spring through early autumn, the interior region (1–2 m deep) of the marsh was dominated by dense monospecific beds of sago pondweed, *Potamogeton pec-*

*tinatus* Linn. (Balling and Resh 1984). The extent of pondweed canopy has been greatly reduced in recent years because of grazing by crayfish (Feminella and Resh 1989). The intertwining stems and leaves of pondweed create a lush floating mat of vegetation at the water surface that has a high amount of intersection line and a complex submergent canopy. The pondweed canopy provides habitat for a variety of invertebrates (Lamberti and Resh 1984), including larvae of 2 species of *Anopheles* mosquitoes, *An. freeborni* and *An. occidentalis* Dyar and Knab (Collins et al. 1983, Balling and Resh 1984). A more detailed description of this site is given in Collins et al. (1983).

The second site, Searsville Lake, is a 40 ha complex of open water and wetlands located on Stanford University's Jasper Ridge Biological Preserve (San Mateo Co., CA). Our study was conducted in the inner marsh, a 4.5 ha, shallow (2.5 m maximum depth) basin at the southwestern end of the lake. The interior region of the marsh is dominated by dense monospecific beds of parrotfeather, *Myriophyllum aquaticum* (Vell.) Verdc. (= *M. brasiliense* Camb.). Parrotfeather produces submergent stems that typically rise vertically from the bottom sediments to within 10–20 cm of the water surface, where they may spread horizontally for 1 m or more before terminating in vertical stem apices that typically rise 5–30 cm above the water surface (Sutton 1985). This growth pattern produces a dense floating mat of stems and leaves, with moderate to high levels of intersection line and a complex subsurface canopy structure that provides habitat for anopheline larvae and a variety of other invertebrates (see Table 10 in Otieno 1977).<sup>1</sup> The dominant species of *Anopheles* at the site was previously identified as *An. freeborni* but has recently been diagnosed as *An. hermsi* Barr and Guptavanij (G. Fritz, personal communication). A detailed description of this site is given in Smith (1963).<sup>2</sup>

Our third study site was a small pond (0.1 ha, 2 m maximum depth) located in an abandoned gravel pit just west of Livermore, CA (Alameda Co.). During mid to late summer the surface of the pond is typically covered by a thick canopy of Eurasian watermilfoil, *Myriophyllum spicatum* Linn., with only scattered patches of other

macrophytes (*P. pectinatus* and *Elodea canadensis* Michx.) present. Eurasian watermilfoil is a submergent plant with loosely twining stems, the apices of which emerge only slightly (1 cm) above the water surface. This type of plant architecture typically creates a moderate amount of intersection line and a moderate degree of structural complexity in the subsurface canopy. The study pond typically supports dense larval populations of *Anopheles* spp. (primarily *An. franciscanus* McCracken with some *An. freeborni* also present) during summer and early autumn.

*Experimental design:* The effects of *Gambusia* density and macrophyte cover on the survival of *Anopheles* larvae were examined for each plant species in a series of enclosure experiments. A factorial design combining 3 (for *P. pectinatus*) or 4 (for both *Myriophyllum* spp.) levels of *Gambusia* density with 3 levels of plant cover provided a total of 8 or 12 treatments for each experiment. Each treatment was replicated 4 times. Enclosures were cylindrical cages (60 cm diam × 122 cm high; surface area 0.25 m<sup>2</sup>) that enclosed a complete column of water, which extended from bottom sediments to the water surface. Enclosure walls were made of vertical panels of 6 mil polyethylene sheeting and fiberglass window screen (1-mm mesh) sewn together and positioned over a cylindrical frame of welded-wire fencing. The 2 screen panels (each 30-cm wide) allowed exchange of water and small invertebrates between enclosures and the surrounding macrophyte beds. When in position, the enclosures were embedded approximately 10 cm into the sediment. The enclosure walls were adjusted so that they extended 5–10 cm above the water surface, thereby preventing fish from jumping into or out of the enclosures. The enclosure walls also reduced the amount of surface disturbance caused by wind. The upper 20 cm of each screen panel was covered by a band of polyethylene sheeting to prevent emigration of surface-dwelling *Anopheles* larvae.

Preliminary studies showed no substantial differences in temperature or dissolved oxygen vertical profiles within enclosures of this design compared to the surrounding undisturbed macrophyte bed.

Enclosures were installed in fully developed monospecific beds of *P. pectinatus*, *M. aquaticum*, and *M. spicatum* (located in water 35–40, 80–85 and 85–90 cm deep, respectively) approximately 2 weeks prior to experimentation. All mosquito larvae and fish (and more rarely, notonectids and belostomatids) found in the enclosures were removed by repeated netting. Treatments were assigned randomly to enclosures, and stems of each macrophyte species were cut

<sup>1</sup> Otieno, W. A. 1977. The pathology and host-parasite relationship of entomogenous nematode *Romanomermis culicivora* Ross and Smith (Mermithidae) in a mosquito host *Culex pipiens* Say (Culicidae): its application in integrated control. PhD dissertation, University of California, Berkeley.

<sup>2</sup> Smith, F. W., Jr. 1963. A comparison of the fish species composition of Searsville Reservoir, 1950–51 and 1957–58. MA thesis, Stanford University.

approximately 25 cm below the water surface and removed as necessary to achieve the desired levels of plant canopy. All plant stems were shaken vigorously before removal from the enclosure to dislodge epiphytic invertebrates. The highest naturally occurring level of cover observed for each plant was considered to be 100% cover. The high cover experimental treatments approximated the amount and structure of surface canopy and the above ground plant biomass (approximately 4.9, 7.3 and 2.4 kg m<sup>-2</sup> for *P. pectinatus*, *M. aquaticum* and *M. spicatum*, respectively) present in natural macrophyte beds with 100% cover. Medium (33 or 50%) and low (0 or 10%) cover treatments corresponded to levels of canopy structure and cover observed in macrophyte beds with moderate to sparse or no surface canopy. Thus, the cover treatments represented the full range of actual conditions (0–100% cover) found at each site. The fish densities used in experiments were well within the range of *Gambusia* densities observed at the study sites.

Enclosures were left undisturbed for 10–12 days to allow for recovery from the initial disturbance caused by installation and plant removal. Medium-sized *Gambusia* (35–40 mm standard length for *P. pectinatus* and 25–30 mm for both *Myriophyllum* spp.) were then added at the desired densities (0, 1, 2 or 8 fish per enclosure) and allowed to acclimate to the enclosures for 24 h. Experiments began on August 23, 1986, for *P. pectinatus*, August 20, 1987, for *M. aquaticum* and September 2, 1987, for *M. spicatum*; 100 first instar *Anopheles freeborni* larvae that had hatched in the laboratory within the previous 24–48 h were added to each enclosure to begin each experiment. All larvae used were offspring of field collected adults. Preliminary observations indicated that within 15 min after addition to enclosures most larvae had stopped moving and were entrained within menisci associated with the intersection line created by plants or the enclosure walls. No predation by *Gambusia* was observed during this initial entrainment period. Each experiment ran for 10 days, at which time most surviving larvae were in the fourth instar (a few pupae and third instar larvae also were present). Surviving larvae and pupae were isolated by depressing the floating plant canopy; surfacing larvae and pupae were then collected with a fine mesh net. This procedure was repeated until 5 consecutive sweeps yielded no additional *Anopheles* larvae or pupae.

**Data analysis:** Survivorship (S) was calculated as the percent of the initial number of larvae that survived to the end of the experiment. Since the initial number of larvae was 100 in all experiments, percent survivorship equals the number of larvae surviving. S was used in tests of

hypotheses H<sub>1</sub> and H<sub>2</sub>. The refuge hypothesis (H<sub>3</sub>) predicts that predation on anopheline larvae will decrease as plant cover increases. An estimate of predation rate is thus required to test this hypothesis. Total larval mortality (M = 100 - S) is not a good estimate of actual predation rate in this case, however, because substantial mortality occurred in all control (0 fish) treatments, especially at low cover levels (Fig. 1). To account for mortality due to predation we used the following equation:

$$P = (M - C)/(100 - C)$$

where P = proportion of available larvae killed by fish, M = total mortality and C = mortality due to factors other than fish predation (no. of dead larvae in the appropriate control treatment). Three values of C were calculated for each experiment, one for each experimental cover level. P was then calculated for each replicate of the treatments containing fish, using the appropriate value of C in the calculations. The refuge hypothesis (H<sub>3</sub>) prediction was then tested using P.

The means and standard errors for S and P shown in Figs. 1 and 2 were calculated for untransformed data. All statistical tests (2-way analysis of variance (ANOVA) and Duncan multiple range tests (DMRT)) were conducted using angular transformations (Zar 1974) of the proportion of larvae surviving (S/100) or the proportion of available larvae killed (P).

Data and preliminary statistical analysis for experiment 1 were presented in Orr and Resh (1987) and are reproduced here (Fig. 1A and Table 1), along with additional data and statistical analysis (Fig. 2A and Table 2), to facilitate comparison with experiments 2 and 3.

## RESULTS

The pattern of *Anopheles* larval survivorship was similar in each of the 3 experiments (Fig. 1). Survivorship of *An. freeborni* larvae was significantly affected by both *Gambusia* density and amount of plant cover in each experiment (Table 1). As predicted by H<sub>1</sub>, plant cover was positively correlated, and fish density negatively correlated, with larval survivorship. In each experiment, survivorship was highest in the high cover, no fish treatment; in contrast, survivorship was lowest in the low cover, high fish treatment (Fig. 1). Larval survivorship tended, in general, to be highest for *P. pectinatus* (experiment 1) and lowest for *M. spicatum* (experiment 3) (Fig. 1).

*Anopheles* larval survivorship tended to be negatively correlated with *Gambusia* density for each plant species and level of plant cover (Fig. 1). For example, the treatments without fish

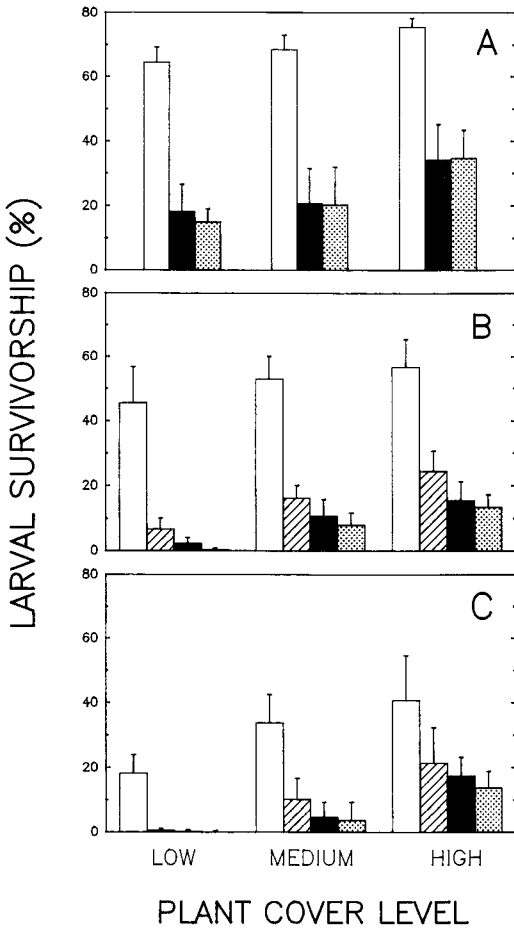


Fig. 1. The influence of amount of aquatic macrophyte cover and *Gambusia* density on the mean percent of *Anopheles* larvae surviving to the end of the experiment. Vertical lines indicate 2 SE. A. Experiment 1 -*Potamogeton pectinatus*, B. experiment 2 -*Myriophyllum aquaticum*, and C. experiment 3 -*Myriophyllum spicatum*. Fish density treatments are 0 fish (open bars), 1 fish (diagonal bars), 2 fish (solid bars), and 8 fish (stippled bars). Experiment 1 lacked the 1 fish treatment.

always had significantly more larvae surviving than did any of the treatments with fish (DMRT,  $P < 0.05$ ). An increase from 1 to 8 fish per enclosure within a given cover level always resulted in decreased larval survivorship, although the difference was statistically significant in only 4 of the 6 comparisons (DMRT,  $P < 0.05$  for low, medium and high cover in experiment 2, and for medium cover in experiment 3). Smaller increases in fish density (from 1 to 2 or from 2 to 8 fish per enclosure) also resulted in increased mortality, although the change was usually not statistically significant. For example, the difference in larval survivorship between 2 and 8 fish per enclosure was significant in only

1 of the 9 possible comparisons (DMRT,  $P < 0.05$  for low cover in experiment 2).

For a given density of fish, larval survivorship was positively related to amount of plant cover (Fig. 1). This trend resulted in significant differences between low and high cover treatments for 10 of 11 comparisons (DMRT,  $P < 0.05$ ), with the 0 fish treatments in experiment 1 (*P. pectinatus*) being the exception. The trend toward increased survival with increasing levels of plant cover in the absence of fish (comparison of 0 fish treatments) supports  $H_2$ , the microhabitat hypothesis. The lack of a significant effect for *P. pectinatus* might be due to the low cover treatment in experiment 1 containing

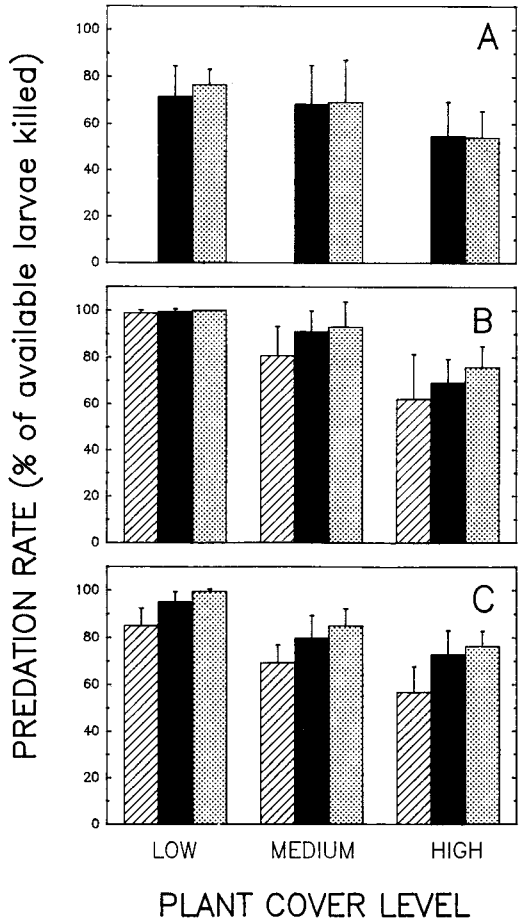


Fig. 2. The influence of amount of aquatic macrophyte cover and *Gambusia* density on the mean predation rate (proportion of available larvae killed) on *Anopheles* larvae. Vertical lines indicate 2 SE. A. Experiment 1 -*Potamogeton pectinatus*, B. experiment 2 -*Myriophyllum aquaticum*, and C. experiment 3 -*Myriophyllum spicatum*. Fish density treatments are as in Fig. 1.

Table 1. Summary statistics (F-statistics with degrees of freedom and P-values) from 2-way analysis of variance on the proportion of *Anopheles freeborni* larvae surviving to the end of each experiment. Angular transformation of the proportion of larvae surviving was used in the analysis.

Experiment 1: <i>Potamogeton pectinatus</i>		
Fish density	$F_{2,27} = 102.31$	$P < 0.001$
Plant cover	$F_{2,27} = 11.55$	$P < 0.001$
Interaction	$F_{4,27} = 0.41$	$P = 0.797$
Experiment 2: <i>Myriophyllum aquaticum</i>		
Fish density	$F_{3,36} = 144.99$	$P < 0.001$
Plant cover	$F_{2,36} = 45.05$	$P < 0.001$
Interaction	$F_{6,36} = 1.90$	$P = 0.108$
Experiment 3: <i>Myriophyllum spicatum</i>		
Fish density	$F_{3,36} = 37.96$	$P < 0.001$
Plant cover	$F_{2,36} = 48.11$	$P < 0.001$
Interaction	$F_{6,36} = 0.96$	$P = 0.466$

Table 2. Summary statistics from 2-way analysis of variance on the rate of predation on *Anopheles freeborni* larvae (i.e., proportion of available larvae killed by *Gambusia*, see text). Data were angularly transformed for analysis.

Experiment 1: <i>Potamogeton pectinatus</i>		
Fish density	$F_{1,18} = 0.09$	$P = 0.763$
Plant cover	$F_{2,18} = 4.33$	$P = 0.029$
Interaction	$F_{2,18} = 0.08$	$P = 0.921$
Experiment 2: <i>Myriophyllum aquaticum</i>		
Fish density	$F_{2,27} = 19.50$	$P < 0.001$
Plant cover	$F_{2,27} = 45.56$	$P < 0.001$
Interaction	$F_{4,27} = 0.91$	$P = 0.475$
Experiment 3: <i>Myriophyllum spicatum</i>		
Fish density	$F_{2,27} = 3.49$	$P = 0.045$
Plant cover	$F_{2,27} = 40.50$	$P < 0.001$
Interaction	$F_{4,27} = 0.43$	$P = 0.786$

10% cover instead of 0% cover, as in the other two experiments. Results of additional experiments with *P. pectinatus* using 0% instead of 10% cover for the low cover level (Orr and Resh 1987) are consistent with the results of experiments 2 and 3 in this study and provide additional evidence that the microhabitat hypothesis applies to *P. pectinatus* as well as to the two species of *Myriophyllum*.

Predation rate, P, was negatively correlated with cover level, as predicted by H<sub>3</sub>, the refuge hypothesis. P also tended to increase as fish density increased, although the cover effect was stronger than the fish density effect (Fig. 2, and compare F-values in Table 2). Cover level was highly significant in all 3 experiments, while fish density was statistically significant in only 2 of the 3 experiments (Table 2). The lack of a statistically significant effect of fish density with *P. pectinatus* might be due to the absence of the 1 fish per enclosure treatments in experiment 1. In the other 2 experiments the influence of fish density was much more pronounced, especially among the low fish densities (1 vs. 2 fish per enclosure).

## DISCUSSION

The results of this study clearly demonstrate that aquatic macrophyte cover enhances the survival of *Anopheles* larvae by creating favorable microhabitat and providing refuge from predation by *Gambusia*. As we had initially hypothesized (Orr and Resh 1987, and H<sub>1</sub> of this study), larval survivorship was positively related to amount of plant cover and negatively related to *Gambusia* density. Larval survivorship was lowest in treatments with no plant cover and high fish density, and highest in treatments with high plant cover and no fish. The generality of this relationship is suggested by the similarity in the pattern of larval survivorship in the 3 experiments, despite species-specific differences in plant canopy structure and site-specific differences in various environmental factors.

Several mechanisms may act in conjunction to produce the benefits associated with submerged and emergent macrophytes. First, the physical structure provided by the intertwining stems and leaves found in the surface canopy of many macrophytes creates a refuge from predation by

*Gambusia* (Balling and Resh 1984, Collins et al. 1983, Orr and Resh 1987). The trend toward higher survivorship with increasing levels of macrophyte cover indicates that macrophyte surface canopy reduces the effectiveness of *Gambusia* as a biological control agent for *An. freeborni* larvae. This supports the refuge hypothesis ( $H_3$ ). A laboratory study by Curtin et al. (1984) demonstrated that predation rates on *An. occidentalis* larvae decrease when *P. pectinatus* cover is increased. Our study shows that this pattern also occurs under more natural conditions, and extends this finding to two additional macrophyte species. Our results also suggest that increasing *Gambusia* density beyond a certain threshold (between 2 to 8 fish per enclosure, = 8 to 32 fish  $m^{-2}$ , in our experiment) is unlikely to produce a significant increase in larval mortality when macrophyte cover is abundant. This effect might be an artifact of the relatively small scale of our enclosures, but it is consistent with conditions frequently observed in the field. For example, anopheline larval populations in densely vegetated habitats often reach maximum densities in late summer at the same time that *Gambusia* abundance peaks. This is the common pattern at our study sites: we have measured larval densities in excess of 30 per dip when local *Gambusia* density exceeded 100 fish  $m^{-2}$ .

The value of aquatic plant cover as a refuge from predators of anopheline larvae will be influenced by the structural complexity of the plant canopy. The amount of intersection line produced and the degree of complexity below the water surface are 2 components of plant architecture that should influence the effectiveness of macrophyte cover in reducing rates of predation on *Anopheles* larvae. Preliminary investigations by Collins et al. (1988) into the relationship between intersection line value (ILV) and predation rate demonstrated that predation rates do decrease as ILV increases. They found that increasing ILV reduced predation on *Anopheles* larvae by 2 types of predators: *Gambusia*, which is primarily a visual predator, and 2 species of *Notonecta*, which rely primarily upon mechanoreception to detect prey. They concluded that a major advantage of the intersection line as anopheline microhabitat is the concealment of larvae from predators. The meniscus associated with the intersection line appears to interfere with both visual and mechanical detection of prey. Further research is needed to determine the relative importance of ILV and subsurface structural complexity in reducing predation rates on anopheline larvae.

Rates of predation by *Gambusia* on *Anopheles* larvae might also be affected by indirect factors. For example, alternative prey are often more

abundant in densely vegetated regions than in adjacent regions of sparse vegetation or open water (Gerking 1962, Collins et al. 1983). Increased availability of alternative prey in dense vegetation should tend to reduce predation rates on anopheline larvae. Dense vegetation might also reduce the availability of alternative prey via the refuge effect, as has been shown for chironomid larvae (Diehl 1988).

A second major benefit of plant cover, independent of its role in providing refuge from predation, is the creation of favorable microhabitat for anopheline larvae. For example, microbial food resources for filter-feeding *Anopheles* larvae may be richest in the surface film and intersection line associated with aquatic vegetation, both in terms of amount of food per unit of water volume (Hess and Hall 1943, Walker et al. 1988) and in terms of dietary composition or quality (R. Dadd, personal communication). Plant canopy may also provide protection from physical disturbance, such as water turbulence caused by wind and rain, and affect water temperature through thermal stratification and shading (Hess and Hall 1943, Collins et al. 1985, Orr and Resh 1987). Our results support the microhabitat hypothesis ( $H_2$ ) in that for all 3 macrophyte species tested, higher levels of surface canopy favored increased larval survivorship even when *Gambusia* were absent. We did not test directly the relative importance of food enhancement versus protection from physical disturbance. It seems likely, however, that the benefits of plant cover in our experiments were more the result of enhanced food quality or quantity than of protection from physical disturbance because enclosure walls minimized surface disturbance.

The influence of macrophytes on *Anopheles* oviposition could also account for positive associations between macrophyte surface cover and egg or larval densities, such as those found by Hess and Hall (1943), Balling and Resh (1984), Walker et al. (1988) and Collins and Resh (1989). Recent field experiments indicate that *An. hermsi* females selectively oviposit in specific types of vegetation, and at specific cover levels within beds of preferred macrophyte species (Orr and Resh, unpublished data). The interaction of oviposition and subsequent mortality undoubtedly governs the larval distribution patterns we see in nature.

We have experimentally demonstrated the relationship between aquatic macrophyte cover and *Anopheles* larval survivorship. Our findings show that macrophytes create favorable microhabitat and provide a partial refuge from predation for anopheline larvae. All 3 of our hypotheses were supported; this indicates that the interplay between plant cover and predator den-

sity has an important role in regulating anopheline larval abundance. Further research is needed to elucidate fully the mechanisms underlying the beneficial effects of aquatic plant cover on the survival of *Anopheles* larvae. An improved understanding of the mechanisms responsible for the patterns we have observed will allow us to predict which macrophyte species provide habitat conditions most conducive to outbreaks of anopheline mosquitoes.

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