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Sexual selection in mosquito swarms: may the best man lose?

KIJA R. NG'HABI*†, BERNADETTE J. HUHO*†, GAMBA NKWENGULILA†, GERRY F. KILLEEN*‡, BART G. J. KNOLS§ & HEATHER M. FERGUSON*§**

*Public Health Entomology Unit, Ifakara Health Research and Development Centre
†Departments of Zoology and Wildlife Conservation, University of Dar es Salaam
‡School of Biological and Biomedical Sciences, Durham University
§Laboratory of Entomology, Wageningen University and Research Centre
**Division of Immunity & Infection, and Division of Environmental & Evolutionary Biology, University of Glasgow

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One of the greatest paradoxes in evolutionary biology is the continued maintenance of genetic variation for phenotypic traits that appear to confer strong fitness advantages. Of these traits, body size is perhaps the one that has been most consistently linked to increased longevity and reproductive success in males. We investigated two hypotheses for how events occurring during mating in the mosquito *Anopheles gambiae* could impede directional selection on male body size: (1) whether male copulation probability is random with respect to body size in aerial swarms, and (2) whether large males are less competitive during mating than smaller, shorter-lived rivals. By manipulation of larval nutritional conditions (low, intermediate and high food allocation), we generated cohorts of male *A. gambiae* mosquitoes that differed in adult body size and energy reserves (body size and energy reserves being positively correlated with larval nutrition). When competing against one another in aerial swarms, males from the intermediate food treatment were six and two times more successful at acquiring mates than those from the high and low food treatments, respectively. The median survival of males from this most sexually competitive group was approximately 13% lower than that of the larger males with high larval nutrition. We conclude that phenotypic determinants of long-term survival and mating success may not be correlated in this system, and thus that stabilizing selection as well as environmental condition-dependent expression of traits could account for the maintenance of variation in male body size in this species and in other swarming insects.

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In animal taxa ranging from insects to large mammals, the male phenotypic trait that has been most consistently associated with enhanced longevity and/or reproductive success is body size (Andersson 1982, 1994; Thornhill & Alcock 1983; Blanckenhorn 2000; Jennions et al. 2001; Blanckenhorn et al. 2004; Maan et al. 2004; Malo et al. 2005). Additional phenotypic traits that are correlated with body size (e.g. energy reserves) have also been linked to enhanced longevity and reproduction (Yuval et al. 1994; Sparkes et al. 1996). On the basis of this near ubiquitous demonstration of the fitness advantages of being large, one would predict directional selection on male body size to exist in most animal species. The continued existence of

Correspondence: K. Ng'habi, Ifakara Health Research and Development Centre (IHRDC), P.O. Box 53, Off Mlabani Passage, Ifakara, Tanzania (email: kija@ihrdc.or.tz; ndyai@yahoo.com). G. Nkwengulila is at the University of Dar es Salaam, P.O. Box 35064 Dar es Salaam, Tanzania. B. G. J. Knols is at the Laboratory of Entomology, Wageningen University and Research Centre, P.O. Box 8031, 6700 EH Wageningen, The Netherlands. G. F. Killeen is at Ifakara Health Research and Development Centre Coordination Office, P.O. Box 78373, Kiko Avenue, Dar es Salaam, Tanzania. H. M. Ferguson is at the Division of Immunity & Infection, and Division of Environmental & Evolutionary Biology, University of Glasgow, Glasgow G12 8TA, U.K.

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variation in male body size in nature suggests that this does not occur. Three general classes of hypotheses have been put forward to explain this apparent paradox, the first being that the efficiency of selection is impeded by plasticity in the expression of phenotypic traits caused by environmental variation occurring during development (condition dependence). A well-known example of this phenomenon is the 'silver spoon' hypothesis which proposes that nutritional resources provided during early life history are an equal if not greater determinant of adult fitness than genotype (Metcalfe & Monaghan 2001). A second, but not necessarily conflicting, paradigm is that traits apparently under strong sexual selection such as body size may be negatively correlated with other aspects of male fitness (e.g. predation risk and/or energy requirements) which, in combination, generate stabilizing selection (Blanckenhorn 2000). These costs have been demonstrated in some species (Blanckenhorn 2000), although a thorough cross-taxa meta-analysis suggested that male traits under sexual selection such as size are often positively correlated with longevity (Jennions et al. 2001).

An additional factor that may influence the efficiency of sexual selection on male traits is the predictability of encounter rate between males and females. Where the probability of a male coming into contact with receptive females is determined primarily by randomly occurring environmental variation, male mating success may be ultimately determined by chance events rather than particular phenotypic characteristics. One system where this may occur is aerial mating swarms of insects. Unlike communal courtship grounds (leks) where groups of males congregate on a fixed habitat feature or area and compete for a territory within it (Kruijt & Hogan 1967), an aerial swarm is a three-dimensional arena whose physical structure is determined by the flight activity and abundance of males themselves (Downes 1969; Yuval et al. 1993; Yuval 2006). A wide variety of insects, including dipterans, trichopterans, lepidopterans, hymenopterans, hemipterans, ephemeropterans and odonates, mate in aerial swarms, which are typically maintained in a fixed position above a marker by the constant 'figure-of-eight' flight of males within them (Downes 1969). Females may be attracted to a swarm from a relatively long distance (Clements 1992). Some of the most detailed observations of swarming behaviour have been made on mosquitoes in which males have been observed to dart out and grab approaching females before they reach the 'body' of the swarm and quickly mate with them (Reisen et al. 1977; Charlwood et al. 2002a, 2003). Consequently, males flying on the edge of the swarm when a female arrives may have the highest chance of copulation. As males repeatedly fly through the swarm, it may not be possible for them to predict their location (centre or edge) when a female arrives and/or secure a fixed 'optimal' position on the periphery. This being the case, male mating success with respect to phenotypic traits such as body size may indeed be random: a phenomenon that has been proposed for some swarming insects (Charlwood et al. 2002b) but dismissed in other studies where both large (Yuval et al. 1994) and small size (Marshall 1988; Fyodorova & Azovsky 2003; LeBas et al. 2004) have been associated with higher success.

To assess the impact of phenotypic traits on mating success under the competitive conditions of an aerial swarm, we manipulated the larval nutrition of male *Anopheles gambiae* sensu stricto mosquitoes. Our principal aim was to test whether male mating success was enhanced by commonly proposed indexes of male fitness such as body size and energetic reserves or random as has been speculated for aerial swarms. We also investigated the association between traits that may be under sexual selection and long-term survival to assess the possibility of trade-offs between different components of male fitness that could generate stabilizing rather than directional selection on body size.

Elucidating the strength and direction of sexual selection can have strong applied relevance to the implementation of disease control programmes that are based on the mass release of sterilized or genetically modified male insect vectors that can drive infection-blocking genes into natural populations (Ferguson et al. 2005). The creation of genetically modified mosquitoes that are refractory to infection by malaria parasites (Capurro et al. 2000; Ito et al. 2002) and dengue virus (Franz et al. 2006) has fuelled optimism that these diseases could be controlled by releasing a sufficiently large number of transgenic males into the wild that could drive these genes into natural populations. The *A. gambiae* mosquitoes studied here are the principal vectors of malaria in sub-Saharan Africa (WHO 1993) and a primary candidate for disease control programmes based on the mass release of transgenic males (Ito et al. 2002; Tabachnick 2003; Moreira et al. 2004; Andreasen & Curtis 2005). Crucial to the success of this approach is the ability of transgenic males to compete successfully for females in the presence of their wild counterparts (Scott et al. 2002; Ferguson et al. 2005). Information on the existence and phenotypic determinants of heterogeneity in male *A. gambiae* mating success could thus provide useful guidance for the design of rearing protocols for the mass production of sexually competitive males whose release could reduce malaria transmission.

METHODS

Rearing

We studied *A. gambiae* sensu stricto mosquitoes from a population at the Ifakara Health Research and Development (IHRDC), Tanzania. This population was established from a wild population near Njage village, Tanzania, in 1996. We randomly assigned first-instar larvae to one of three food quantity treatments: 0.1 mg, 0.2 mg or 0.4 mg of dried fish food (Tetramin, Melle, Germany) per larva per day (Lyimo et al. 1992). Larvae of the same food treatment were housed together in groups of 100 in standard rearing trays (37 × 14 cm and 13 cm high). Each tray was filled with 1 litre of river water and supplied with a total daily mass of fish food sufficient to feed all 100 larvae (a total of 10 mg, 20 mg and 40 mg of Tetramin added to low, medium and high food treatment trays, respectively). We used river water as it is most representative of the natural aquatic substrate in which larvae develop in the wild. To synchronize

pupation, we established cohorts of larvae in the lowest food treatment 4 days before the other two food treatments (this time gap was chosen on the basis of pilot studies of pupation rates). Once established, we inspected larval trays twice a day for the presence of pupae. When detected, pupae were collected and held individually in vials to allow for emergence. Batches of males from all three food treatments that emerged on the same day competed against one another in mating trials, for females from the medium food treatment. We used females from only one larval background to minimize variation in female body size and energy reserves, and thus standardize the 'resource' for which males were competing across trials.

Marking

From emergence, males were pooled according to larval food treatment and held in separate cages. On the second day after emergence, cohorts of adult males from two of the three food treatments were marked with green or pink fluorescent powder, respectively. The third group was left unmarked. We alternated marking treatments between food treatments across trials to ensure no systematic bias in performance from dusting. Pilot studies and previous investigations (Ng'habi et al. 2005) indicated that males from different backgrounds could be reliably identified in this way, with no evidence that dust was transferred between males during the swarming process. In swarms, males make contact with females during copulation but not with one another during flying. As copulating pairs were removed from the mating arena before separating (see below), marking dust could not be transferred from one male to another through a female.

Mating Experiment

On the third day after emergence, 30 males (10 males from each treatment) were put together in one cage (15 × 15 cm and 10 cm high). The cage was exposed to natural light a few hours before dusk. We placed a black plastic disk on the bottom of each cage to act as a swarm marker (Ng'habi et al. 2005). In nature, *A. gambiae* males form mating swarms approximately 15–20 min before sunset (Charlwood & Jones 1980; Charlwood et al. 2002a, 2003), a behaviour that is also observed in our laboratory population (Ng'habi et al. 2005). We began monitoring activity inside each experimental cage approximately 10 min before sunset.

Once swarming was underway, 10 females from the medium food treatment were simultaneously released into the cage (making a 3:1 male to female ratio). We observed mating activity within cages under a low-intensity red light bulb. Pairs observed to form copula were immediately aspirated out of the cage and put together into a holding cup. As a consequence of this design, females could not be mated more than once in a swarming event. As the frequency of polyandry in *Anopheles* populations is known to be very low (<3%, Tripet et al. 2003), this restriction on multiple mating in females is not inconsistent with nature. In addition, a male and female in copula normally fly away from the swarm, reducing the likelihood of returning for multiple mating.

On each evening of the experiment, we finished observations of mating after all males had stopped swarming (usually 40–45 min after first initiation). The following morning, a cotton wad soaked with chloroform was put on top of each holding cup, and all mosquitoes captured in copula were anaesthetized and killed. Males were then observed under a fluorescent lamp to identify from which larval food treatment they originated. We measured the wing lengths of both male and female partners under a dissecting microscope. Males that did not mate were also killed and their wing lengths measured for comparison with those caught in copula. We conducted eight replicates of this competition experiment.

Quantification of Energy Reserves

A subsample of newly emerged males from each larval food treatment were killed and transferred individually into glass test-tubes for the quantification of lipids, sugars and glycogen. Once in the tubes, the mosquitoes were crushed with a glass rod. We added 100 µl of 2% sodium sulphate (which adsorbs glycogen) and 600 µl of a 1:2 chloroform–methanol mixture (which dissolves lipids and sugars, respectively) to each tube, then incubated them for 24 h at room temperature. We analysed 93 males: 28 from low, 26 from medium and 39 from high food treatments. For each batch of males analysed, we prepared one blank by adding the same chemicals to a tube that contained no mosquitos. Lipids, glycogen and sugars of each male were then quantified by using a colorimetric technique adapted for mosquito analysis (Van Handel 1988).

Longevity Experiment

In a separate experiment, males emerging from each larval food treatment were denied access to females but held in groups of 32–47 in standard holding cages (15 × 15 cm and 10 cm high) to monitor their longevity. At least two cage replicates of each larval food treatment were monitored. While in cages, adult males received a 10% glucose solution ad libitum. We observed each cage daily, and removed and counted all dead males found within it.

Statistical Analysis

The main aim of our statistical analysis was to test for differences in male mating competitiveness within a swarm as a function of the larval food-set adult phenotype. We considered whether male larval food treatment was a predictor of: (1) the first male to mate and (2) the total number of males that obtained copulations across all trials. In both cases, chi-square tests were used to evaluate the relation between the dependent variable (proportion of matings) and larval food treatment. General linear models (GLM) were used to test whether larval food treatment influenced male body size (as indexed by wing size) and/or

the abundance of energy reserves (sugars, glycogen and lipids) on emergence. Finally, we used Kaplan–Meier survival analysis to test whether the survival of adult males varied in response to larval food treatment. The SPSS statistical package version 11.5 for Windows (SPSS Inc., Chicago, IL, U.S.A.) was used for all analyses. Unless otherwise stated, means are given ±1 SE.

RESULTS

By varying larval food, we succeeded in generating three distinct adult male phenotypes, which differed with respect to each of the male traits we measured. First, body size varied substantially between groups (GLM: $F_{2,234} = 94.128$, $P < 0.01$; Fig. 1), with males from the high food treatment being 18% and 9% bigger than those in the low and medium food treatments, respectively (low food: 2.49 ± 0.015 mm; medium food: 2.69 ± 0.014 mm; high food: 2.94 ± 0.01 mm). The average size of females used in these experiments was 2.83 ± 0.02 mm.

The abundance of teneral energy reserves in adult males also varied in response to larval food treatment. As with body size, males emerging from the high food treatment had the most abundant lipids, glycogen and sugars (GLM: lipids: $F_{2,26} = 15.76$, $P < 0.001$; sugars: $F_{2,24} = 22.05$, $P < 0.001$; glycogen: $F_{2,37} = 27.05$, $P < 0.001$; Fig. 2). Furthermore, male survival was similarly influenced by the abundance of food received during development (log rank$_2$ = 136.69, $df = 2$, $N = 294$, $P < 001$; Fig. 3). The median survival of males from the low food regime (12 days) was 8 and 11 days lower than that of males from the medium (20 days) and high (23 days) larval food treatment, respectively. Thus the larval food treatment we imposed yielded three distinct phenotypic groups of males that varied substantially in condition as indexed by body size, teneral reserves and survival.

In the mating experiment, males originating from the medium food treatment with intermediate body size, energy reserves and survival were the first to obtain a mate in seven of eight trials (chi-square test: $\chi^2_2 = 18.50, P < 0.01$). Considering all mating events and not just the first to occur in an evening, we found that copulations were not distributed randomly among males from different larval treatment groups ($\chi^2_2 = 13.59$, $P < 0.01$; Fig. 4). Males from the intermediate larval food treatment dominated mating activity, being six times more likely to acquire a female than males from the higher larval food regime (odds ratio, 95% CI = 6.33, 2.05–19.52, $P < 0.01$). Males from the intermediate group tended to accrue more matings than those in the low larval food treatment, although this difference was not statistically significant (odds ratio 95% CI = 2.09, 0.93–4.71, $P = 0.08$).

Within each of the three larval food treatments, males that obtained mates were larger than those that did not (GLM: $F_{1,234} = 29.49$, $P < 0.01$; Fig. 1). This observation contrasts with the between-treatment observation that males from

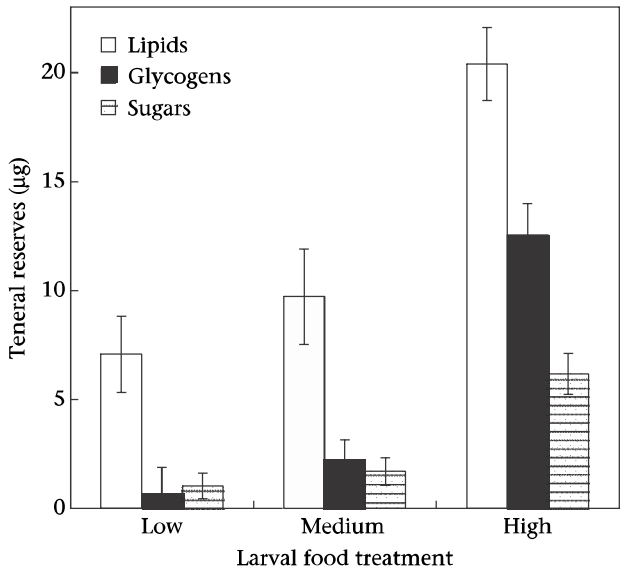


Figure 2. The mean amounts of lipid, glycogen and sugars in newly emerged *A. gambiae* males reared in low, medium and high larval food conditions. Vertical lines indicate ±1 SE.

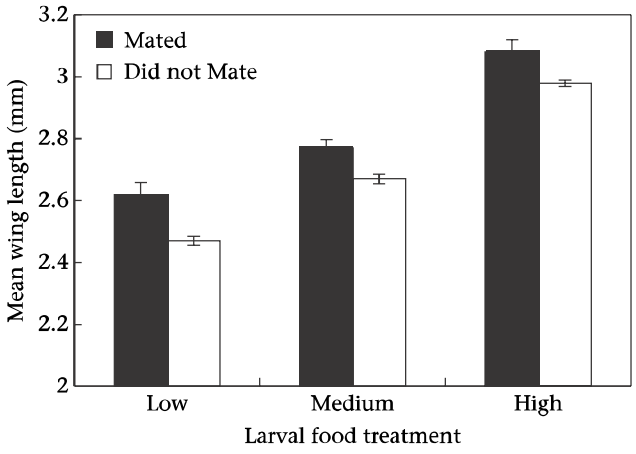


Figure 1. The mean wing length (an indicator of body size) of *A. gambiae* males that mated and those that did not from each larval food treatment. Vertical lines represent ±1 SE.

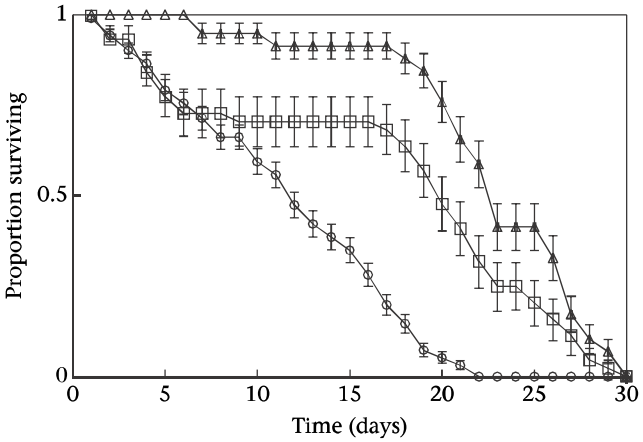


Figure 3. The survival of adult male *A. gambiae* maintained on high ($N = 58$), medium ($N = 44$) and low ($N = 192$) food provision during larval development. ○: Males from the low food treatment; □: males from the medium food treatment; △: males from the high food treatment.

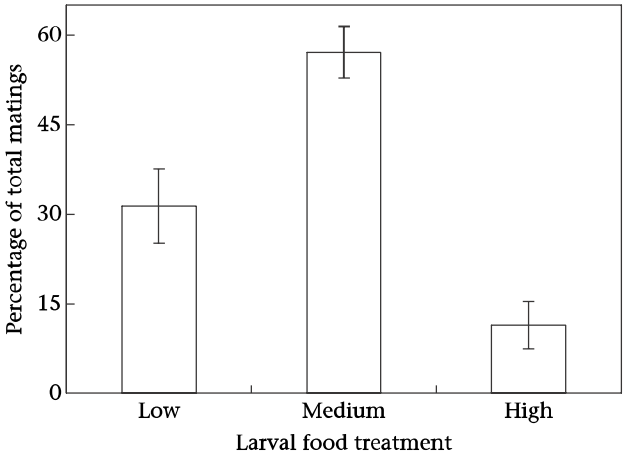


Figure 4. Percentage of total matings ($N = 35$) going to adult male *A. gambiae* from low, medium and high larval food treatments, pooled over eight experimental replicates. Vertical lines indicate ±1 SE.

the group having intermediate body size were more successful than males from the higher and lower larval food groups. In our statistical analysis of the total number of matings, inclusion of male body size as an additional explanatory factor did not diminish the significance of larval treatment (chi-square test: $\chi^2_1 = 40.64$, $P < 0.01$), indicating that adult body size and larval nutrition were important and possibly independent predictors of mating competitiveness.

DISCUSSION

The mating success of male *A. gambiae* mosquitoes within swarms in our study was not random, but depended on nutrient uptake during larval development and its impact on adult body size. Contrary to expectation, we observed that the 'best male' with respect to frequently observed predictors of male insect fitness (longevity, body size and energy reserves) was not the most competitive in acquiring females within a mating swarm. Instead, we observed that males most likely to win females were those of intermediate body size and energy reserves. Despite being an average of 10% larger and having two to four times more energy reserves (lipids, glycogens and sugars), males from the high larval food background were only one-sixth as likely to obtain a copulation in one swarming event as males from the intermediate treatment. There was also no evidence of a correlation between male mating success within a swarm and long-term survival. The median survival of males from high larval food conditions was 13% greater than that of males from intermediate food backgrounds, but their probability of obtaining a mate per swarming event was much lower (84% reduction). We caution that these results were obtained under artificial conditions in the laboratory, in which an equal ratio of males from all three distinct phenotype categories competed against one another, and may not be representative of the full range of competitive pressures experienced by *A. gambiae* swarming in the wild. However, as variation in male body size and energy reserves generated in this experiment falls within the range observed in wild populations from the surrounding Kilombero valley (1.9–3.1 mm, Huho et al. 2007), we are reassured that our results are representative of natural phenotypes and not simply an artefact of aberrant extremes produced in the laboratory. The females we used were also within the range observed in the local population (2.34–3.62 mm, Lyimo & Takken 1993).

Although this is not the first report of males of intermediate size having high mating success (e.g. Delisle & Hardy 1997), it does stand in contrast to most of the evidence from other arthropods that indicates that male mating success increases with body size (Partridge & Farquhar 1983; Thornhill & Alcock 1983; Alcock 1996; Blanckenhorn 2000, 2005; Crean et al. 2000). What could thus account for the strong mating advantage of males of intermediate size in this study? One possibility is that smaller males are more agile in flight and can make contact with females more quickly, a phenomenon that has been observed in other swarming insects (Neems et al. 1998; Crompton et al. 2003). The potential fitness advantage that small males may have in flight could be eroded by their lower energy reserves which may compromise their ability to sustain swarming (e.g. Yuval et al. 1994), and thus render males with intermediate body size and reserves the most competitive. Another explanation for the differential mating success of males in this study could be size-assortative mating. Post hoc observation confirmed that males from the intermediate larval food group were closer in body size to available females than males from other treatment groups. Size-assortative mating has been found in several arthropod species (Crespi 1989), and anecdotal evidence suggests it may occur in *Anophelines* (Gomulski 1985). This phenomenon could arise as a by-product of size-dependent competition between males for large females and/or higher copulation and insemination success in male–female pairs with similarly sized genitalia (Arnqvist 1997). Our study was not designed to test for size-assortative mating, and indeed we tried to minimize size variation in the females offered to males to standardize their perceived 'quality' across trials. Although we are currently logistically limited in our ability to rear the large numbers of size-varying males and females that would be required to confirm this phenomenon, we recommend it as a valuable line of inquiry for future studies of mating biology in this species.

A final hypothesis for the success of intermediate-sized and energetically provisioned males is that they may be most in concordance with natural patterns of sexual size dimorphism in this species. Field data indicate that female *Anopheles* are generally larger than males, although there is considerable overlap in body size between the sexes (Charlwood 2002; Lehmann et al. 2006). Although the body sizes of all males used in this study were within the natural range of this species, not all were smaller than the females on offer. In particular, males from the high larval food group were slightly larger than females. If females follow a rule where they prefer large males, as long as they are not larger than themselves, intermediate-sized males would be favoured over large males, as observed here. This hypothesis, however, would require that mating is driven by female choice rather than male–male competition, which, although possible, seems unlikely in a system such as this where males physically grab females in flight to copulate.

We initially set out three hypotheses for why traits such as body size that apparently confer strong fitness benefits to males in many animal taxa show substantial phenotypic variation in nature: (1) environmental condition-dependent expression of traits; (2) opposing influences of phenotypic traits on different aspects of total lifetime fitness that lead to stabilizing selection; and (3) randomness in the encounter rate between males and receptive females. While our study was not designed to test each of these hypotheses exhaustively, it provides valuable insights into the likely constraints on male body size evolution in swarming mosquitoes. We can reject the hypothesis that chance plays a strong role in determining male mating success in this system: one group of males was consistently more successful at acquiring females than others, and, furthermore, success within these groups was not random as larger males within groups were most likely to mate. Thus, even though it may appear that mosquitoes adopt similar flight behaviour in swarms and cannot guard particular territories, they are not equally likely to obtain copulations. This finding contrasts with Charlwood et al.'s (2002b) conclusion that male size does not influence mating success in natural *A. gambiae* swarms. These authors based their conclusion on the fact that the average body size of males in copula was not statistically different from that of other males in the swarm, but ambiguity remains as to whether the body size of copulating males was less variable than that of all other males. If it was less variable, it would suggest intermediate-sized males may also have had an advantage in this study. Alternatively, the discrepancy between these studies could arise from variation in additional male traits such as age and previous mating experience in natural swarms, which may make it more difficult to detect clear associations between male phenotype and mating success of the type we found under controlled laboratory conditions.

In addition, we have shown strong condition dependence in the expression of both adult body size and energy reserves. Within our laboratory population, we were able to generate substantial variation in male body size and energy reserves by moderate manipulation of larval nutrition. Although body size in *A. gambiae* is known to be heritable in nature (Lehmann et al. 2006), variation in larval habitat quality may ultimately be a stronger determinant of adult phenotype than genetic background and thus impede the speed of selection towards an optimum.

In addition to these environmental effects, our results also suggest the potential for stabilizing selection to act on male body size. We found that longevity increased with body size, but that large males were less successful at obtaining mates than those of intermediate size. Although the extended survival of males from the high food background indicates they will have more mating opportunities than those from other groups, this advantage may not sufficiently compensate for their reduced competitiveness within a swarm to make them the most successful in the long run. Other potential costs of large body size that were not measured here include increased vulnerability to predation (Allan et al. 1987; McLachlan et al. 2003) and higher energy requirements which increase the risk of starvation in resource-poor environments (Blanckenhorn 2000). These factors have been proposed to explain why intermediate body size may be favoured in natural populations of other swarming insects (Neems et al. 1998; Peckarksy et al. 2002), and may operate to maintain genetic variation for body size in mosquitoes.

Finally, our findings have relevance for the design and implementation of malaria control programmes based on the mass release of laboratory-reared, genetically modified and/or sterilized *Anopheles* mosquitoes. A commonly cited weakness of this approach is that it generally fails to consider ecological and behavioural aspects of target species (Scott et al. 2002). Ignorance of mating biology in particular has been implicated in the failure of numerous attempts to control disease vectors by the mass release of sterile males in trials during the 1960–1970s, and little progress to increase knowledge has been made since then (Ferguson et al. 2005). Our results clearly show that male mating success, at least in experimentally generated swarms, is highly dependent on larval nutrition and associated adult phenotype. Failure to consider the strong influence of phenotype on *A. gambiae* males in mass-rearing facilities could therefore lead to the production of millions of transgenic insects with little chance of passing on their genes in the wild. The range and nutritional quality of larval habitats used by *A. gambiae* in the wild varies substantially (Ye-Ebiyo et al. 2003; Minakawa et al. 2005). We emphasize the importance of identifying the phenotypic and genetic determinants of male *A. gambiae* mating success within target malaria-endemic settings before attempting any mass-release trial so that rearing protocols in mass-rearing facilities can be optimized to produce competitive individuals.

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