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Density-Dependent Development of *Anopheles gambiae* (Diptera: Culicidae) Larvae in Artificial Habitats

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ABSTRACT The growth and development of *Anopheles gambiae* Giles larvae were studied in artificial habitats in western Kenya. Larvae responded to increasing densities by extending their development time and by emerging as smaller adults, although survival was not significantly affected. Addition of nutrients in the form of cow dung collected near the study site had no impact on larval growth and development. Regression analysis showed that female development time increased by 0.020 d and female dry mass decreased by 0.74 μg with each additional larva. By fitting the data to the pupation window model, the estimated minimum dry mass to achieve pupation was 0.130 mg and the estimated minimum time to pupation was 5 d. The most likely food source for *An. gambiae* larvae was algal growth, which was significantly reduced by the presence of larvae. Bacterial densities were not significantly affected by the presence of larvae although total bacteria counts were lower at the higher densities indicating they may provide a secondary food source when algal resources are depleted. Similarly, the levels of nitrogen and phosphorus in the habitats were not significantly affected by the presence of larvae although there was evidence of decreasing nitrogen levels occurring with increasing larval densities suggesting that nitrogen may be a limiting resource in the larval environment. The data indicate that competition within the larval environment may indirectly regulate *An. gambiae* populations by reducing adult body size, which may in turn reduce adult survivorship and fecundity. The potential impact of density-dependent interactions among *An. gambiae* larvae on the transmission of *Plasmodium falciparum* is discussed.

KEY WORDS *Anopheles gambiae*, larvae, density-dependence, algae, bacteria, nutrients

Anopheles gambiae GILES is the most important vector of human malaria in sub-Saharan Africa. Production of adults of this species occurs in small, temporary, sunlit, turbid pools of water. Habitats are often created by human or animal activity wherein larvae are found in small depressions such as foot or hoof prints, the edges of bore holes and burrow pits, roadside puddles formed by tire tracks, irrigation ditches and other artificial bodies of water (Gillies and DeMeillon 1968, White et al. 1972, Charlwood and Edoh 1996, Minakawa et al. 1999, Gimnig et al. 2001). Recent studies (Minakawa et al. 1999, Gimnig et al. 2001) showed that small size, the presence of turbid water and algae, and the absence of emergent vegetation were associated with the presence of *An. gambiae* larvae. However, these studies were descriptive surveys that did not determine the adult productivity of the habitats observed.

The number and productivity of larval habitats ultimately determines the density of adults. Conditions of larval development also affect adult body size (Lyimo et al. 1992, Koella and Lyimo 1996), which can influence adult survival (Hawley 1985b, Ameneshewa and Service 1996) and vector competence (Paulson and Hawley 1991, Nasci and Mitchell 1994). In container habitats of *Aedes aegypti* (L.) and *Aedes sierrensis* (Ludlow), biotic processes such as intraspecific competition have been linked to the dynamics of adult populations and overall population regulation processes, through effects on fecundity (Hawley 1985a) and survival (Dye 1984). However, despite the confined nature of *An. gambiae* larval habitats, there is little information about the effects of density-dependent processes such as larval crowding or larval competition for food on *An. gambiae.* In laboratory studies under conditions of extreme larval crowding and defined larval diets, larval development is slowed, survivorship is reduced, and adult body size is decreased as larval density increases (Schneider et al. 2000). The

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only field studies of *An. gambiae* complex larval dynamics are those of Service (1973, 1977) on *Anopheles arabiensis* Patton. However, those studies were conducted in rice fields where *An. gambiae* was rare or absent.

A critical factor that is often ignored in the study of larval growth and development is the quantity and quality of food sources available to larvae. Bacteria are abundant in the treehole habitats of *Aedes triseriatus* (Say) (Walker et al. 1991) and the marsh habitats of *Anopheles quadrimaculatus* Say (Walker and Merritt 1993, Smith et al. 1998). Gut content analyses have shown that bacteria are common in the food bolus of both species (Wallace and Merritt 1999) and feeding by *Ae. triseriatus* has been shown to reduce bacterial density (Walker et al. 1991) and alter the microbial community structure (Kaufman et al. 1999). These observations indicate that bacteria serve as the primary food source for larvae, converting carbon and inorganic nutrients into forms that can be assimilated by larvae. However, nothing is known about the microbial community of *An. gambiae* habitats and how the microbial community contributes to the growth and development of larval mosquitoes.

We conducted the following studies to examine whether density-dependent larval development occurs, and how it affects survivorship, development time, and body size of emergent adults. We also began investigations into the availability of bulk nutrients and the microbial community that occupy the habitats of larval *An. gambiae.*

Materials and Methods

Artificial Habitats. This study was conducted in artificial habitats constructed at the Vector Biology Control and Research Center of the Kenya Medical Research Institute near Kisumu, in western Kenya. Forty-five artificial habitats were formed in plastic wash basins (35 cm diameter, 13 cm deep). Five liters of local "black cotton" soil were placed into each basin and mixed with 2–3 liters of water. Mud was packed into the basins, and then dried in the sun, to replicate natural soil-lined habitats found in this area (Gimnig et al. 2001). The habitats were set in the ground inside an enclosure constructed to keep out animals. The enclosure had a roof constructed of corrugated metal that was removed each morning to expose the habitats to sunlight and replaced each evening to prevent rain from falling into the habitats. Three liters of rainwater or deionized water was added on the first day of each experiment and an additional liter was added the next day and water level marked. Most of the water was absorbed into the soil; the average surface area and volume of water available to larvae in the artificial habitats was 600 cm^2 and 1.0 liter. On the day the habitats were initially flooded, eggs were hatched and larvae counted into cups, and then later distributed to the habitats at the desired densities. The eggs were mixed Asembo and Kisumu strains that had originally been colonized from wild mosquitoes collected within 50 km of the study site. Screens were set over each habitat to prevent predators or feral mosquitoes from ovipositing in the habitats. On each day following, water was added to replenish the amount lost to evaporation.

Experimental Design. Two experiments were conducted. The first experiment employed a 2×2 factorial design. Habitats were randomized to receive 20 or 60 larvae per habitat. Half of the habitats at each density received 1 g of partially dried and ground cow dung as a food supplement whereas the other half received no dung. There were nine replicates for each treatment combination. An additional nine habitats were flooded with water but not seeded with larvae to serve as controls for the microbial analyses. Four of these received 1 g of cow dung. In the second experiment, habitats were randomly assigned 20, 50, 100, 150, or 200 larvae per habitat. No habitat received nutrient supplemention in this experiment. The day before each experiment began, habitats were flooded with 3 liters of water. The next day, an additional liter of water was added to each habitat and newly hatched *An. gambiae* were counted and distributed to each habitat at the appropriate densities. Twice daily, habitats were checked visually for presence of pupae, and these were collected, counted, and held in cups to allow for emergence and separation of adults by sex. All adults were killed by freezing and desiccated over anhydrous calcium sulfate for dry mass determinations (0.001 mg sensitivity on a Cahn electrobalance, Cahn Instruments, Cerritos, CA). Dried adult females that emerged from some replicate artificial habitats of each larval density in experiment 2 were pooled and their total lipid content assayed spectrophotometrically using vanillin-phosphoric acid reagent (Van Handel 1985).

Chemical and Microbial Characterizations. On day 0 and day 5 of the first experiment, a series of water samples were taken for water chemistry and microbiologic analyses. Surface microlayer (0–1 mm) samples were collected from the central portion of each habitat using a syringe and 16-gauge needle. For algal and bacterial counts, the water samples were preserved with formalin (final concentration in sample 3–5% formaldehyde) and refrigerated until analysis. Water samples for chlorophyll analysis were collected independently using the above method and filtered on site through glass fiber filters (nominal pore size 1 μm). Filters were then kept frozen until analysis. Because of time and equipment constraints, chlorophyll *a* samples were taken from treatments with 0 or 60 larvae only. Subsurface water samples for nitrogen and phosphorous analysis were collected from 1 to 2 cm below the surface microlayer with syringe and needle, and stored frozen until analysis.

Bacterial abundance was measured using direct counting methods with epifluorescent microscopy and DAPI (4′6-diamidino-2-phenylindole) staining procedures (Porter and Feig 1980). A minimum of 200 cells/filter were counted at 1,600×. Rod, cocci, and filament subgroups of total bacteria also were counted, with filaments being defined as cells or chains of cells exceeding 9 μm long. For algal counts, samples were

Table 1. Average survivorship, development time, and adult mass of *An. gambiae* grown as larvae in artificial habitats in experiment 1

	20 larvae		60 larvae	
	No nutrients	Nutrients added	No nutrients	Nutrients added
Replicates	9	8	9	9
Proportion surviving	0.606 (0.045)	0.494 (0.095)	0.535 (0.050)	0.600 (0.050)
Male development time, d	8.4 (0.51)	7.9 (0.28)	9.9 (0.27)	10.4 (0.29)
Male mass, mg	0.245 (0.010)	0.239 (0.014)	0.222 (0.010)	0.214 (0.007)
Female development time, d	8.5 (0.44)	8.1 (0.31)	9.9 (0.26)	10.3 (0.33)
Female mass, mg	0.264 (0.009)	0.291 (0.025)	0.235 (0.009)	0.224 (0.005)
Total biomass, mg	3.111 (0.237)	2.492 (0.494)	7.259 (0.664)	7.806 (0.582)

Standard errors are in parentheses.

concentrated by settling. Then, algae, other microeukaryotes, and cyanobacteria were counted using inverted light microscopy at 400× (Wetzel and Likens 1991). Predominant microeukaryotic subgroups were determined after initial examination and were defined as "small" green algae (single celled, <20 μm diameter or length), "large" green algae (colonial forms and/or exceeding 20 μm diameter or length), "euglenoids" (*Euglena*-type organisms), and "other" (encompassing any nongreen algal forms, ciliated and flagellated heterotrophic protists, amoeba tests, and unidentified organisms).

Chlorophyll *a* content of filter-collected particulates was determined fluorimetrically after overnight extraction in 95% ethanol (Welschmeyer 1994). Total phosphorous (P) was determined using persulfate oxidation techniques on unfiltered samples. In this procedure, all phosphorous moieties present are converted to phosphate (Menzel and Corwin 1965) followed by subsequent colorimetric assay of total phosphate (Murphy and Riley 1962). Total nitrogen (N) was determined using persulfate oxidation of all nitrogen moieties to nitrate followed by subsequent analysis of total nitrate using second derivative spectroscopy (Crumpton et al. 1992, Bachmann and Canfield, Jr. 1996).

Statistical Analysis. For experiment 1, the effects of larval density and nutrient supplementation on survivorship, development time and adult mass were analyzed by two-way analysis of variance (ANOVA) using the GLM procedure in SAS (SAS Institute, 1990). The effects of larval density and nutrient supplementation on microbial densities and on the availability of N and P were analyzed similarly using SYSTAT (Wilkinson 1989). For experiment 2, the effect of larval density on survivorship, development time and adult mass was analyzed by regression using the GLM procedure in SAS. The relationship between development time to, and body mass at, metamorphosis for larvae of *Aedes* mosquitoes has been described by a curvilinear relationship, the so-called pupation window model, where body mass decreases with increasing development time (Gilpin and McClelland 1979, Carpenter 1984, Walker et al. 1997). It is not known whether a similar relationship exists for *An. gambiae.* Therefore, data of mean development time and mean female mass from experiments 1 and 2 were fitted to the pupation window model using least squares, nonlinear regression with the NLIN procedure of SAS, following the methods described in Walker et al. (1997). The fitted form of the equation was adapted from an equation for a hyperbola (Carpenter 1984) as follows:

$$mass = [(time*b) + a*(b - c)]/(time - a)$$

where a is a development time minimum, b is a body mass minimum, c is the degree of sharpness of the bend in the hyperbola, and *time* and *mass* are parameters estimated from experimental data. Initial estimates used as input for parameters a and b for the fitting procedure were set within the range of the minimum to average values for the observed data.

Results

In experiment 1, the density of larvae significantly affected body mass and development time of males and females, but did not affect survival (Tables 1 and 2). The time to pupation was longer by 2.0 d (19.9%) for males and 1.8 d (17.5%) for females with initial conditions of 60 larvae per habitat compared with 20 larvae per habitat. Male and female larvae pupated as early as day 5 of the experiment; the latest pupation

Table 2. Summary of ANOVA for effects of density and nutrient addition on *An. gambiae* growth and survival in experiment 1

	Error mean square	*F*-statistics			R^2
		Density	Nutrients	Density × Nutrients	
Survivorship	0.033	0.09	0.15	2.09	0.068
Male development time	1.109	30.9***	0.05	1.46	0.514
Male mass	0.001	5.68*	0.49	0.00	0.176
Female development time	1.031	26.2***	0.00	1.60	0.470
Female mass	0.002	12.7**	0.37	1.99	0.320
Total biomass	2.382	82.0***	0.00	1.24	0.727

Degrees of freedom for error terms were 30 for male development time and male mass. Degrees of freedom for error terms were 31 for all other comparisons; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

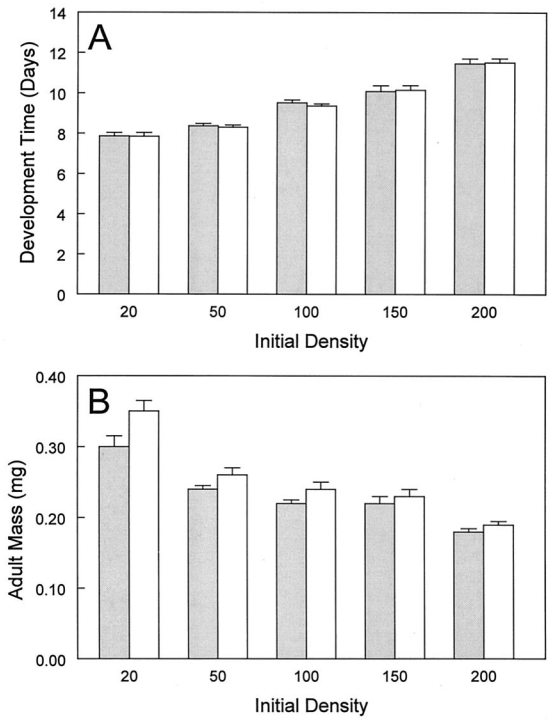

Fig. 1. Effect of increasing larval densities on *An. gambiae* growth and development in experiment 2. (A) Development time from egg to adult for males (gray bars) and females (open bars). (B) Average individual dry mass of adult males (gray bars) and females (open bars). Vertical lines represent the standard errors of the means.

events occurred on day 12. Overall, adult males weighed 0.024 mg (9.9%) more and adult females 0.048 mg (17.3%) more under initial conditions of 20 larvae per habitat compared with adults emerging from initial conditions of 60 larvae per habitat. Total biomass of adults produced was significantly higher in habitats with an initial density of 60 larvae. The addition of cow dung did not have a significant effect on survivorship, development time, adult mass or total biomass. There were no significant interactions between larval density and the addition of cow dung.

In experiment 2, male and female body mass declined, and development time increased, as larval density increased (Fig. 1; results of statistical tests are shown in Table 3). Overall, adult males weighed 0.12 mg (40.0%) more and adult females 0.16 mg (45.7%) more under initial conditions of 20 larvae per habitat compared with adults emerging from initial conditions of 200 larvae per habitat. The time to pupation was longer by 3.6 d (31.4%) for males and 3.7 d (31.9%) for females with initial conditions of 200 larvae per habitat compared with 20 larvae per habitat. By linear regression analysis, female body mass decreased 0.74 μg and female development time increased 0.020 d for each additional larva. Further, female lipid content decreased by 0.078 μg for each additional larva, but the decrease was not significant when expressed as percentage of body mass (Table 3). Although there was a trend toward decreasing survival with increasing larval density, the effect was not statistically significant (Table 3). Overall survival per habitat ranged from 45.0 to 91.0%. The initial density of larvae significantly affected total biomass. Average total biomass of adults produced increased from 4.19 mg in habitats with initial densities of 20 larvae to a maximum of 20.35 mg in habitats with initial densities of 150 larvae. Average total biomass in habitats with an initial density of 200 larvae was 20.31 mg. The water temperature in the habitats was measured in a subset of habitats every 2 h from 0800 to 1600 hours each day. Temperature varied considerably throughout the day in both experiments from an average low of 24.6°C at 0800 hours to an average high of 36.0°C at 1400 hours.

Data of adult female body masses and days to pupation were fitted to the pupation window model, and a scatter plot of the data with the fitted curve was graphed (Fig. 2). The estimated minimum mass to achieve pupation was 0.130 mg (95% CL, 0.105–0.154 mg). The estimated minimum development time was 5 d (there was no 95% confidence interval; the estimate was precise). The estimated parameter describing the curvature of the fitted parabola was 0.20 (95% confidence interval, 0.17–0.23). The fit of the curve as indicated by the R^2 was 0.38. Male development data were not fitted.

From initial conditions to 5 d after larval addition, total bacteria in the surface layer of the habitats declined in abundance, however, neither nutrient addition nor larval density significantly affected bacterial densities. Filamentous bacteria were significantly

Table 3. Summary of linear regression analysis testing for effects of *An. gambiae* larval density on growth and survival in experiment 2

	Error mean square	Estimate	*F*-statistic	*P*	R^2
Survivorship	0.015	−0.0004	2.13	0.152	0.051
Male development time	1.402	0.019	47.7	<0.0001	0.544
Male mass	0.002	−0.59	27.7	<0.0001	0.409
Female development time	1.032	0.020	69.6	<0.0001	0.635
Female mass	0.004	−0.74	25.6	<0.0001	0.390
Female total lipids content	569.68	−0.078	9.65	0.006	0.337
Female lipid content/mg[a]	3.023	−0.0092	2.58	0.125	0.119
Total biomass	20.516	0.094	77.4	<0.0001	0.659

Degrees of freedom for error terms were 40 for all comparisons, except female lipid content where degrees of freedom were 20.
[a] Lipid content expressed as percentage of body mass.

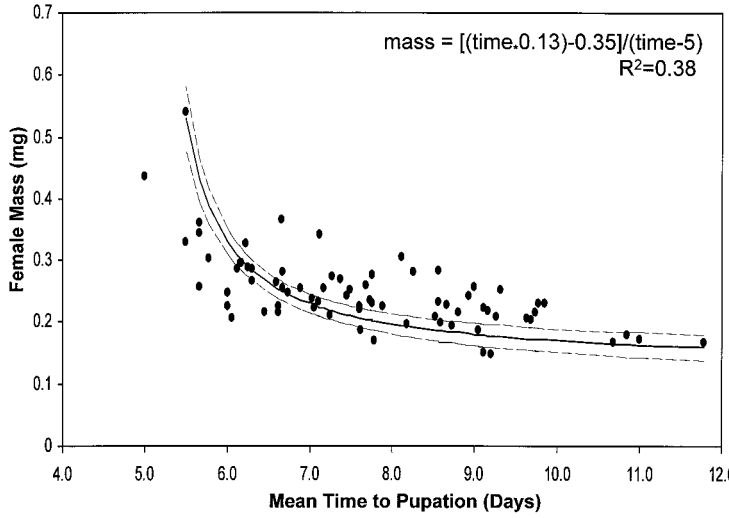

Fig. 2. Fit of pupation window model to *An. gambiae* female growth data from experiments 1 and 2. Data points are coordinates of the means of development time and mean individual female mass per artificial habitat. Solid line is predicted curve. Dashed lines are 95% CL to fitted curve.

more prevalent in habitats with nutrients added but were not affected by the presence of larvae (Table 4). All algal/microeukaryote categories examined with the exception of euglenophytes were significantly affected by the presence of larvae but not by nutrient addition (Table 5). Compared with initial levels and treatments without larvae, total algae, both categories of green algae, and undefined microeukaryotes all decreased in treatments with larvae present, while cyanobacteria increased in the 20 larvae treatment. The small green algae group was dominated by single-celled organisms presumed to be *Chlamydomonas* and *Chlorella* spp. The large green algal group was represented mainly by colonial forms in the order Volvocales (e.g., *Gonium, Volvox*). In contrast to the other algal groups, euglenophyte (*Euglena* spp.) abundance was not affected by larvae or nutrient addition. The ill-defined "other" microeukaryotes included many ciliated protists, but no particular group of organisms was predominant. Cyanobacteria were dominated by solitary filamentous forms, most of which contained heterocysts (sites of nitrogen fixation).

The order of magnitude-level differences between no larvae and larvae-present treatments, particularly in the green algal groups, was reflected by an order of magnitude difference in chlorophyll *a* levels between zero larvae treatment and the 60 larvae treatment (Table 6). By ANOVA, total phosphorous and nitrogen levels in the subsurface water were not significantly affected by larvae or nutrient addition (Fig. 3; Table 6). However, by regression analysis, a trend for decreasing total N with increasing larval density was observed with a probability level of 0.06.

Table 4. Densities (log #/ml) of different bacterial classes in experiment 1 on day 5

	0 larvae		60 larvae	
	No nutrients	Nutrients added	No nutrients	Nutrients added
Filaments	2.09 (1.28)	5.16 (0.12)	1.13 (0.76)	3.38 (0.84)
Bacilli	5.86 (0.15)	6.33 (0.16)	5.67 (0.77)	5.34 (0.72)
Cocci	7.29 (0.15)	7.39 (0.12)	7.09 (0.25)	6.97 (0.15)
Total	7.31 (0.14)	7.43 (0.12)	7.24 (0.26)	7.06 (0.16)

Overall ANOVA significant for Nutrient main effect on filament category only ($P = 0.012$, error term $n = 23$). Standard errors are given in parentheses.

Discussion

In laboratory studies, Schneider et al. (2000) found that survivorship decreased at higher densities of *An. gambiae.* In the current study, larval survivorship was not affected significantly by the initial density of larvae. Rather, *An. gambiae* larvae responded to high larval densities by extending their development time

Table 5. Algal densities (log #/ml) observed on days 0 and 5 during experiment 1

	Time zero	0 larvae	20 larvae	60 larvae
Small green algae	4.18 (0.06)	5.19a (0.08)	2.93b (0.39)	3.09b (0.11)
Large green algae	3.11 (0.14)	4.17a (0.10)	1.44b (0.38)	0.51b (0.23)
Euglenoids	2.39 (0.10)	3.15a (0.41)	2.16a (0.40)	2.70a (0.27)
Cyanobacteria	1.43 (0.42)	1.03a (0.51)	2.74b (0.37)	1.29a (0.32)
Other	2.56 (0.17)	3.42a (0.10)	0.78b (0.30)	0.14b (0.14)
Total	4.24 (0.06)	5.26a (0.07)	3.98b (0.13)	3.53c (0.08)

Nutrient addition had no effect on algal densities and values were averaged across nutrient and no nutrient treatments. Time zero algal densities were not included in statistical analysis but are presented for comparison. Within a row, means followed by the same letter were not significantly different by Scheffe's multiple comparison procedure ($P > 0.05$). ANOVA included day 5 data only. Standard errors are in parentheses. Degrees of freedom for error terms were 37 for all comparisons.

Table 6. Summary of ANOVA for effects of density and nutrient addition on levels of chlorophyll *a*, nitrogen, and phosphorus at day 5 of experiment 1

	Error mean square	*F*-statistics			R^2
		Density	Nutrients	Density × Nutrients	
Chlorophyll *a*	0.156	43.428***	1.047	0.699	0.668
Nitrogen	0.011	2.235	1.325	0.252	0.168
Phosphorus	0.001	0.295	0.077	1.142	0.075
N/P	0.002	1.658	0.084	0.278	0.113

Degrees of freedom for error terms were 23, 34, 36, and 33 for chlor. *a*, N, P, and N/P, respectively. Analyses performed on log transformed values for chlor. *a*, N, and P, and arcsine/square root transformed values of N/P. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

and emerging smaller as adults. *Anopheles gambiae* body sizes varied considerably: the largest females were up to five-fold larger than the smallest females. In contrast, Lyimo et al. (1992) demonstrated in laboratory studies that development time decreased with increasing densities and that adult body size was dependent upon the interaction between density and temperature; at certain temperatures, increasing larval density could result in larger adults. Comparison of the current study with laboratory studies is difficult as larvae were reared at different densities and, more importantly, the quantity and quality of food sources differed. Laboratory reared mosquitoes are generally provided a standard diet of liver powder or fish food whereas no food was provided to larvae in the current study. In the study by Lyimo et al. (1992), larvae were provided a constant 0.2 mg of food per surviving larva. Under these conditions, density-dependent effects would not be expected unless larvae somehow interfere with each other at higher densities.

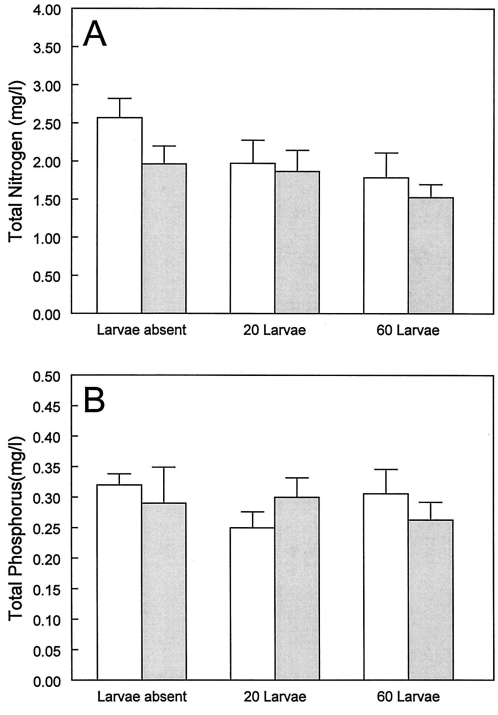

Fig. 3. Nutrient levels within artificial habitats. (A) Nitrogen. (B) Phosphorus. The shaded bars represent habitats to which nutrients were added in the form of partially dried and ground cow dung. The open bars represent habitats to which no nutrients were added. Vertical lines represent the standard errors of the means.

In their study, Schneider et al. (2000) observed competitive interactions among *An. gambiae* larvae at densities up to 30 times higher than those in the current study. In a study on the effects of female body size on gonotrophic development, Takken et al. (1998) produced large and small mosquitoes by rearing the small mosquitoes at densities eight times those of the large ones and by giving the smaller ones less food per larvae. In the current study, differences in mosquito size and development time were observed when larval densities differed by as little as three-fold. At the highest densities in the current study, total biomass began to plateau, suggesting that the density-dependent effects would become even more pronounced if initial densities were increased further. A direct impact on survivorship might be observed by increasing densities beyond those used in the current study. The observation of competitive interactions at lower densities in the artificial larval habitats indicated that food resources were lower in quantity and/or quality than the standard food provided to larvae in laboratory settings.

The pupation window model describes the relationship between development time and mass at pupation. The curve fitted to *An. gambiae* development time and mass at pupation is similar to that observed in other mosquito species, although the shape of the curve is not as sharp as that observed for *Ae. triseriatus* (Walker et al. 1997). The pupation window model was fitted using aggregated data from each habitat. Interestingly, a different pattern was observed within habitats, with larger mosquitoes often emerging later. This phenomenon was likely due to the release of slowly developing individuals from competition after the majority of larvae had emerged.

Although survivorship was not directly affected, increasing larval densities could still act to regulate *An. gambiae* populations in at least two ways. First, larvae of *An. gambiae* occupy small, temporary habitats that can dry rapidly (Gimnig et al. 2001). By extending their development time, *An. gambiae* larvae are more likely to be stranded as their habitat dries (J.E.G., unpublished data). Second, smaller females often require two to three blood meals to generate a batch of eggs and lay fewer eggs each batch (Lyimo and Takken 1993). The size distribution of females from the two experiments is shown in Fig. 4. Assuming a relationship between dry mass and wing-length as described by Koella and Lyimo (1996) and assuming a size threshold of 3.0 mm below which females would require two to three blood meals (Lyimo and Takken 1993), the majority of females developing in the artificial habitats would require at least two blood meals to develop an egg batch. Furthermore, there are dif-

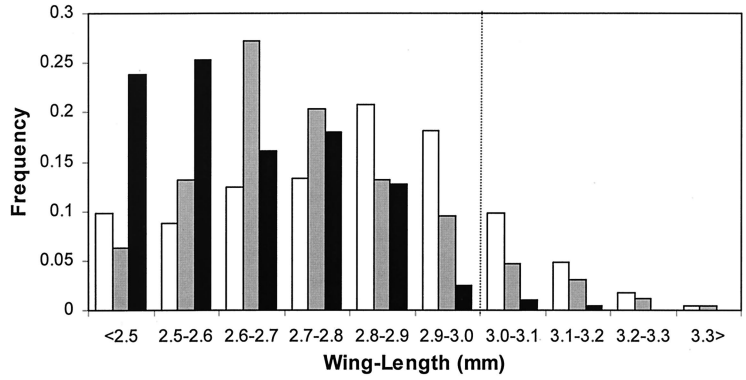

Fig. 4. Frequency distribution of the wing lengths of *An. gambiae* females from experiments 1 and 2 combined relative to larval rearing density and the estimated number of blood meals required for the first egg batch. Wing lengths were estimated from body mass using the relationship reported by Koella and Lyimo (1996). Open bars represent mosquitoes reared at densities ≤50 larvae per habitat, shaded bars represent mosquitoes reared at densities of 50–100 larvae per habitat and the closed bars represent mosquitoes reared at densities >100 larvae per habitat. The dashed vertical line indicates the cut off reported by Lyimo and Takken (1993) for females requiring a second blood meal to mature an egg batch.

ferences in the distribution among the different treatment groups. For females reared at initial densities <50 larvae, 75% would require more than one blood meal to develop an egg batch, whereas 84% reared at initial densities between 50 and 100 females and 97% reared at initial densities >100 larvae would require at least two blood meals to develop an egg batch. Smaller females also have lower total fecundity as they do not live as long and, therefore, lay fewer egg batches compared with larger adults (Hawley 1985b, Ameneshewa and Service 1996). Females reared at higher densities may also lay fewer eggs per oviposition cycle. Assuming a similar relationship between wing length and fecundity as described by Lyimo and Takken (1993), females reared at 200 larvae per habitat were estimated to mature nearly 50% fewer eggs than females reared at 20 larvae per habitat. Combined with the shorter life expectancy of smaller females, the total fecundity of females developing at high larval densities could be significantly reduced compared with those developing at lower densities.

Our analysis of lipid content of newly emerged female mosquitoes showed that it was at the low end of the range previously reported in laboratory studies with *Aedes* and *Anopheles* mosquitoes (Van Handel 1985, Briegel 1990, Clements 1992, Timmerman and Briegel 1999), and that it declined with increasing larval density. However, total lipid content was strongly correlated with female body size and, when expressed as a percentage of body weight, the trend for decreasing lipid content was not statistically significant. *Anopheles gambiae* females emerge as adults with deficits of lipid, carbohydrate, and protein and frequently require multiple blood meals as they shunt some blood meal protein from yolk synthesis to extraovarian lipid and protein stores (Briegel 1990, Timmerman and Briegel 1999). Indeed, Briegel and Horler (1993) have proposed this process as a basic reproductive strategy in *Anopheles* generally. Our results reinforce this hypothesis as most females, even when reared at low densities, would require at least two blood meals for egg maturation based on the size thresholds determined by Lyimo and Takken (1993). The importance of lipid reserves in egg maturation is not clear but the low levels observed in females emerging from all treatments may partially explain the need for multiple blood meals in most *An. gambiae* females.

While the current study has demonstrated that density-dependent processes can impact the growth and development of *An. gambiae* under artificially manipulated conditions, it is unclear how important these processes are in regulating their populations under natural conditions. Washburn (1995) argued that larvae in containers are limited more by resource availability while larvae in ground pools are limited more by natural enemies. Laird (1988) classified the larval habitats of *An. gambiae* in the "Intermittent Ephemeral Puddles" category, i.e., small, ephemeral ground pools. In the rice field habitats, predation and parasitism were shown to be important factors limiting populations of *An. arabiensis* (Service 1973, 1977). However, the effects of density-dependent processes were not measured and the invertebrate community of rice fields likely differs from that of small temporary pools where *An. gambiae* larvae are often found. A key consideration in elucidating the importance of density-dependent factors in the regulation of *An. gambiae* populations is the density of larvae in natural habitats. Remarkably, it is not known what larval densities are common in nature. Part of the problem lies in the dearth of studies on larval biology and ecology. Contributing to this is the difficulty in measuring the surface area of the habitats and the abundance of larval anophelines. Anecdotal reports indicate that larval densities can be high (Gillies and DeMeillon 1968). Our own observations suggest that extremely high densities are not common, at least in this area of western Kenya. We recently conducted a study of natural larval habitats in the area near the site of our artificial habitat arena. Using an area sampler, we

found that there were an average of 0.13 *An. gambiae* s.l. larvae per square centimeter of habitat (E.D.W., unpublished data). Given that our artificial habitats had a surface area of ≈600 cm^2, the experimental densities we established in our artificial habitats overlap with densities observed in natural habitats nearby. In addition, habitat size and mosquito populations fluctuate considerably and density-dependent effects may be observed seasonally, particularly at the end of the rainy season or beginning of the dry season when mosquito populations are highest and habitats begin to dry. The wide variation in adult mosquito size observed in natural populations (Lyimo and Takken 1993, Ameneshewa and Service 1996) suggests that density-dependent processes may play a role in the regulation of *An. gambiae* populations.

Density-dependent effects on the growth of *An. gambiae* larvae could influence the transmission of malaria. Density-dependent processes may act to limit the population of adult females, thereby limiting transmission. Density-dependent effects on the size of adult females could affect malaria transmission in several ways. First, smaller adults may have lower daily survival rates than larger adults (Hawley 1985b, Ameneshewa and Service 1996) and are therefore less likely to survive longer than the extrinsic incubation period of the *Plasmodium falciparum* and become infectious to humans. Body size also may affect the probability of a mosquito becoming infected. Among wild caught females, Lyimo and Koella (1992) found that intermediate sized females were more likely to be infected in nature than smaller or larger females. Body size also affects the pregravid rate of *An. gambiae.* Smaller females often require two to three blood meals to develop their first batch of eggs. The need for frequent, repeated blood meals may increase the probability that smaller females acquire an infectious blood meal. Although the impact on survivorship due to decreased body size is likely to have the strongest impact on malaria transmission, the competing effects of body size on vectorial capacity need to be investigated more thoroughly to fully assess the influence of density-dependent effects on the transmission of malaria in nature.

This study clearly demonstrated the importance of algae as food items for *An. gambiae* larvae. Larval grazing reduced algal abundances and biomass by an order of magnitude, and changed microeukaryote community structure. Changes in this algal food resource due to larval consumption almost certainly led to the observed density-dependent responses in larval development. The results were obvious from casual observation of the experimental habitats—those with larvae were noticeably clearer at the surface layer within a few days of larval addition. Though algae are known food items for anopheline larvae (Laird 1988), our study represents the first quantitation of larval grazing potential. It is noteworthy that large green algal groups and other large microeukaryotes declined most precipitously in the presence of larvae as they likely represent the most valuable food items present. Green algae of ingestible size classes are considered preferred food items for freshwater zooplankton (Sterner 1989) and ciliated protists are known to be grazed heavily by culicine larvae (Eisenberg et al. 2000). The increased relative abundance of nitrogen-fixing cyanobacteria in larvae-present treatments is consistent with this algal group's relative indigestibility for invertebrates (Martin and Kukor 1984, de Bernardi and Giussani 1990) and with the trend toward lower available nitrogen in treatments with larvae. However, cyanobacteria were less prominent in the highest larval density treatment, suggesting larvae may resort to grazing unpalatable cells as more digestible resources decline.

Chlorophyll *a* values and algal counts illustrate the high biomass available for larval consumption and growth. Values for chlorophyll *a* are consistent with those found for eutrophic Kenyan ponds previously investigated (Sarnelle et al. 1998). Because conversion factors of chlorophyll *a* to algal biomass vary widely with algal species and physiological status (Cloern et al. 1995), it is difficult to estimate algal biomass in the experimental habitat surface layers. However, using published conversion factors of chlorophyll *a*, algal cell carbon (Cloern et al. 1995) and a cell carbon content of 53% (Wetzel 1983), mosquito feeding potentially reduced dry algal biomass by 100 μg/ml, or a total of 7 mg per habitat surface layer. Based on total adult production of 7.5 mg and 2.8 mg per 60 larvae and 20 larvae treatments, respectively, the decline in algal biomass goes far in explaining density-dependent larval growth in the experimental habitats. Obviously, this oversimplification ignores algal turnover rates, larval consumption and growth efficiencies, and other nutritional sources (i.e., bacteria and noncellular organics in the surface layer). Nonetheless, we believe it emphasizes the key role of algal food resource in *An. gambiae* habitats. The above calculations also ignore any contribution of algae to the detrital pool (only intact cells were counted and chlorophyll *a* is a measure of living algal biomass). Because detritus is a major food item for anopheline larvae (Merritt et al. 1992, Wallace and Merritt 1999), it represents an additional contribution of algal biomass to *An. gambiae* nutrition. We did not quantify detritus in this study, but noticed substantially higher levels in the surface layer samples from experimental habitats without larvae. Presumably, some of this came from the soil used to construct the habitats, but a major portion was likely derived from algal senescence.

The general lack of response by total bacteria to larval presence or nutrient addition is somewhat surprising given laboratory studies demonstrating the importance of surface layer bacterial activity to anopheline growth (Wotton et al. 1997) and surface-associated bacteria population and productivity declines in response to culicine grazers (Kaufman et al. 1999). Other evidence, however, suggests that all bacterial populations do not respond to larval mosquito grazing uniformly (Kaufman et al. 1999) and varying responses (decrease, increase, or no change) in bacterial abundance (Walker et al. 1991, Cochran-Stafira and von Ende 1998, Paradise and Dunson 1998) might

be explained by nutrient dynamics, alternative predators, or other factors. Our observations that a nutrient addition increased the numbers of filamentous bacteria, but that larval grazing appeared to have no effect on bacterial morphotypes (Table 4) indicates that bacterial food webs in *An. gambiae* surface layer habitats are different from previously investigated planktonic systems (Güde 1989, Jürgens 1994).

The lack of discernible nutrient addition effects in most aspects of this study likely was a consequence of the initial richness of the soil in the habitats and the form of nutrients added (i.e., dried cow dung). The two key nutrients known to stimulate production in freshwater systems, N and P, were evidently present in high quantities initially. That bacterial numbers were high at day zero, and then declined significantly by day 5 in both nutrient treatments also indicates an initial pulse of nutrients when the habitats were first flooded. Cow dung, although an obvious form of nutrient input in natural *An. gambiae* habitats, is not necessarily rich in immediately available N and P (Sarnelle et al. 1998). Nitrogen levels would be expected to be low in dried manure because of losses to ammonia volatilization and denitrification, while phosphorous forms are mainly particulate and would be released slowly through decompostion processes (Loehr 1974). Thus, our cow dung nutrient addition more likely represents a source of recalcitrant carbon, nitrogen and phosphorous that would support more heterotrophic production (i.e., bacteria) as the habitats matured beyond the short duration of our study.

The presumed immaturity of the experimental habitats raises questions about their representation of natural *An. gambiae* habitats. Many natural habitats are newly formed by human and animal activity, but many tend to cycle through flood and dry periods over a period of years, promoting leaching of nutrients and export via insect emergence. Additionally, our artificial habitats were formed using local "black cotton soil" which is likely high in humus and micronutrient content relative to the red clay soil that is common in many parts of tropical Africa. Interestingly, N and P levels measured in our experimental habitats were within ranges reported for more permanent Kenyan ponds (Sarnelle et al. 1998) and are well within values we have measured for natural habitats in areas near the study site (M.G.K., unpublished data). Thus, key nutrients supporting the food base for *An. gambiae* larvae in natural habitats were effectively mimicked in our experimental habitats. Although N and P values in this study were within ranges of natural *An. gambiae* habitats and more expansive ponds in Kenya (Sarnelle et al. 1998), the ratio of N to P we measured was generally lower than previously reported. Values of <14 (mass N to mass P) are considered indicative of nitrogen limitation to algal production. Our N/P values averaged from 6 to 8. Sarnelle et al. (1998) concluded, in part based upon N/P ratios, that phosphorous was the element limiting algal production in a range of Kenyan ponds. Indications from this study that available N pools are reduced with increasing larval densities, and our initial investigation of N and P concentrations in natural larval habitats, suggest that nitrogen may be the key element in the trophic dynamics of *An. gambiae* larvae and an algal community food source.

A key question that needs to be addressed is how representative our larval habitats are of natural habitats of *An. gambiae.* The habitats proved capable of producing adults without the input of additional food sources. However, it was necessary to add water each day to prevent the habitats from drying. Development time from egg to adult averaged between 8.4 and 11.5 d, within the ranges reported by Gilles and DeMeillon (1968) and, assuming a relationship between adult dry mass to wing lengths as reported by Koella and Lyimo (1996), the size of adult females emerging from the artificial habitats was similar to those captured in the wild (Lyimo and Koella 1992, Lyimo and Takken 1993). However, direct comparisons should be viewed with caution as temperature may also influence development time and adult mass. Preliminary studies indicate that the bacterial fauna and the amount of micronutrients available was similar to that observed in natural habitats found nearby (M.G.K., unpublished data). Lastly, when the screens were removed after all larvae had pupated, wild *An. gambiae* rapidly colonized the habitats (J.E.G., unpublished data).

In summary, this study demonstrated that density-dependent processes may affect larval *An. gambiae* under quasi-natural conditions. Larval mosquitoes respond to density-dependent pressures primarily by extending their development and emerging smaller; the impact upon larval survivorship was not significant. The primary food source for the larval stages is likely derived from algal growth, with bacterial growth forming a secondary food source. Although survivorship does not appear to be strongly affected by increasing larval densities, competition among larvae may regulate *An. gambiae* populations through dampening effects upon adult survivorship and fecundity. Density-dependent processes may also indirectly affect the vectorial capacity of *An. gambiae* by reducing adult survivorship and increasing the need for additional blood meals for the development of eggs.

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