

BIOLOGY OF THE MALARIA VECTOR *ANOPHELES GAMBIAE*: BEHAVIORAL
AND REPRODUCTIVE COMPONENTS OF SUGAR FEEDING

DISSERTATION

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the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

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ABSTRACT

Feeding on sugar from floral and extra-floral nectaries is an important, if less well known, part of mosquito biology. Male mosquitoes, as well as females of a few species, feed only on nectar. Females of blood feeding species feed on sugar to provide energy for flight and survival as well as to improve fecundity. This is the case for every species so far examined. One possible exception to this rule is *Anopheles gambiae*, the world's most important malaria vector. This endophilic, anthropophilic species is thought to forego sugar-feeding, instead taking multiple blood meals when necessary. However, this belief is based largely on observations that nectar sources and fructose-positive females are too infrequent to suggest that nectar feeding is obligatory. My laboratory experiments show that extra-floral nectar-producing plants found commonly near homes in Africa, as well as homopteran honeydew, contributed significantly to the survival of male and female *An. gambiae* when kept in their presence and that males require early access to sugar sources in order to survive and have any reproductive success. Results of a sugar digestion study show that *An. gambiae* digest even large sugar meals at a more rapid rate than other species, and the rate is even higher if metabolic reserves are low or if sugarmeal concentration is low, indicating the need for exercising restraint when interpreting field fructose results. Experiments focusing on the first gonotrophic cycle suggest that sugar feeding increases fecundity more than blood feeding alone. In

addition, multiple blood meals, which occurred mostly after females were gravid, increased fecundity but delayed oviposition. There was no evidence of overlapping gonotrophic cycles due to multiple blood meals. The final set of experiments showed that, while males feed daily on sugar, females do not feed on sugar while developing their eggs. Instead, they sugar-feed after ovipositing and before taking their next blood meal. If blood or oviposition is delayed, they continue to sugar-feed daily for survival. Females can survive equally well on either blood or sugar, but feed less frequently on blood when sugar is available, suggesting that plants might be used to impede malaria transmission.

Dedicated to my son

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FIELDS OF STUDY

Major Field: Entomology

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Chapter 1

INTRODUCTION

1.1 Biology of *An. gambiae*

According to the World Health Organization, malaria is the most common and deadly parasitic disease in the world. It is an ancient disease that is as serious a problem now as it has ever been. Among malaria vectors, *Anopheles gambiae s.s.* is unusually efficient. It is the primary vector in Africa, where 90% of the world's 300,000,000 annual malaria cases occur. It is largely due to this mosquito that over 50% of Africa's population has malaria at any given time. While it is by far the most important malaria vector (Collins and Paskewitz 1995), *An. arabiensis* (a member of the *An. gambiae* species complex) and *An. funestus* are both also important vectors in tropical Africa. They tolerate drier conditions than *An. gambiae* (Gibson 1996, Takken 1999) and may be more important in those conditions. However, *An. arabiensis* tends to be zoophilic (Braak et al. 1994, Githeko et al. 1996) and therefore less efficient a malaria vector. *An. funestus* is not as susceptible to infection by *Plasmodium spp.*, and its importance lies in its numbers (Charlwood et al. 1997).

An. gambiae s.s. Giles females are nocturnal, endophilic, and endophagic (Gibson 1996). The females spend most of their time resting indoors and blood feeding when human hosts are sleeping. This affords freedom from host defensive behavior and is

probably important to anopheline females, which will continue to blood feed even after the midgut is full, concentrating erythrocytes and expelling serous fluid. This phenomenon is called prediuresis and may occur due to limitations of gut expansion and small midgut capacity. Vaughan et al. (1991) showed that host-contact time is increased almost twofold beyond that required for gut filling, when *An. gambiae* females are allowed to feed to repletion. This is particularly important when considering that infection with *P. falciparum* lowers a female's blood-feeding efficiency, increasing the number of partial blood meals required to reach repletion (Wekesa et al. 1992).

In addition to feeding indoors, *An. gambiae* is unusually anthropophilic (Gibson 1996). For most mosquitoes, CO₂ and octenol are attractants used for orienting to hosts (Takken and Kline 1989, Kline et al. 1991). However, *An. gambiae* is not as attracted to CO₂ or octenol (Takken et al. 1997) as are other, more zoophilic species. It is more attracted to volatiles associated with human skin and the microflora present on human skin (Cork and Park 1996, Knols and DeJong 1996), perhaps explaining *An. gambiae*'s host-specificity.

It is assumed that mosquitoes have a gonotrophic cycle that begins with a blood meal and ends with oviposition (Clements 1992). Knowing the duration of the cycle allows one to calculate biting frequency, an important component of vectorial capacity (Garrett-Jones 1964). However, lab (Briegel and Hörler 1993, Klowden and Briegel 1994, Takken et al. 1998) and field studies (Beier 1996) have demonstrated that *An. gambiae* females will take multiple bloodmeals within a single gonotrophic cycle

(gonotrophic discordance). Among the proposed explanations for this behavior is an adaptation for independence from sugar feeding (Beier 1996), where blood meals take the place of sugar meals.

Anophelines, relatively speaking, emerge with low teneral metabolic reserves (Briegel 1990b). Multiple blood meals taken within the first gonotrophic cycle seem to be necessary for small teneral females. The primary ovarian follicles of undersized female mosquitoes may be arrested in the previtellogenic phase, at the stage 1 gate, until a meal of sugar or blood is taken (Clements 1992). This is sometimes known as the “pregravid” state (Gillies 1955) and is linked to juvenile hormone suppression, which is required to stimulate development of follicles to the previtellogenic gate (resting stage) (Clements 1992). After the sugar or blood meal, the follicle develops to the resting stage.

Takken et al. (1998) found that, unlike *Ae. aegypti* (Feinsod and Spielman 1980), sugar feeding was not sufficient to bring follicles of undersized *An. gambiae* to resting stage, and blood was required. The first blood meal helps build protein reserves and develops follicles to the resting stage. The second meal is then used to initiate vitellogenesis and yolk uptake by the oocytes, to develop them completely (Takken et al. 1998). Even well fed, normal-size females show no inhibition to host seeking during the first oogenesis (Klowden and Briegel 1994), though they are able to develop oocytes without a 2nd blood meal (Takken et al 1998).

An anomaly in the development of oocytes has been observed that is somewhat contrary to the above scenario and is worth further investigation. Takken et al. (1998), found, like Briegel and Hörler (1993), that small females with low reserves took a second meal prior to oviposition. However, an initiation of the next cycle resulted in this case,

causing overlapping gonotrophic cycles. This has not been observed in other mosquitoes and, while the implication here is no different from the above scenario for biting frequency, it could have an impact on fecundity.

While these findings have important implications for malaria transmission, the females in these studies were either in their 1st gonotrophic cycle (Briegel and Hörler 1993, Klowden and Briegel 1994), or age was not determined (Beier 1996). Therefore, it is not known if multiple blood feeding and overlapping gonotrophic cycles continue for the life of the mosquito or if it is a phenomenon of small nulliparous females. Gillies (1954) found little evidence of multiple blood feeding in parous females.

1.2 Sugar as a resource for mosquitoes

Sugar derived from plants or honeydew is an essential part of the mosquito diet. For males, and females of some species, nectar is the only food (Van Handel 1984, Foster 1995); and whereas hematophagous females generally utilize protein from blood meals to develop eggs, they still use sugar to help meet their metabolic needs and increase survivorship (Nayar and Sauerman 1975a, Foster 1995). In addition, sugar provides females with a ready source of flight energy (Nayar and Van Handel 1971) and can, in some cases, improve fecundity, both by helping to develop follicles to the resting stage in small females (Magnarelli 1978, Nayar and Sauerman 1975b) and by increasing the number of follicles undergoing vitellogenesis (Mostowy and Foster 2004).

Inferences from field data suggest that there is a lot of variation in the frequency of sugar feeding by mosquitoes. Some species feed on small amounts of sugar frequently (Nasci and Edman 1984), while others may sugar-feed once per gonotrophic cycle (Holliday-Hanson et al. 1997). Whichever is the case, many mosquitoes do not survive

and reproduce as well without sugar in their diet (Nayar and Sauerman 1971, Briegel and Kaiser 1973, Nayar and Pierce 1980, Foster unpublished). However, I will discuss the exceptions to this in the next section.

Currently, the evidence for sugar feeding in *An. gambiae* is inconclusive. In the field, females are thought to sugar-feed rarely, if at all (Gillies 1968, Muirhead-Thomson 1951, Beier 1996), though one study concluded that sugar feeding forms part of their normal feeding behavior (Laarman 1968). The belief that sugar feeding rarely occurs is based largely on collections of indoor resting and biting females that either contained no fluid in the oesophageal diverticulum (Gillies 1968) or mostly tested negative for fructose (Beier 1996). McCrae (1989) stated that sugar sources in Africa were too restricted in time or attractiveness to be of importance to vectors. If so, then male *An. gambiae* must mate soon after emergence. This might be possible, considering that females have higher fecundity when mated with 2-day-old males than with older males (Chambers and Klowden 2001). However, males must emerge with enough teneral reserves to sustain them through mating.

In the malaria vector *An. freeborni*, Holliday-Hanson et al. (1997) demonstrated that fructose is usually lacking in resting and host-seeking females, yet present in gravid females. Given the endophilic nature of *An. gambiae* (Gibson 1996), this is the best time for them to seek sugar, since they must go outdoors to oviposit. Gravid females make up less than 5% of indoor catches (Beier 1996), and there are no field data to demonstrate that sugar feeding is rare when gravid females move outdoors for oviposition or just after oviposition, prior to returning indoors. Therefore, an important portion of the reproductive cycle is missed with respect to determining whether or not they feed on sugar. If one can

assume sugar meals to be relatively small (Smith and Kurtz 1994) and digestion rate to be rapid (Nayar and Sauerman 1975a, Smith and Kurtz 1994), then the potential exists that field-caught females could test mostly negative for sugar when sampling is restricted to only a portion of the reproductive cycle.

It is common laboratory practice to maintain mosquitoes on sugar between blood meals, but Muirhead-Thompson (1951) suggested that sugar is an unnatural diet for *An. gambiae* and may be responsible, combined with restricted blood meals, for life expectancy being shorter in the lab than what is expected in the field. However, we found (Gary and Foster 2001), like Straif and Beier (1996), that sugar, in combination with daily blood meals, did increase laboratory life span over that of daily blood meals alone.

Aedes aegypti (L.), although not closely related, is ecologically similar to *An. gambiae* and seems to have a fitness advantage when feeding on blood alone (Scott et al. 1997, Costero et al. 1998b, Morrison et al. 1999). Like *An. gambiae*, *Ae. aegypti* is anthropophilic and endophilic (Macfie 1915, Foster 1995, Gibson 1996), living where sugar is scarce and blood is readily available. Adapting to this domestic environment may have led, at least in *Ae. aegypti*, to exclusive blood feeding (Macfie 1915, Van Handel et al. 1994, Foster 1995) and the associated higher biting frequency (Foster and Eischen 1987, Scott et al. 1993). The supplemental blood is used to meet metabolic needs (Nayar and Sauerman 1975b) as well as to improve fecundity (Scott et al. 1997).

Nonetheless, sugar feeding in *Ae. aegypti* is more common in the field when nectar sources are abundant (Van Handel et al. 1994, Martinez-Ibarra et al. 1997), despite the

presence of human blood hosts (Martinez-Ibarra et al. 1997) and the apparent cost to fecundity (Morrison et al. 1999), suggesting that exclusive blood feeding is a function of sugar scarcity.

If *An. gambiae* rarely sugar-feed in nature (Beier 1996), then it is not known whether a lack of sugar feeding in nature is due to a lack of available sugar (Martinez-Ibarra et al. 1997) or to a preference for blood (Edman et al. 1992). If the latter is true, it is difficult to reconcile with Beier's (1996) findings that 14.4% of host seeking females contained fructose when the preferred host was constantly available. Furthermore, females in the current study sugar-fed even when offered daily blood meals (Straif and Beier 1996). The continued existence of sugar feeding, both in the laboratory and in the field, indicates that sugar meals still may provide a fitness advantage to this species in some natural situations.

1.3 objectives of this project.

My goal for this research was to investigate (within the limits of laboratory work) the effect of sugar on the reproductive biology, survivorship, and behavior as well as the responsiveness to and handling of sugar by *An. gambiae*. The underlying principle being tested is that sugar feeding is a fundamental characteristic of mosquito life. A new dogma is developing regarding anthropophilic endophilic species, such as *An. gambiae*, and *Ae. aegypti*, that sugar feeding is at most facultative or, in some cases, detrimental to them (Scott et al 1997). Considering these alternate hypotheses, I hope to fill in gaps of knowledge and determine what the potential role of sugar is in this mosquito's life.

Biting frequency and survivorship are key features of vectorial capacity (Garrett-Jones 1964). The equation for vectorial capacity follows:

$$C = ma^2p^n / -\ln p, \text{ where:}$$

C = # new infections per day / original case

m = vector density / host

a = biting frequency

p = survival probability

n = extrinsic incubation period

The extrinsic incubation period (EIP) is the time required for a pathogen to develop in the mosquito host before becoming infective to the vertebrate host. In most mosquitoes, survivorship is increased through sugar feeding (Foster 1995), while biting frequency is increased when sugar is unavailable (Foster and Eischen 1987). The relative importance of biting frequency and survivorship is determined by the EIP of the pathogen. Within the natural range of *An. gambiae*, the EIP of *P. falciparum* is as short as 9-11 days (Lines et al. 1991), making biting frequency more important. Therefore, a readily available sugar source might actually lower vectorial capacity of *An. gambiae* if it were attractive to females. The continued existence of sugar feeding in the laboratory, and possibly in the field, indicates that sugar meals still may provide a fitness advantage to this species in some natural situations. Even if sugar does not provide a fitness advantage, but the females are attracted to it, this behavioral relic makes *An. gambiae* susceptible to manipulation of its vectorial capacity and to attraction to sugar-related stimuli. Sugar-related attractants might therefore be useful in traps to monitor vector populations.

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Chapter 2

Anopheles gambiae Giles (Diptera: Culicidae) Experimental Feeding and Survival on the Extra-floral Nectar of Two Common Tropical Plants

2.1 Abstract

It is widely believed that *Anopheles gambiae* rarely or never feeds on sugar in nature. If so, the need for supplemental blood feeding may be increased. This may help to explain why it is such an efficient malaria vector. Nonetheless, both sexes of this mosquito species readily imbibe and digest sugar solutions, and sugar is a staple of laboratory colonies. In this study, we investigated whether *An. gambiae* will feed on the extra-floral nectar of three common peridomestic plants in Africa, and on mealybug honeydew, and how this affects survivorship. We found that both males and females provided with vegetative parts of cassava (*Manihot esculenta*) survived as well ($\bar{x} = 26.3$ and 19.2 days, respectively) as they did on 50% sucrose solution ($\bar{x} = 29.7$ and 24.3 days respectively) and much longer than they did on water alone ($\bar{x} = 1.8$ days, both sexes). Females provided with mealybug honeydew also lived substantially longer ($\bar{x} = 16.5$ days) than those on water alone. Males and females provided with vegetative parts of castorbean (*Ricinus communis*) also survived much longer ($\bar{x} = 12.7$ and 7.8 days, respectively) than on water, but those provided with flowering lantana (*Lantana camara*) did not. Anthrone

tests of females after 1 night of exposure to these potential energy sources confirmed that they obtained fructose from cassava, from mealybug honeydew, and from non-flowering castorbean, but not from lantana or from castorbean lacking its petiolar nectaries. Previous laboratory studies had shown that sugar availability affects the survival and biting frequency of *An. gambiae*. It now appears that this mosquito can locate natural sources of plant sugar readily and utilize them effectively. Nectar-producing plants in the domestic environment may play a significant role in this mosquito's energy budget and malaria vectorial capacity.

2.2 Introduction

There appears to be much variation in the frequency of sugar feeding and size of sugar meals ingested by female mosquitoes, judging from the contents of their esophageal diverticula (= crops). Females of some species often feed more than once during each gonotrophic cycle, while others may sugar-feed only between gonotrophic cycles or even less often, or ingest only small amounts of sugar at a time (Yuval, 1992; Foster, 1995; Holliday-Hanson et al., 1997; Costero et al., 1998). Whichever is the case, many anautogenous species survive longer and/or produce more eggs with sugar as well as vertebrate blood in their diet (Nayar & Sauerman, 1971,1975; Briegel & Kaiser, 1973; Nayar & Pierce, 1980; Gary & Foster, 2001; Briegel et al., 2002). They also use sugar as a ready source of flight energy (Clements, 1955; Nayar & Van Handel, 1971) and as a nutrient that allows the ovarian follicles of small females to reach the gonoactive resting

stage (Mer, 1936; Feinsod & Spielman, 1980; El Akad & Humphreys, 1990). For males of nearly all species, sugar appears to be essential for them to achieve more than minimal mating activity and early death.

Around 90% of the world's malaria cases occur each year within the range of *Anopheles gambiae* Giles, a highly endophilic, and anthropophilic species. Its efficiency as a vector is due in large part to its high biting frequency. Its gonotrophic cycles are short, and in the laboratory females have been reported to take very frequent blood meals, leading to overlapping gonotrophic cycles (Briegleb & Hörler, 1993). In host-seeking catches and in resting collections of recently blood-fed females in western Kenya, Beier (1996) found that most had oocytes in advanced stages of vitellogenic development (Christophers' stages IV and V), supporting the idea that gonotrophic discordance is not restricted to the laboratory. Among hypotheses for how overlapping cycles or multiple blood feeding within a cycle could evolve, given the obvious risks involved, one probable scenario stands out: Due to a paucity of available sugar sources (McCrae, 1989) but an abundance of exposed and sleeping human hosts, blood has replaced plant sugar as the source of energy for the female, or at least it has become the dominant one.

The case for *An. gambiae* having largely dispensed with sugar feeding, however, is not entirely compelling. In its support, apparently there are no documented reports of directly observed sugar feeding by this species in nature. We are aware only of A.W.R. McCrae's observation of 6 female and 1 male *An. gambiae s.l.* feeding on the extra-floral nectaries of the leaves of *Acacia macrostachya* in The Gambia, among 315 mosquitoes in 31 species or species groups (pers. comm. to W.A.F.). Some field studies have suggested that females sugar-feed rarely, if at all (Gillies, 1968; Muirhead-Thomson, 1951; Beier

1996). These conclusions were based largely on collections of indoor resting and biting females that either contained no fluid in their crops (Gillies, 1968) or mostly tested negative for fructose (Beier, 1996), a plant sugar that is completely converted to other carbohydrates and lipids after it leaves the crop. Muirhead-Thompson (1951) was convinced that sugar is an unnatural diet for *An. gambiae* and may be responsible, in addition to restricted blood meals, for a life expectancy shorter in the laboratory than expected in the field. However, we found (Gary & Foster, 2001), as did Straif & Beier (1996), that sugar, in combination with daily blood meals, increased laboratory life span beyond that afforded by a diet of daily blood meals alone. In addition, one field study presented evidence that sugar-feeding forms part of their normal feeding behaviour (Laarman, 1968).

If *An. gambiae* females sugar-feed regularly in the laboratory but rarely in nature (Beier, 1996), then it remains unknown whether a lack of sugar feeding in nature is due to a lack of available sugar sources or to a preference for blood hosts. If the latter is true, it is difficult to reconcile with Beier's (1996) finding that 14.4% of host-seeking females contained fructose when the preferred host was readily available. In the anthropophilic species *Aedes aegypti* L., the female preference for blood is supported by the high fructose-positive rate among males in populations where that rate is low among females (Edman *et al.*, 1992; Van Handel *et al.*, 1994; Costero *et al.*, 1998). Yet, sugar feeding is much more common in the field when nectar sources are abundant (Van Handel *et al.*, 1994, Martinez-Ibarra *et al.*, 1997), despite the presence of human blood hosts (Martinez-Ibarra *et al.*, 1997) and despite the apparent cost to fecundity (Morrison *et al.*, 1999).

In Mbita Point, Kenya, several species of plants were observed (W.A.F., pers. comm.) occurring commonly around human habitations that might serve as nectar sources for local mosquitoes. These plants included cassava, *Manihot esculenta* Crantz, an important crop plant throughout the tropics. *M. esculenta* can produce copious amounts of extra-floral nectar at the leaflet tips. Other potential nectar sources were two common tropical-subtropical shrubs: castorbean, *Ricinus communis* L., and lantana, *Lantana camara* L. The extra-floral nectaries (EFNs) on the petioles of castorbean leaves have been documented as a feeding site for *Culex* spp. mosquitoes in Florida, U.S.A. (Taylor & Foster, 1996), and a variety of short-tongued insects were observed probing among the flower bracts and other foliage of lantana at Mbita Point. In cages outdoors, unfed males and females of a Tanzanian strain of *An. gambiae*, provided with excess water from paper towels, were observed probing or apparently feeding on these plants during the first half of the night. In the study described below, we placed male and female *An. gambiae* in laboratory cages with cassava, castorbean, lantana, and also with mealybug-generated honeydew, to determine directly and under carefully controlled conditions whether the mosquitoes would feed upon them and ingest fructose, and if so, what effect that had on survivorship.

A similar study was initiated at Mbita and was conducted totally independently during the laboratory investigation described here. It is reported separately (Impoinvil *et al.*, 2003).

2.3 Materials and Methods

2.3.1 Rearing and maintenance.

An. gambiae used in all experiments were of the Suakoko strain, established by M. Coluzzi in 1987 from material originating in Suakoko, Bong, Liberia. Colony adults were maintained on a diet of 10% sucrose, water, and periodic human blood meals. Oviposition cups were placed with caged adults 2 days following each blood meal, and eggs were collected the following day. The laboratory conditions were $27 \pm 1^\circ\text{C}$, $85 \pm 5\%$ RH, and 12:12 (L:D), with 75-min gradual crepuscular transitions between photophase and scotophase.

Larvae for both the colony and experiments were reared as follows: Eggs were disinfected with 0.05% sodium hypochlorite solution and hatched in flat enamel-coated pans of aged tap water. First instar larvae were placed, 100 each, into 22.8 x 33.0-cm aluminium pans with 450 ml of aged tap water. The larvae were fed a standard diet of finely ground TetraMin[®] flakes, following a daily installment schedule that produced a nearly synchronous pupation 7-8 days after hatching. Pupae appearing on the 8th day were transferred to plastic cups and placed in a 41-liter cage supplied with water wicks. Emerging adults were collected the following morning. For all experiments, males and females were separated at that time.

2.3.2 Long-term survival on plant nectar.

Within 12 h of eclosion, 5 females were placed in each of seven 41-liter acrylic plastic cages containing a water-soaked cotton wick and either a) no other food source (water only), b) 50% sucrose via cotton wick, c) a lantana cutting with flower heads, d) a castorbean cutting without a flower head, e) a castorbean cutting without flowerhead and

with EFNs removed, f) a cassava cutting without a flower head, or g) a mulberry leaf, *Morus* sp., coated with mealybug honeydew from *Pseudococcus maritimus* (Ehrhorn). In five of these treatments 5 males also were included. The EFNs of castorbean cuttings were blocked by removing the protruding glands with a blade and covering the wound with fingernail polish, which was allowed to dry for many hours before their exposure to mosquitoes. Cuttings for all experiments were placed in 250 ml Erlenmeyer flasks filled with tap water. Sucrose wicks were replaced every 5 days, and cuttings and honeydew-covered leaves were replaced every 3-4 days or as needed to keep them fresh. Dead mosquitoes were counted and removed daily. This experiment was replicated 3 times.

For statistical analysis of survivorship, some mosquito deaths were censored, because they resulted from predators (Neuroptera: Chrysopidae and Hemiptera: Nabidae) or accidental crushing. The predators were removed immediately upon detection. These extraneous deaths included 5 females and 4 males on cassava, 7 females and 4 males on castorbean, and 1 male on sucrose. Censored data were accounted for by using the Kaplan-Meier test to detect overall treatment-group differences in survival and the Gehan modification of the Wilcoxon test to make pairwise comparisons between diets, using Statistica software (StatSoft 1995). Bonferroni adjustments to the α -level of significance were applied to the Gehan Wilcoxon tests to allow for experimentwise error rates. Results from all replicates of treatment groups were pooled and analyzed as single data sets.

2.3.3 *Nectarivory and resource availability.*

A further step was taken to determine whether mosquitoes derive fructose, a hexose moiety found in nearly all plant nectars and most honeydews, from selected plants

and to determine which of these plants had the most fructose available. Ten females were placed in each of six 41-liter cages prepared as above, containing either a) water only, b) castorbean cuttings with blocked EFNs as described above, c) unaltered castorbean cuttings, d) lantana cuttings, e) cassava cuttings, and f) a mulberry leaf coated with honeydew from the mealybug. The mosquitoes were left with the plants from 1 h prior to scotophase to 3.5 h into the next photophase. Then they were removed and frozen and stored at - 40°C, pending tests for fructose. Mosquitoes were tested individually by the cold-anthrone method of Van Handel (1967, 1972), modified as follows: 0.1 ml of supernatant was placed in 96-well micro-well plates and evaporated; then 0.1 ml anthrone reagent was added and the plate held at 23°C for 1 h, then optical density at 650 nm was read with a micro-well plate reader. Results were compared to standards prepared according to the methods of Haramis & Foster (1983), to convert optical density to µg of fructose.

To determine whether mosquitoes probed for nectar on the surface of extra-floral nectaries or whether they penetrated the tissues of the plant to obtain sap, we made time-lapse video recordings of mosquito behavior on castorbean leaves at 0.5-sec intervals throughout one scotophase, by training an infrared-sensitive video camera on each of two leaves held in flasks under 25-watt red light illumination. Two cages were prepared with castorbean leaves and their petioles. In one cage the mosquitoes were presented with a leaf in which the extra-floral nectaries were blocked as described above, and in the other the leaf was unaltered. Fifty female mosquitoes were placed in each cage. This

arrangement was repeated with 50 males. Direct observations of mosquitoes on the leaves were made during the crepuscular period, which was well before peak feeding activity.

2.4 Results

2.4.1 Survivorship.

There was a significant difference in survivorship for both male and female *An. gambiae* in cages with sucrose ($\bar{x} = 29.7$ and 24.3 days, respectively), cassava ($\bar{x} = 26.3$ and 19.2 days, respectively), honeydew ($\bar{x} = 15.5$ days, females only), castorbean ($\bar{x} = 12.7$ and 7.8 days, respectively), lantana ($\bar{x} = 3.5$ and 2.4 days, respectively), and water alone ($\bar{x} = 1.8$ days each, both sexes) ($P = 0.001$) (Table 2.1, Fig. 2.1).

Female survival on castorbean dropped significantly when the EFNs were removed and the wounds were blocked ($\bar{x} = 2.35$ days vs. 7.8 days on castorbean with unaltered EFNs) ($P < 0.001$), but was greater than on water alone ($\bar{x} = 1.8$ days) ($P < 0.001$). Of the plants tested, females survived longest on cassava ($\bar{x} = 19.2$ days), significantly longer than on castorbean ($\bar{x} = 7.8$ days) ($P = 0.001$).

Post hoc comparison between male and female data by Gehan Wilcoxon test, with $\alpha = 0.02$ after Bonferroni adjustment, showed that although males had consistently higher mean and median survivorship than females, the differences in survival between males and females with access to 50% sucrose, cassava, and castorbean were not significant ($P = 0.03, 0.25, \text{ and } 0.27$, respectively).

2.4.2 Nectarivory and resource availability.

Females that had been exposed overnight to plants and honeydew and tested for presence of fructose provided confirming evidence that they had ingested plant sugars. Anthrone tests of groups of 10 females per plant yielded the following proportion positive for fructose and the amount of fructose present: cassava, 80 %, $\bar{x} = 107 \mu\text{g}$, honeydew 90 %, $\bar{x} = 96 \mu\text{g}$, and castorbean 80 %, $\bar{x} = 68 \mu\text{g}$ (Fig. 2.2). For plant nectars and honeydew that consist primarily of the monosaccharide glucose, the disaccharide sucrose (which is only half fructose), or larger oligosaccharides, the fructose value would be an underestimate of the total sugars ingested. Mosquitoes housed with lantana or with castorbean having blocked extra-floral nectaries were uniformly negative for fructose.

Infra-red recordings of mosquitoes on healthy, non-nectariferous parts of castorbean leaves showed no sustained probing and no evidence of any plant-tissue penetration. Mosquitoes of both sexes aggregated around extra-floral nectaries of a cutting with exposed nectaries. One aggregation of mosquitoes on a leaf with blocked petiolar nectaries was observed. After reviewing the videotape and examining the plant carefully, it was discovered that the mosquitoes were aggregating in an area of leaf damage, where plant juices may have been exposed at the surface.

2.5 Discussion

In the laboratory, the presence of cassava, castorbean, and lantana plants increased the survivorship of male and female *An. gambiae*. Mealybug honeydew increased the survivorship of females. While there was no honeydew available for males,

it is safe to assume it would have increased their survivorship as well. Of these treatments, the mosquitoes survived longest when in cages supplied with cassava, though this was still lower than survival on 50% sucrose. The lower survival on castorbean may be attributed to the inadequate production of sugar by nectaries of the cut plants, relative to the numbers of mosquitoes feeding on them. Cassava is an important crop in tropical Africa and is commonly found near human dwellings where *An. gambiae* breed, rest, and seek blood meals. Castorbean is a common roadside shrub that occurs in a variety of marginal and disturbed habitats in many parts of the world, including communities where *An. gambiae* and malaria co-exist. These plants, along with any available honeydew, are potential sources of energy for both sexes.

The importance of plant sugar to the performance of males of *An. gambiae* is unquestioned. At 27°C they live only 2-3 days (Takken *et al.*, 1998, Foster & Takken, 2003) and fail to inseminate any females (Gary & Foster, unpubl.). At lower temperatures they may perform slightly better, but even so, without sugar their ability to inseminate would be so compromised that a high proportion of females in a population might remain uninseminated in an environment with limited numbers of nectariferous plants. Females, on the other hand, can derive energy for flight and survival from human blood (Briegel, 1990) and therefore may remain relatively productive in the absence of sugar sources. Nevertheless, the fact that they do sugar-feed in nature suggests that it provides females with a competitive advantage, even though that advantage is not apparent in laboratory cages (Gary & Foster, 2001). The relatively low fructose-positivity rates reported in some female *An. gambiae s.l.* samples (4.1% of females resting indoors, 16.9% of host-seeking females) (Beier, 1996) may indicate that sugar-

feeding is uncommon in females. However, a preliminary sample collected by B.G.J. Knols in Ifakara, Tanzania, and tested by us yielded 6 fructose-positives among 14 host-seeking females. And in samples of indoor resting females in The Gambia, A.W.R. McCrae found that up to 30% contained refractile fluid in their crops (pers. comm. to W.A.F.). Thus, it seems possible either that females of this species are highly variable in their utilization of plant sugar, depending on age or environmental circumstances, or that they feed on it frequently but digest it quickly. The presence of sugar sources close to breeding sites may be particularly important, in view of the finding that during the night after emergence, when energy reserves already are dangerously low, both sexes strongly prefer the odour of honey over the odour of human feet (Foster & Takken, 2003).

The presence of nectar-producing plants close to the ambit of *An. gambiae* breeding and adult activities provides males with sustenance and energy for swarming and females with the opportunity to increase their chances of becoming inseminated, finding blood, and living long enough to allow completion of the *Plasmodium* extrinsic cycle and post-extrinsic blood feedings (Gary & Foster, 2001; Okech *et al.*, 2003). At the same time, these plants may reduce human-mosquito contact by reducing the females' need for energy (Gary & Foster, 2001). Either way, these plants have the potential to affect the density, behaviour, and age structure of the adult vector populations and therefore the inoculation rate of malaria.

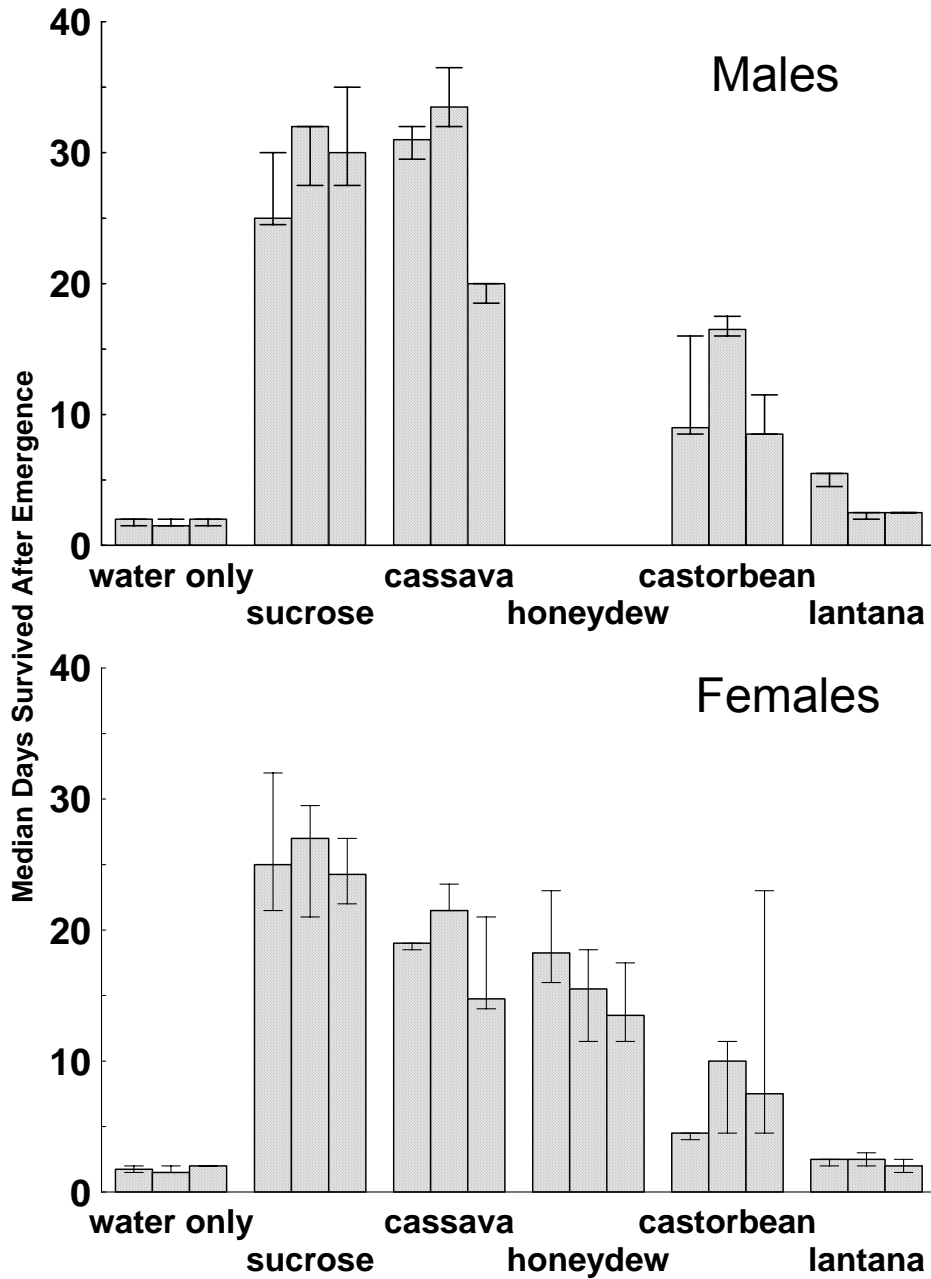


Figure 2.1. Median survival of three replicates of adult male and female *An. gambiae* when placed in cages with various nectar sources. Error bars represent ± 25 percentile.

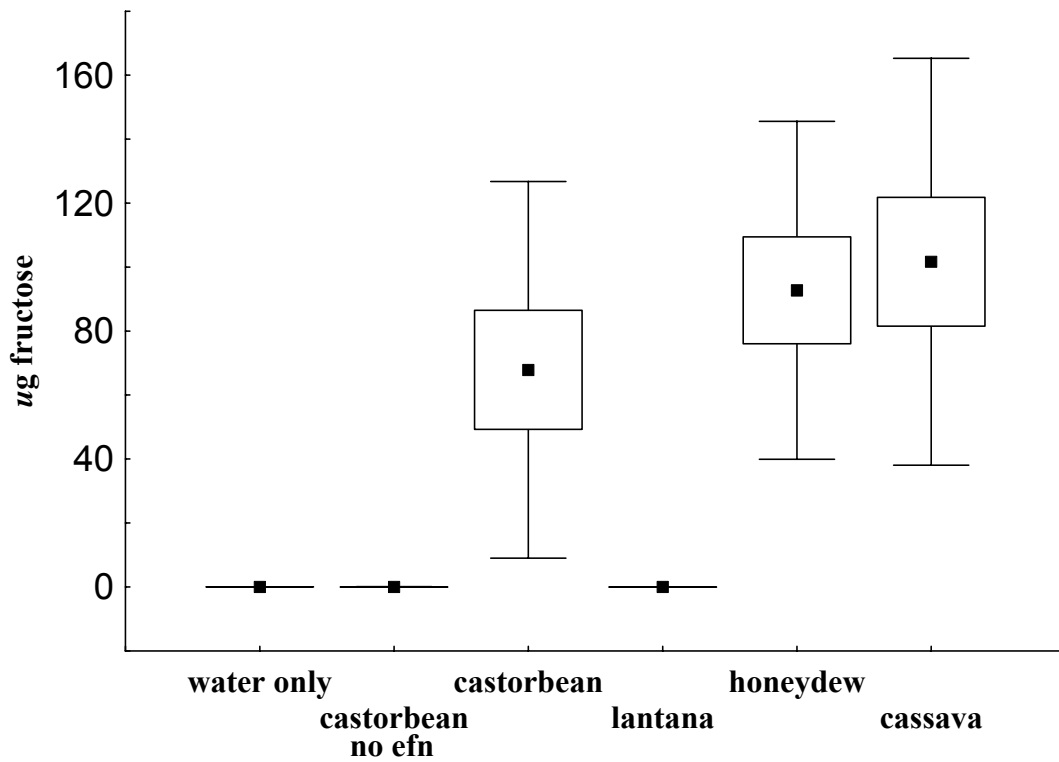


Figure 2.2. Mean μg fructose detected in female *An. gambiae* when placed in cages with various nectar sources over 1 scotophase. Mean includes mosquitoes that had $0\mu\text{g}$ fructose.

Treatment	n	Median	Mean	± S.D.
		days survived	days survived*	
Females				
Water only	15	2.0	1.8 _a	± 0.3
50% sucrose	15	26.0	24.4 _b	± 7.0
castorbean (no efn)	15	2.5	2.4 _c	± 1.2
castorbean	16	5.5	7.8 _d	± 5.9
cassava	15	19.0	19.2 _e	± 6.2
lantana	15	2.5	2.4 _c	± 0.8
honeydew	15	16.5	15.5 _f	± 0.8
Males				
Water only	15	2.0	1.8 _A	± 0.3
50% sucrose	15	28.8	29.7 _B	± 4.8
castorbean	20	11.5	12.7 _C	± 5.5
cassava	15	29.5	26.3 _B	± 9.4
lantana	15	2.5	3.5 _D	± 1.5

Table 2.1. Mean survival times of pooled replicates of adult male and female *An. gambiae* fed various diets.

* Means with different letters are significantly different at $\alpha = 0.002$ for females and $\alpha = 0.005$ for males, Bonferroni adjustment to Gehan Wilcoxon test.

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Chapter 3

Effect of Sugar on Male *Anopheles gambiae* Giles (Diptera: Culicidae)

Mating Performance, as Modified by Temperature and Space

3.1 Abstract

Past studies suggest that sugar feeding is rare for *Anopheles gambiae*, the primary vector of malaria in Africa, because sugar sources are infrequent in space and time. To determine the importance of available sugar on male reproduction, the onset of male mating behaviors and insemination ability were evaluated at two extremes of male body size and two typical environmental temperatures. Males were able to erect their antennal fibrillae and swarm without sugar within 2 days after emergence, but their performance and survival quickly diminished after 2 days. Sugar-deprived males were able to inseminate females in small cages on the second (27°C) and third (23°C) nights. However, in larger cages and under more semi-natural conditions, sugar-deprived male mating capacity was virtually nonexistent. It can be concluded that male performance is closely tied to sugar availability and therefore to plant communities found in their habitat.

3.1 Introduction

Sugar from plant juices is the only food resource of adult male mosquitoes. While both sexes feed on sugar to build energy reserves (Foster 1995), males probably feed on it

more frequently because 1) females get some of their nourishment from blood feeding and 2) males are relatively poor at building metabolic reserves (Van Handel and Lum 1961, Van Handel 1965, O'Meara and Van Handel 1971). Males that emerge with low teneral reserves are especially vulnerable to starvation if they are unable to feed on sugar soon after emergence.

Low teneral energy reserves are typically associated with crowded larval conditions because of the increased competition for limited food. However, some species inherently build a very small teneral reserve, even when larval conditions are ideal. One such species is *Anopheles gambiae* Giles sensu stricto (Briegel 1990b), the principal vector of malaria in tropical Africa. Both sexes emerge with little available energy and, not surprisingly, both sexes are strongly attracted to nectar-related volatiles when newly emerged and often prefer them to host-related volatiles under laboratory conditions (Foster and Takken 2004). This preference suggests that sugar feeding is an early priority, probably due to the risk of starvation. Despite this vulnerability to energy depletion, *An. gambiae* is believed by researchers to feed on sugar in nature only facultatively and rarely (Muirhead-Thomson, 1951; Gillies, 1968; McCrae, 1989; Beier, 1996). McCrae (1989) suggested that sugar sources in tropical Africa are too restricted in time, place, or attractiveness to imply more than facultative feeding. The solution to the energy-deficit problem for females is to feed more frequently on blood, mostly from humans (Gillies 1968, Briegel and Hörler 1993, Beier 1996, Takken et al 1998), which may help to explain the unusual importance of this mosquito in malaria transmission. Males, however, cannot take blood, yet they would be confronted with the same problem as females if nectar sources are scarce. They emerge from the same breeding sites as females, form outdoor swarms in the vicinity of the huts

(Charlwood et al. 2002b), where females get their blood meals, and many rest inside houses along with females (Marchand 1984). It seems unlikely that males would share the females' habitat and at the same time travel unknown distances to find the rare sources of sugar. Therefore, if natural sugar is generally scarce, then the males should be able to survive long enough to swarm and mate without it. Possibly, they are adapted to fast reproductive maturation and frequent early mating, in the likely event of a short lifespan. This is consonant with the report that *An. gambiae* males achieve maximum mating capacity as early as 3 days after emergence (Charlwood and Jones 1979). Additionally, females are more likely to oviposit when mated with 2-3 day old adult males than with older males (Chambers and Klowden 2001). The question remains whether they can successfully accomplish mating without taking a sugar meal.

As part of a larger exploratory project to determine if plant-sugar feeding is a significant part of the natural biology of this species, the present study was designed to determine the importance of nectar sources to male *An. gambiae*. Newly emerged males were observed and tested to determine the effect of sugar availability on reproductive behavior and mating success, as expressed by antennal fibrillar erection, swarming flight, and ability to inseminate females, during and after the evening crepuscular period. In order to gain information applicable to field conditions, large and small males (representing natural extremes) were evaluated at different temperatures. Also, insemination success was measured in three enclosure sizes, ranging from typical laboratory cages to screened greenhouse enclosures that approximated more natural conditions by maximizing space for swarming.

3.2 Materials and Methods

An. gambiae used in all experiments were of the Suakoko strain, established by M. Coluzzi in 1987 from material originating in Suakoko, Bong, Liberia. Colony adults were maintained on honey-soaked sponges, water, and periodic human blood meals. Blood feeding was conducted in accordance with The Ohio State University's Biosafety protocol No. 2005R0020 and Biomedical protocol 200440193. Oviposition cups were placed with caged adults 2 days after each blood meal, and eggs were collected the following day. The laboratory conditions for the colony were $27 \pm 1^\circ\text{C}$, $80 \pm 5\%$ RH, and 13:11 (L:D), with 75-min gradual crepuscular transitions between photophase and scotophase. Laboratory temperatures did not fluctuate as they would under natural field conditions. Instead, two different laboratory temperatures ($23 \pm 1^\circ\text{C}$ and $27 \pm 1^\circ\text{C}$) were used in the experiments, each of which occurs within the natural temperature range at times when breeding is common in equatorial Africa.

Larvae for both the colony and experiments were reared as follows: Eggs were disinfected with 0.05% sodium hypochlorite solution and hatched overnight in flat enamel-coated pans of aged tap water. To produce adults of distinctly different body-size classes, first-instar larvae were placed, 100 each (for large-bodied) or 1000 each (for small-bodied), into 22.8 x 33.0-cm aluminum pans with 450 ml of aged tap water. The larvae were fed a standard diet of finely ground TetraMin[®] fish flakes, following a daily schedule that provided 0.5 mg (for large) or 0.01 mg (for small) of food per larva per day. Pupae were collected daily and segregated in separate 41-liter cages, according to rearing regime, and supplied with water wicks. Males and females emerging overnight were separated the following morning.

Survival. Newly emerged males were divided into three groups of 50 individuals. Each group was placed into a 82-liter acrylic cage supplied with two water-soaked cotton wicks. The first group received no sugar. The second group was supplied with two additional cotton wicks soaked with 20% sucrose and allowed to sugar feed ad lib. The third group was given a single meal of 20% sucrose before they were placed in the experimental cage. To accomplish this, the group of males was placed in a screen-lidded 500ml plastic cup, lined with a paper towel soaked with 20% sucrose. After mosquitoes were allowed to feed for 10 min, by which time all were engorged and had ceased feeding, they were transferred to the experimental cages. Survival was recorded twice daily. This experiment was repeated in two replicates each, with both large and small males, and both at 23°C and at 27°C.

Reproductive behavior. After eclosion, males were transferred, 20 each, into two 41-liter cages supplied with 4 water-soaked cotton wicks each. One cage also was supplied with four wicks soaked with 20% sucrose. A 15-watt red lamp provided ambient light during the crepuscular period and onset of scotophase, when the number of males with extended antennal fibrillae and the number of males swarming were recorded at 5-min intervals through the crepuscular period and first hour of darkness. Although the activity of males approximated the swarming flight described by Charlwood and Jones (1980), the observation cages were too small to determine whether every male was involved in swarming behavior. Therefore, for the purposes of this experiment, the number of males in flight (total number of males, less the number resting), once antennal fibrillae were extended, was considered to be the number swarming. This experiment was replicated four times each, at 23°C and at 27°C, within each size class.

Insemination success. Three separate experiments were conducted to determine the effect of sugar on male insemination success. In the first experiment, 60 newly emerged large males were placed into each of two 82-liter acrylic cages supplied with four water soaked cotton wicks each. One cage was also supplied with four wicks soaked with 20% sucrose. Thirty sugar-fed virgin females, 3-5 days old, were placed in each cage at least 4 hours prior to the start of the crepuscular period. The females were allowed to sugar-feed from emergence until they were put in the experimental cages. To determine cumulative insemination rate, different groups of females in separate cages were left with newly emerged males for 1, 2, 3, 4, or 5 nights, or until most males were dead, after which females were removed and dissected to check for the presence of sperm in the spermathecae. This experiment was replicated four times at 27°C.

In the second experiment, the procedure was the same except that 41-liter acrylic cages were used, and the experiment was replicated four times, both at 23°C and at 27°C, with both large and small males.

In the third experiment, 100 males were released into each of two greenhouse mesocosms, described below, the morning after emergence. One mesocosm was supplied with 10 hanging yellow cellulose sponges (~ 10 x 6 x 1 cm) covered with honey. In each replicate, the two treatments groups were alternated between mesocosms to control for possible local environmental effects. Fifty 5-6 day old females, allowed to acclimate in the mesocosm after emergence in small (15 x 21 x 27 cm) acrylic cages supplied with cotton wicks soaked with 20% sucrose, were released each morning, and most were recaptured the following morning by backpack aspirator (for host-attracted females) and mouth aspirator (for resting females), prior to the release of the next group of females. It

was not possible to recover every released female on each subsequent morning, so it is possible that some females captured on subsequent mornings spent 2 or more nights in the mesocosm. However, this proved to be equally probable in both treatment groups, so systematic errors were not a concern. Captured females were frozen, then dissected to determine daily insemination success. An attempt was made to count resting males each day to determine survivorship. After night 5, all mosquitoes were aspirated for a final account of male survival and female insemination.

The mesocosms used in this experiment were screen enclosures built within two separate greenhouse rooms located in the Ohio State University Biological Sciences Greenhouse facility. Each enclosure was constructed by assembling a lumber frame, covered with surplus mosquito netting. Both mesocosms had the same dimensions of 2.84 x 3.63 x 2.08 m (w,l,h) (21.4 m³). An antechamber served as a safe room to prevent mosquitoes from escaping. The mesocosms were illuminated primarily by direct and indirect sunlight, although to simulate an equatorial photoperiod (12:12, L:D), a portion of the morning was lighted by an incandescent light on a timer. The minimum temperature was maintained by wall radiators heated by steam and regulated by a sheltered, centrally positioned thermostat. Temperatures varied between 23° and 30° C. Humidity cycled between 50% and 90% RH by mist-spraying devices and humidifiers. Walls of concrete blocks, holding beds of wet sand, stood along one wall of each mesocosm to provide resting sites for the mosquitoes and increase moist surface area that helped to stabilize humidity and provide water for drinking.

Statistical analysis. Survivorship data were analyzed by a Kruskal-Wallis test for multiple treatments. For the purposes of this analysis, “deathday” for males with ad-lib

sugar were recorded as time of censoring. Post-hoc multiple comparisons between treatments and against a control were performed using Statistica (StatSoft, Inc.), applying the method of Siegel and Castellan (1988). Other results (sugar effects on fibrillar erection, swarming, and insemination) were analyzed by Chi-square test.

3.4 Results

Wing-length measurements for males reared at densities of 100 per pan ($\bar{x} = 2.87$ mm) and 1000 per pan ($\bar{x} = 2.61$ mm) demonstrated that rearing procedures resulted in two significantly different body-size classes (*t*-test, $P < 0.0001$). Based on field measurements (Lyimo and Takken 1993, Charlwood et al. 2002a), these size classes are representative of natural extremes of adult size.

Survival. Without sugar, large males maintained at 23°C lived longer ($\bar{x} = 3.72$ days) than small males at 23°C ($\bar{x} = 2.99$ days) ($P < 0.0001$) and longer than large males at 27°C ($\bar{x} = 2.36$ days) ($P < 0.0001$) (Table 3.1, Fig. 3.1). A single 20% sucrose meal increased the survivorship of large males by 1 day ($\bar{x} = 4.94$ days) ($P < 0.0001$) at 23°C. When all other variables were equal, large males lived longer than small males, and males at 23°C lived longer than those at 27°C (Table 3.1). In all survival experiments, the majority of males were dead by 3.5 days. Without sugar, the longest survival time was 4.5 days, achieved by a few large males at 23°C (Fig. 3.1).

Fibrillar erection and swarming. On the first night (night 1, 24 hr after emergence), a small proportion of both large and small males had erect fibrillae at 27°C at some time during the crepuscular period or early scotophase (Fig. 3.2a,b), whether or

not sugar was available, but most fibrillae returned to a recumbent position within 10 min during the crepuscular period. Fibrillae of males at 23°C remained recumbent the first night after emergence (Fig. 3.2c,d). By the second night, up to 100% of sugar-fed large males at both temperatures extended their antennal fibrillae (Fig. 3.2b,d), which occurred just prior to the beginning of scotophase and lasted until just after swarming (ca 1.0 hr). On night 2, at 27°C, only 20% of remaining males without sugar had erect fibrillae (Fig. 3.2), which were sustained less than 40 min. In contrast, at 23°C 100% of large males both with and without access to sugar had fibrillar erections on night 2. On night 3 at 27°C (Fig. 3.2) and night 4 at 23°C (Fig. 3.2), there were no erect fibrillae among starved males, and none lived through the night.

In general, sugar was necessary for swarming in both large and small males at 27°C, but not at 23°C (Fig. 3.3). Swarming was first observed the second night after emergence. When sugar was available, swarming was significantly more common at 27°C in males of both sizes ($X^2 = 73.01$ and 87.13 , respectively, $P < 0.05$ both) and at 23°C in small males ($X^2 = 9.13$, $P < 0.05$), than when sugar was not available. That was not the case for large males at 23°C ($X^2 = 0.54$, $P > 0.05$); those with and without sugar swarmed in similar numbers. By night 3, when sugar was present, a higher proportion of large and small males swarmed at both 23°C ($X^2 = 20.01$ and 71.54 respectively, $P < 0.05$ both) and 27°C ($X^2 = 200$ and 151.65 respectively, $P < 0.05$ both). Without sugar, swarming activity by males of both sizes ceased after the second evening at 27°C, and after the third evening at 23°C (Fig. 3.3). With sugar available, swarming activity was near 100% for both body sizes, starting on night 2 at 27°C and on night 3 at 23°C.

Insemination success. In 41-liter cages, when sugar was present, cumulative insemination rates increased over 5 days from 0 to 88 % (large males at both temperatures), to 60% (small males at 27°C), and to 73% (small males at 23°C) (Fig. 3.4). In the absence of sugar, insemination increased similarly to those with sugar over the first 2 nights at 27°C and over the first 3 nights at 23°C. Sugar had no significant effect after 2 nights of mating opportunity in any treatment group, except among small males held at 27°C, in which sugar-deprived males inseminated slightly fewer females (4%) than males with sugar (12%) ($X^2 = 4.60$, $P < 0.05$).

By day 3, there were no surviving males at 27°C in sugar-deprived groups. However, at 23°C, both large and small sugar-deprived males were still alive and had inseminated females (32% and 15% respectively). This was still significantly less than insemination rates of those with sugar (58% and 41%, respectively) ($X^2 = 14.17$ and 16.58 respectively, $P < 0.05$ both) and there were no inseminations beyond night 3 among sugar-deprived males.

In 82-liter cages at 27°C, the large males with access to sugar produced results were similar to those above. Unlike the experiment above, however, sugar-deprived males had very low rates of insemination after the first (1%) and second (3%) nights, and no inseminations after that (Fig. 3.5). Males with sugar had much higher insemination rates after 2 days (43%) ($X^2 = 44.68$, $P < 0.05$). After 5 nights, males with sugar had inseminated 82% of the females.

In the mesocosms, results were very similar to the 82-liter cage experiment above, except that very few inseminations occurred before night 3 (Fig. 3.6). The insemination rate rose substantially on night 3 if sugar was available (26.5%) but not if sugar was

withheld (0%) ($P < 0.05$). Daily insemination rates remained high after night 3 when sugar was available, but most males without sugar were dead by night 3 when sugar-deprived. Daily counts of resting males suggest that, in each replicate, there was low survivorship the first night, whether or not sugar was present ($\bar{x} = 41.3\%$ and $\bar{x} = 42.3\%$, $P = 0.51$). After the first night, survival remained high in the room with honey while declining in the honey-deprived room. After the 3rd night, 38% of males remained alive in the honey-supplied room and 3.6% remained alive in the honey-deprived room.

3.5 Discussion

The results of this study suggest that *An. gambiae* males require early and frequent access to nectar or other sugar sources in their habitat to survive and mate. In nature, males form daily mating swarms that last for about 0.5 hours at dusk (Charlwood et al. 2002b), during which time females entering the swarm are inseminated. Just prior to taking flight, the antennal fibrillae of males become erect and remain so until the end of swarming (Charlwood and Jones 1979). In the present study, mature male *An. gambiae* performed similarly, but they required a period of maturation after emergence to exhibit these behaviors. Male anophelines of other species rarely swarm before the second night after emergence (Reisen and Aslamakhan 1979, Reisen et al 1981). We found similarly that very few male *An. gambiae* erect their antennal fibrillae or swarm before the second night after emergence. By then, their ability to do so without sugar feeding was associated with larger body size and lower temperature. Presumably, the larger males had higher teneral reserves to rely on, and the lower temperature (23°C) slowed the depletion of those reserves without retarding reproductive maturation to such

a degree. By the third day, the effects of sugar deprivation were evident in the reduced performance of these behaviors, no matter the size class or temperature.

Daily insemination ability dramatically increased on the third night after emergence in the semi-natural conditions of the mesocosms. Other studies have demonstrated that insemination ability peaks at 3 days (Charlwood and Jones 1979, Reisen et al. 1979, Mahmood and Reisen 1982) or even 7 days after emergence (Verhoek and Takken 1994). Females are more likely to oviposit when inseminated by 2-3 day old males than by older males (Chambers and Klowden 2001). Therefore, sugar-deprived males, which survive long enough and exhibit appropriate mating behaviors, might still make important contributions to the next generation. However, we found that in large cages and in greenhouse enclosures, the mating capacity of sugar-deprived males was very low, often nonexistent, and never reached that of sugar-fed males. Only in smaller cages was the mating capacity of sugar-deprived males as high as that of sugar-fed males and then only for the first 2 days. While highly artificial, this small cage experiment probably represents the limits of what is possible. Alternatively, the large cage and greenhouse experiment demonstrate what is probable under more natural conditions. Therefore, while it is possible that sugar-deprived males can mate, especially within the first 2 nights after emergence, it is not likely to happen often in nature, and sugar-fed males will probably have a very large competitive advantage.

An. gambiae females may be able to use human blood (Muirhead-Thomson 1951, Gillies 1968, McCrae 1989, Beier 1996) for survival and performance. Male performance, however, is closely connected to sugar availability and therefore to the composition of plant communities. In contrast to McCrae's (1989) conclusion that plant

sugar sources are too restricted to be of any importance to this species, we conclude that sugar sources must be readily available in areas where this species thrives. If females feed restrictively on sugar (Beier 1996), it is not due to a lack of available nectar sources. Recent studies have shown that males, as well as females, can survive by feeding on the fluids of plants commonly found around villages and homes in Mbita Point, Kenya (Gary and Foster 2004, Impoinvil et al. 2004). One plant that males and females thrived on was *Manihot esculenta*, a food staple grown for its starchy root. This plant produces extrafloral nectar from leaf edges and petioles. It is often grown abundantly in and around villages and homes. Charlwood et al. (2002a) found that the fence around a garden of manioc (*M. esculenta*) provided the swarm marker for the daily occurrence of the largest male mating swarm in the village. Unlike other swarms in the village, this one could not be diverted by experimental markers and ceased once the manioc was harvested. Here, in close proximity to dwellings, is an exceptional example of available nectar within easy reach of both males and females. This is not to imply that the extrafloral nectaries of manioc are an important nectar source for this species. Field evidence is lacking. Newly emerged males and females are known to be attracted to floral odors (Foster and Takken 2004), though preferred plant species have not yet been identified.

It is evident that male *An. gambiae* require early and frequent access to sugar to exhibit normal reproductive behaviors and effectively mate. Small-cage experiments demonstrated that some males are capable of mating on the second night after emergence without sugar, but this is much less likely to occur in the field where energy demands are sure to be much higher than in the laboratory. This species emerges with low teneral reserves (Briegel 1990b), and sugar feeding is likely to be an early priority (Yuval 1992,

Foster 1995, Foster and Takken 2004). In addition, male mosquitoes in general are relatively poor at building metabolic reserves (Van Handel and Lum 1961, Van Handel 1965, O'Meara and Van Handel 1971), so it is not likely they would travel far to obtain sugar. This is particularly true of small males, which make up a substantial proportion of some natural populations and have been shown to mate just as successfully in the field as larger males (Charlwood et al. 2002a). Without sugar, a vast majority of small males in the present study survived less than 3 days and had virtually no mating success.

In an environment with limited nectar sources, a few males might have success inseminating females, but their ability would be so diminished, that a high proportion of females might remain uninseminated. Where preferred nectar-providing plants are present, it may be possible to interfere with male reproductive capacity by genetic manipulation of the plants. Even if female reproductive success is not tied directly to sugar feeding, the reduction in female egg output resulting from delayed insemination, and therefore delayed oviposition, can nevertheless affect a population's vectorial capacity (see chapter 1) by reducing both m , the vector density, and a , the biting frequency.

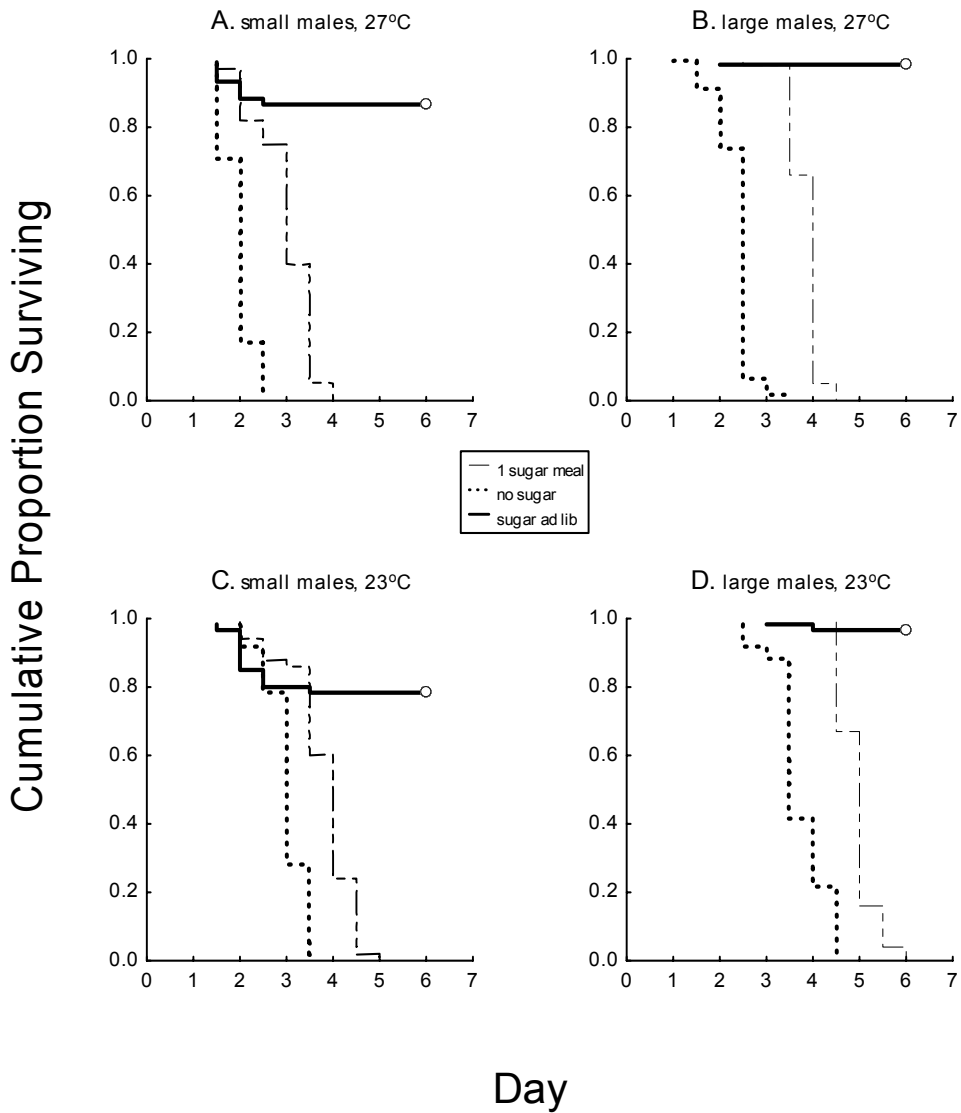


Figure 3.1. Survival of small (A,C) and large (B,D) males at 27°C (top) and 23°C (bottom) given 1 sugar meal (25% sucrose) the evening after emergence, given sugar ad lib, or given water only (all had water). Open circles denote censored data. For each treatment, n = 80.

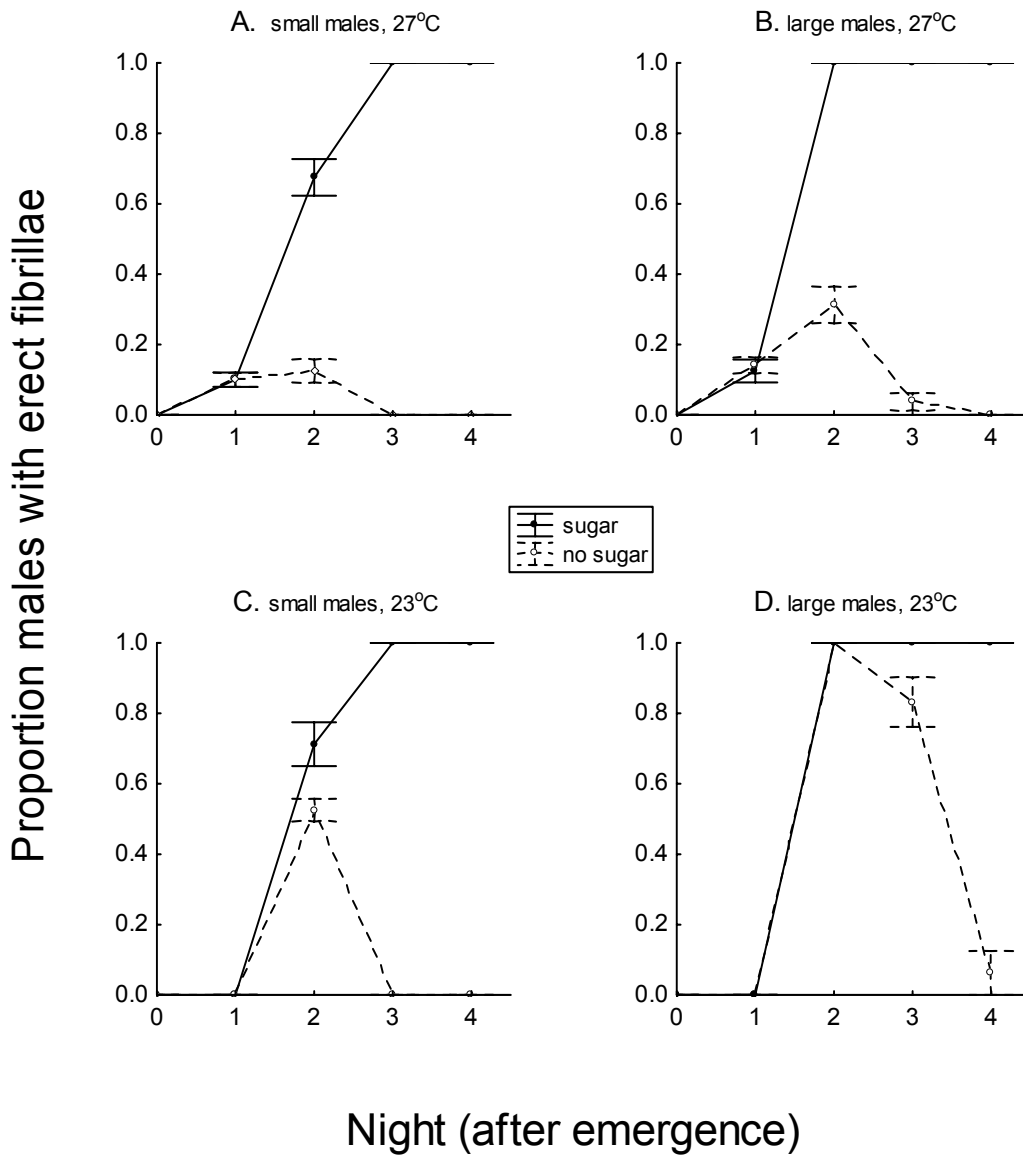


Figure 3.2. Effect of available sugar on the maximum proportion of small (A,C) and large (B,D) males with extended fibrillae each night after emergence at 27°C (A,B) or 23°C (C,D). More males with sugar access erected their fibrillae than males without sugar ($P = 0.05$), starting from night 2 (A,B,C) or night 3 (D) onward.

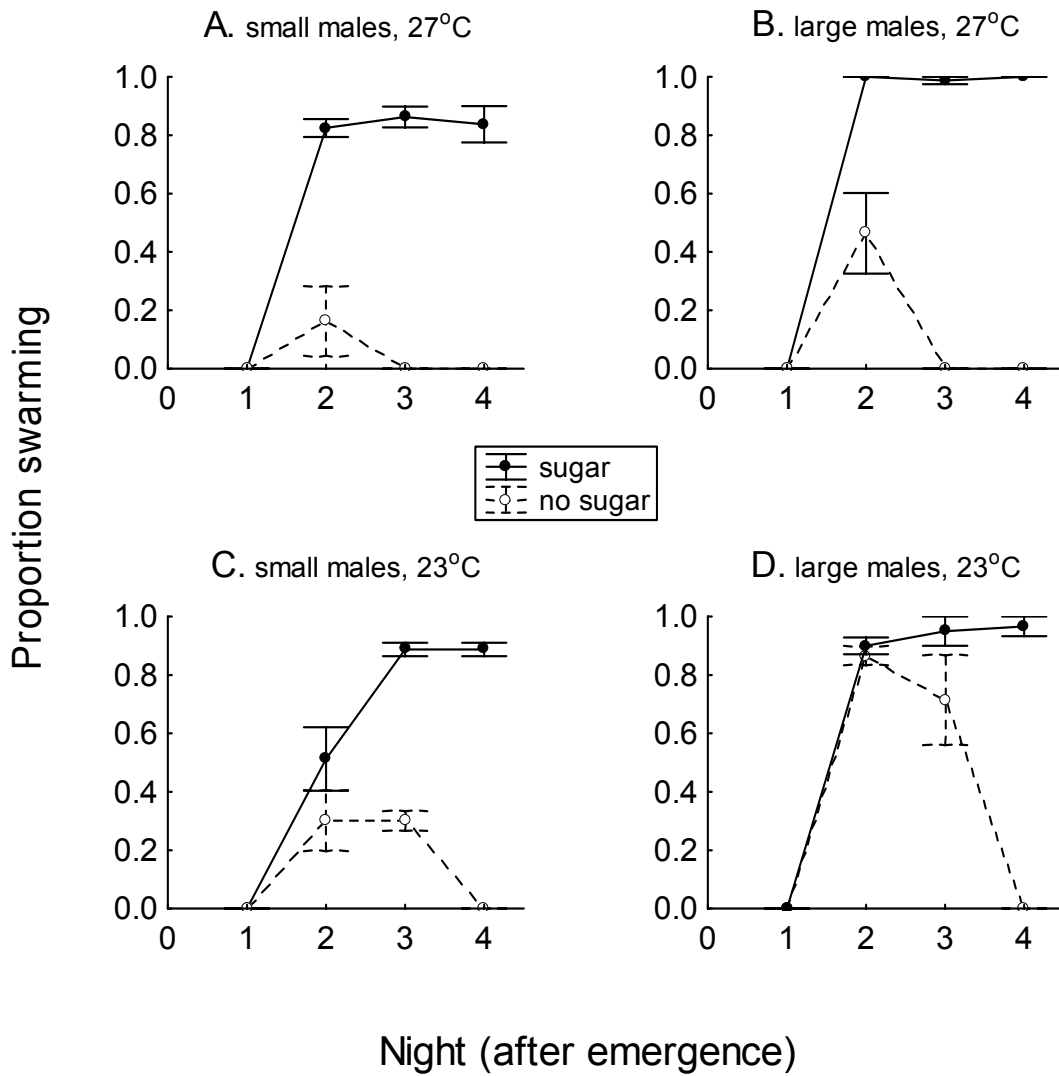


Figure 3.3. Effect of available sugar on the maximum proportion of small (A, C) and large (B,D) males swarming each night after emergence at 27°C (A,B) or 23°C (C,D). More males with sugar access swarmed than males without ($P = 0.05$), starting from night 2 (A,B,C) or night 3 (D) onward.

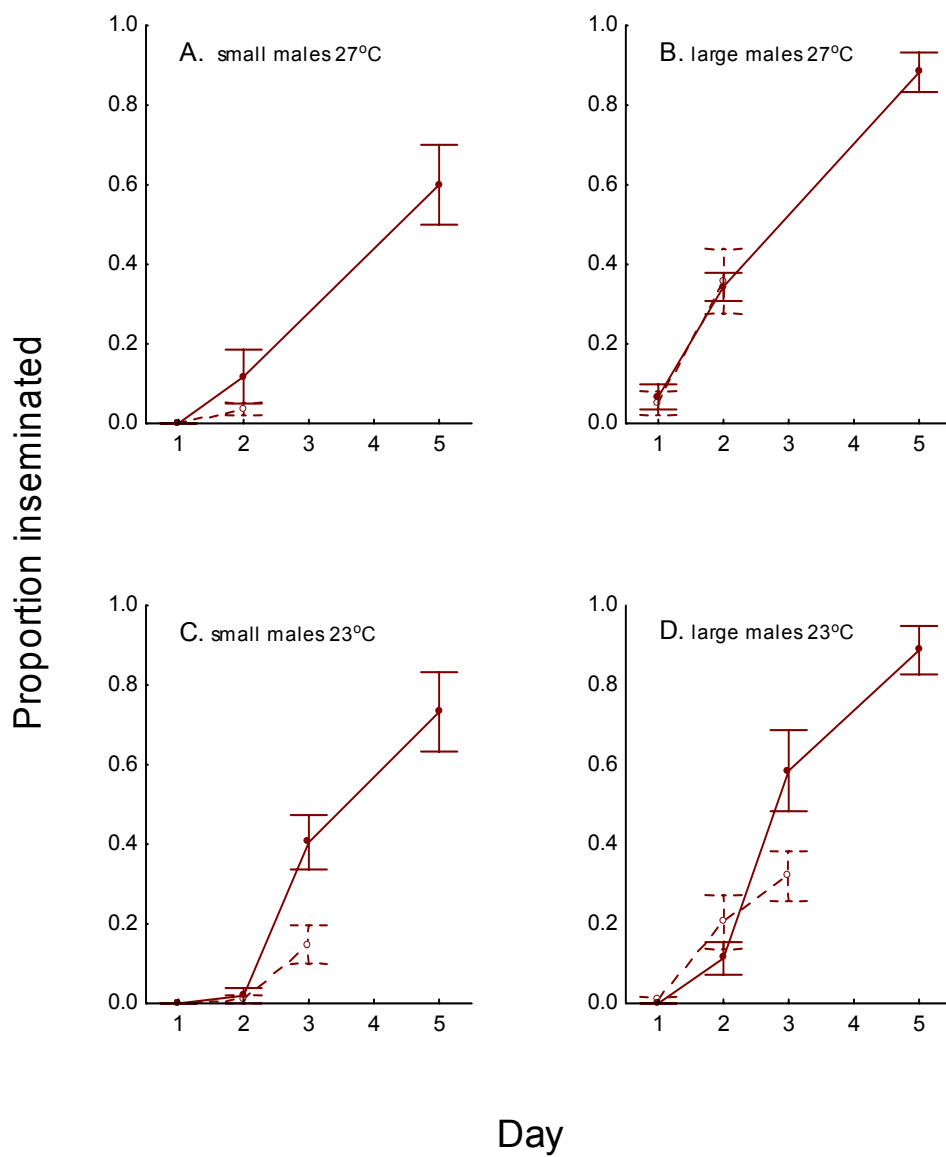


Figure 3.4. Effect of available sugar on the cumulative insemination success of small (A, C) and large (B,D) males in 41-liter cages each night after emergence at 27°C (A,B) or 23°C (C,D).

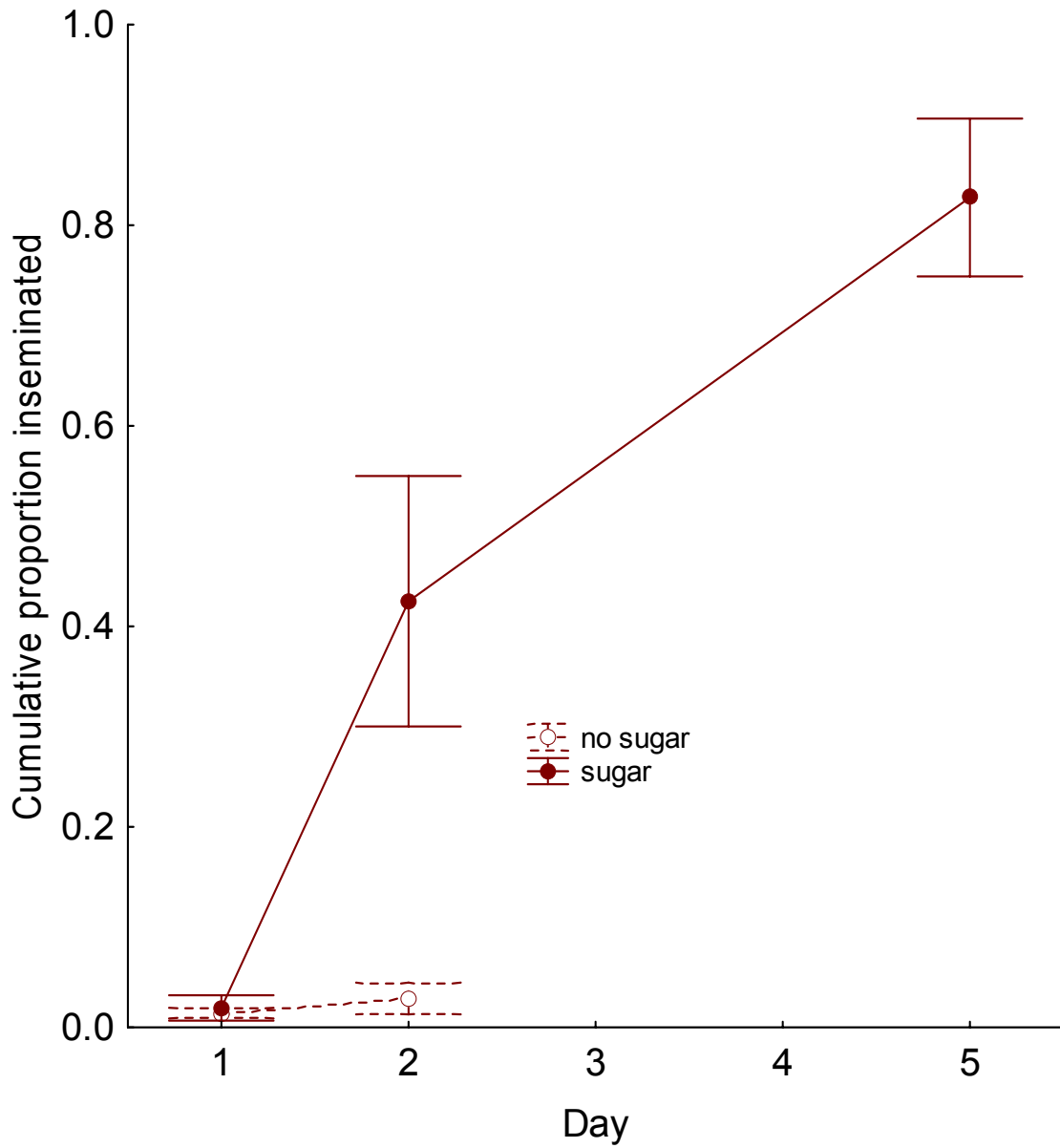


Figure 3.5. Effect of available sugar on the cumulative insemination success of large males in 82-liter cages at 27°C.

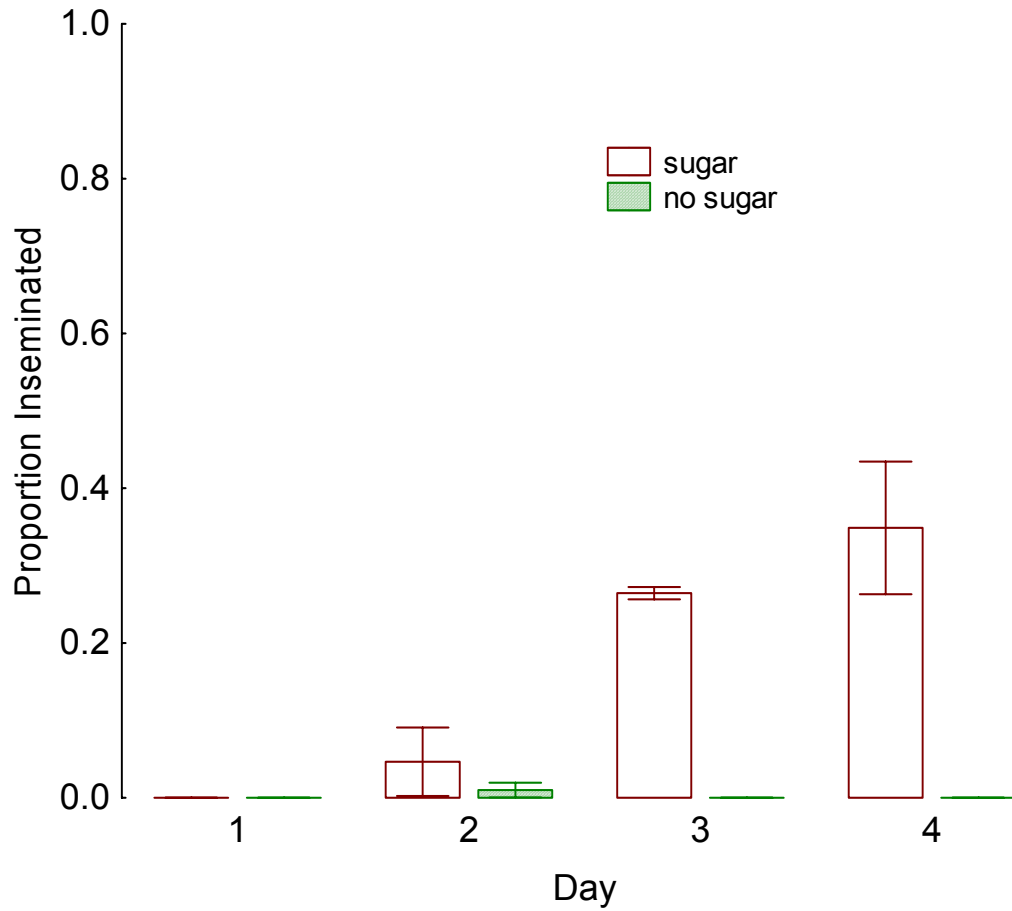


Figure 3.6. Effect of available sugar on the daily insemination success of large males in a greenhouse enclosure with temperatures allowed to fluctuate between 23°C and 30°C.

Mean days survived* ± SD (N)		
Treatment	Large Males	Small Males
23°C		
no sugar	3.72 ± 0.56 (97) _{a,b}	2.99 ± 0.44 (101) _{b,d}
1 meal 20% sucrose	4.94 ± 0.39 (100) _e	3.77 ± 0.70 (100) _a
20% sucrose ad lib**	> 6 days (100) _f	> 6 days (99) _f
27°C		
no sugar	2.36 ± 0.39 (120) _{c,d}	1.94 ± 0.34 (100) _c
1 meal 20% sucrose**	3.84 ± 0.33 (103) _a	3.15 ± 0.66 (100) _b
20% sucrose ad lib	> 6 days (100) _f	> 6 days (100) _f

Table 3.1. Effect of sugar feeding on survival times of large and small virgin male *An. gambiae* at 2 temperatures.

* Means with different letters are significantly different after post-hoc multiple comparisons between treatments and against a control (method of Siegel and Castellan 1988) in a Kruskal-Wallis rank test.

**Data were censored at 6 days.

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Chapter 4

Rate of Sugar Digestion in *Anopheles gambiae* (Diptera: Culicidae) Under Controlled Conditions: Effect of Internal State and Comparison to Other Species

4.1 Abstract

The low proportion of female *Anopheles gambiae* Giles containing fructose in western Kenya suggests that they may seldom feed on plant sugar. An alternative explanation is that they digest sugar meals rapidly, either because the meals are small or because energetic demands are large. To test the last hypothesis, the rate and total time of digestion of *ad lib.* sugar meals were measured in *An. gambiae*, *Aedes aegypti* (L.), and *Ochlerotatus triseriatus* (Say) under controlled conditions. In addition, within *An. gambiae*, females were compared with males, and the effects of high sugar concentration, presence of a digesting blood meal, and high energy reserves were examined. In the species comparison, *An. gambiae* females digested sugar significantly more rapidly than the other two species and reached basal levels much sooner, even though *Ae. aegypti* and *An. gambiae* ingested similar quantities. Males and females digested sugar at approximately the same rate, but females ingested larger meals than males when the sugar concentration was high. Digestion rate was slightly affected by concentration in both sexes, but more concentrated meals were larger, and larger meals took more time to be completely digested, which accounted for most of the difference. However, high

energy reserves, the result of extensive sugar feeding during the previous week, reduced sugar-meal size and slowed its digestion rate considerably. The presence of a blood meal did not significantly affect either the size of a sugar meal or its rate and total time of digestion. Together, these results suggest that a low prevalence of fructose positivity of *An. gambiae* in nature can be a reflection not just of infrequent sugar feeding but also of an intrinsically rapid rate of sugar digestion in this species, integrated with the energy status of the population and with the characteristics of the sugar available.

4.2 Introduction

Sugar provides an important food for mosquitoes. It is the principal food for males of all species and also for females of species that do not feed on blood (e.g. *Toxorhynchites* spp.) (Foster 1995). For females that do feed on blood, sugar provides most of the fuel for flight and maintenance (Nayar and Van Handel 1971, Nayar and Sauerman 1975a, Foster 1995). Blood also can be used for flight fuel, but must first be converted to glycogen, which takes about 24 hours (Nayar and Van Handel 1971) and is therefore not as efficient.

In nature, mosquitoes obtain sugar meals primarily from plant sources such as floral and extra-floral nectaries, damaged fruit, and honeydew (Foster 1995). Sugars from these sources consist mostly of fructose, glucose, and sucrose (Van Handel 1972). Other sugars and amino acids also may be present, but usually in trace amounts only (Auclair 1963, Van Handel et al. 1972, Baker and Baker 1983).

Field caught mosquitoes can be evaluated for recent feeding on nectar or other plant sugars by the cold-anthrone test (Van Handel et al. 1972). This test utilizes anthrone reagent to detect fructose (evidence of recent plant associated sugar feeding) when

conducted at room temperature (Van Handel 1967). The cold anthrone test has been used widely to confirm the pervasiveness of sugar feeding of many species (Foster 1995). It also has been used to provide evidence for infrequent sugar feeding in some anthropophilic species, such as *Aedes aegypti* (L.) and *Anopheles gambiae* (Giles) (Edman et al. 1992, Beier 1996, Costero et al. 1998a). Yuval (1992) suggested that this might be an epidemiologically significant phenomenon caused by having easier access to blood meals than to sugar meals. It is in this setting that *Ae. aegypti* females have been shown convincingly to live without sugar (Edman et al. 1992, Day et al. 1994) while enhancing their reproductive fitness (Scott et al. 1997, Day et al. 1994, Costero et al. 1998b, Naksathit and Scott 1998) in the laboratory. But even this important anthropophilic species utilizes sugar when nectar sources are readily available and human hosts uncommon (Van Handel et al. 1994, Martinez-Ibarra et al. 1997), presumably to optimize fitness. The practical importance of this decision to feed on sugar when available is that, while it may have a fitness benefit, it also reduces biting frequency and therefore, possibly, vectorial capacity.

Not nearly as much information exists for *An. gambiae* sugar feeding, though it is believed to feed on sugar rarely or never in nature (Muirhead-Thomson, 1951; Gillies, 1968; McCrae, 1989; Beier, 1996). Even in the laboratory, Muirhead-Thomson (1951) associated a shortened life of this species with sugar feeding, which he thought was an unnatural food and probably acted as a source of fungal infections. Existing field evidence is based on collections of indoor resting and biting females, which either contained no fluid in the oesophageal diverticula (Gillies, 1968) or mostly tested negative for fructose (Beier 1996). However, this evidence is not convincing, because it does not account for the possible cyclic nature of sugar feeding (Holliday-Hanson et al. 1997). A significant part of

the gonotrophic cycle, when females return outdoors to oviposit, is unaccounted for by these assessments of restricted sugar feeding. This is when the opportunity and need for sugar feeding possibly would be greatest. By this time, the female will have gone at least 2 days without food while developing eggs, depending on the length of the gonotrophic cycle and assuming she did not take additional meals during vitellogenesis. Beier's (1996) data from Kenya show that 14 % of biting and 6 % of resting females were fructose positive. Though he interpreted this as restrictive sugar feeding, that interpretation overlooks the possibility that sugar feeding takes place in association with oviposition. Assuming a female takes a sugar meal while out in the field to lay eggs, she is more likely to test positive before taking her next blood meal than after, depending on the size of the sugar meal and the rate of sugar digestion. Additionally, it is possible that the resulting sugar meal is digested rapidly, becoming undetectable within a short time after the mosquitoes arrive at resting sites.

As a mosquito feeds on a sugar solution, most of it enters the ventral diverticulum (crop), where it is stored. The crop gradually empties while the mosquito is otherwise engaged, and the fructose transferred from it, including the fructose moiety of sucrose, is rapidly absorbed by the midgut and converted to other metabolites. Thus, the rate of depletion of fructose in the mosquito's body is a function of the time since feeding. The rate of sugar depletion, here called "digestion rate," is exponential (Van Handel 1965, Nayar and Sauerman, Jr. 1975), so it is possible to derive the approximate interval between sugar meals from the proportion of a population that is in the process of digesting sugar meals. However, factors such as meal size, sugar concentration, metabolic reserves, and presence of a blood meal may affect the speed of disappearance and total time taken before

fructose becomes undetectable (Foster 1995). Thus, without knowing the approximate rate of sugar digestion in this species under a variety of conditions, the frequency of sugar ingestion cannot be determined with confidence from field data on fructose prevalence. In other species investigated, the time to digest a sugar meal varied from under 20 hours up to 4 days (Reisen et al. 1986, Andersson and Jaenson 1987, Costero et al. 1998a).

Using a modification of the cold-anthrone test to quantify the rate of digestion under controlled conditions, in this study of sugar-feeding frequency we test the hypothesis that *An. gambiae* digests sugar more rapidly than other species. We also address the effects of sex, sugar concentration, energy reserves, and the presence of a digesting blood meal on its sugar digestion rate.

4.3 Materials and Methods

4.3.1 Mosquito rearing and maintenance

An. gambiae used in all experiments were of the Suakoko strain, established by M. Coluzzi in 1987 from material originating in Suakoko, Bong, Liberia. The *Ae. aegypti* colony was started from eggs collected in San Juan, Puerto Rico, in 1997, the *Oc. triseriatus* colony from larvae collected in Columbus, Ohio, U.S.A in 1999. Colony adults were maintained on honey-soaked sponges, water, and periodic human blood meals. Oviposition cups were placed with caged adults 2 days after each blood meal, and eggs were collected the following day. The laboratory conditions for the colony were $27 \pm 1^\circ\text{C}$, $85 \pm 5\%$ RH, and 13:11 (L:D), with 75-min gradual crepuscular transitions between photophase and scotophase. Conditions for *Oc. triseriatus* and *Ae. aegypti* were the same, except that day length was 16:8 (L:D).

Larvae for both the colony and experiments were reared as follows: Eggs were disinfected with 0.05% sodium hypochlorite solution and hatched overnight in flat enamel-coated pans of aged tap water. First-instar larvae were placed, 100 each, into 22.8 x 33.0-cm aluminum pans with 450 ml of aged tap water. The larvae were fed a standard diet of finely ground TetraMin[®] fish flakes, following a daily schedule that produced a nearly synchronous pupation 7-8 days after hatching. Pupae appearing on the 8th day were transferred to plastic cups and placed in a 41-liter cage supplied with water wicks. Males and females emerging overnight were separated the following morning.

4.3.2 Experimental setup

For each experiment, newly emerged mosquitoes were divided into 2 groups, each consisting of approximately 80 individuals. Sugar feeding for experiments was accomplished by placing the group of females into a 500 ml plastic cup with a screen lid and lined with a 10% or 20% or 50% sucrose-soaked paper towel. After mosquitoes were allowed to feed for 10 min, by which time all were engorged and had ceased feeding, they were transferred, 10 each, into 0.45-liter paper-carton cages and supplied with water via a cotton dental wick and held at 27±°C. These cartons were placed in a -40°C freezer at intervals from 0 to 66 hrs after sugar-feeding. After freezing and short-term storage at -40°C, all samples were transferred to a -80°C freezer to prevent all enzymatic activity during storage (Van Handel 1985).

High versus low sugar-meal concentration. One-day-old males and females maintained on water only were separated into two groups each. Each group was allowed to feed for 10 min on either 10% or 50% sucrose. They were then separated into 0.45-liter carton-cages until time of freezing.

High versus low metabolic reserves. Each of two groups of female *An. gambiae* was placed in a 41-liter cage and maintained for 6 days on a diet of either 1.2 or 50% sucrose. The group with 50% sucrose was then switched to 10% sucrose for 2 days. This was done to reduce the amount of high-concentration sucrose in the crop before experimental feeding. The sugar was then removed 18 hr prior to experimental sugar feeding. Anthrone testing revealed that $< 1 \mu\text{g}$ of fructose remained in the crops at this time. The other group was allowed access to 1.2% sucrose the entire time. Females in both groups were then fed 20% sucrose and placed into the 0.45-liter carton-cages until time of freezing.

Blood meal prior to sugar feeding. One group of females was allowed to blood-feed from the arm of a human subject (R.E.G.) on night 1, about 24 hr after emergence. Experimental sugar feeding of 20 % sucrose, as described above, took place the following afternoon, 24 hr after blood feeding. Mosquitoes were then transferred into the 0.45-liter carton-cages and held at $27^{\circ}\pm\text{C}$ until time of freezing.

An. gambiae vs. *Oc. triseriatus* and *Ae. aegypti*. One group, each, of 2-day old *Oc. triseriatus* and *Ae. aegypti* females was fed 20% sucrose for 10 min, as above, then transferred to 0.45-liter cages and held at $27^{\circ}\pm\text{C}$ until time for freezing. After testing, the resulting regression function was compared to female *An. gambiae* fed 20% sucrose.

4.3.3 Modified cold-anthrone test.

Crop fructose was detected using anthrone reagent (Van Handel et al. 1972) and quantitatively assessed using methods of Haramis and Foster (1983) with modifications described in the following sections.

Sample preparation. Each mosquito was placed in a 2 ml graduated, flat cap microcentrifuge tube with a single, copper-clad, steel bead (BB-caliber airgun shot) and 0.2ml absolute methanol. Samples were then homogenized by placing tubes into an MM300 Mixer Mill (QIAGEN, Valencia, Calif.) at 25 Hz for 60 sec. After grinding, the homogenate was centrifuged for 1 min in an Eppendorf® micro-centrifuge at 14000 rpm. All supernatant was extracted by pipet and filtered through a QIAamp spin column (QIAGEN) into a 1.5 ml graduated micro-centrifuge tube by centrifuging at 14,000 rpm for 1 minute. Once filtered, 0.1 ml of supernatant from each tube was added to a 96-well tissue culture plate and allowed to evaporate. Standard sucrose solutions corresponding to 0, 1, 2, 4, 8, 16, 32, 64, 128, 256, and 512 µg/µl (0.1 to 51.2% solutions)(Haramis and Foster 1983) were added, 1 µl each, to the top two rows of each plate.

Cold-anthrone test. One-tenth ml of anthrone reagent was added to each well. The plates were held at 26°C for 1h and agitated at 0, 30, and 60 min by utilizing the shaking feature of a Spectra 96-well microplate reader (Tecan Instruments, Maennedorf, Switzerland). Following the final agitation, each plate was read at 650nm to obtain optical densities of the samples. The resulting optical densities of the test samples were then compared to those of known standards to determine the amount of fructose present.

Blooded females created a problem for the plate reader, because the undigested blood meal, which could not be completely filtered, stained and darkened the samples, increasing light absorbance. To overcome this problem, plates containing blooded-female samples were read twice; once immediately after anthrone reagent was added to

the wells and once after 1 h. Resulting optical density readings of absorbance due to fructose were generated from the difference between these two readings and compared to standards containing no blood.

4.3.4 Data Analysis

Standard solutions were plotted on a line, and the resulting equation was used to determine the μg fructose present in each mosquito tested. Results were then analyzed by regression and plotted on graphs using Statistica (Statsoft, Inc.). Regression functions were compared by ANOVA using methods of Neter et al. (1996) to determine differences in regression function and slope (digestion rate). For tests involving regression function and slope, significance level was 0.01. Differences in the amount of sugar present at any one time were determined by *t*-test.

4.4 Results

In all mosquitoes tested, fructose digestion followed an exponential rate of decline. Only data that were part of the initial decline were used to calculate regression functions. Once μg of fructose approached zero, the rate of digestion slowed, suggesting that a small residue of sugar is difficult to evacuate or is left in reserve. Including these data would artificially affect the regression function, incorrectly showing a decrease in the overall rate of digestion.

Males ingested less high-concentration (50%) sugar than females ($t = 13.163$, $P < 0.001$). However, there was no difference between the sexes when the concentration was low (10%) ($t = 1.55$, $P = 0.139$). This suggests males had a narrower range in the

amount of sucrose they could imbibe, due to their more limited capacity ($t = 6.49$, $P < 0.001$) (Fig. 4.5). A comparison of regression functions showed that higher sucrose concentration increased the amount of fructose imbibed and the amount of time required to digest it for both males and females ($F = 29.75$, $df = 2, 137$, $P < 0.001$ and $F = 7.69$, $df = 2, 146$, $P < 0.001$ respectively). The lower concentration of the sugar also was digested at a slightly faster rate in males ($F = 9.16$, $df = 1, 137$, $P = 0.003$) and marginally so in females ($F = 6.65$, $df = 1, 146$, $P = 0.011$) (Fig. 4.1). Additionally, a prior blood meal did not significantly affect the rate of sugar digestion in females ($F = 5.84$, $df = 1, 82$, $P = 0.018$) (Fig. 4.3).

The effect of metabolic reserves on rate of sugar digestion was apparent (Fig 4.2). Females maintained initially on 50% sucrose (high-reserve females) digested a 20% sucrose meal at a substantially lower rate than females maintained initially on 1.2% sucrose (low-reserve females) ($F = 107.90$, $df = 1, 66$, $P < 0.001$). When compared to other species, *An. gambiae* females digested a replete meal of 20% sucrose at a higher rate than female *Ae. aegypti* ($F = 20.19$, $df = 1, 198$, $P < 0.001$) and female *Oc. triseriatus* ($F = 45.66$, $df = 1, 158$, $P < 0.001$) (Fig. 4.4).

4.5 Discussion

While the conditions are highly artificial, this study provides a yardstick for determining the maximum time of detection after a sugar meal, and therefore the interpretation of field data on prevalence of fructose in crops. The rates of digestion in nature probably vary considerably with temperature, meal concentration, and physiological state of the mosquito. It would be expected to be faster during warm

seasons and in populations where mosquitoes do not develop under optimal conditions and where greater demands are placed on their energy budgets (Day and Van Handel 1986). Regardless, this study demonstrates that *Anopheles gambiae* digests sugar rapidly, with only minute amounts remaining after 1 day in most cases. This is not too surprising, because similar studies have found that *Ae. aegypti* (Nayar and Sauerman 1975, Costero et al. 1998a) and *Oc. triseriatus* (Smith and Kurtz 1994) also digest sugar rapidly, mostly within 1 or 2 days. However, *An. gambiae* did so faster than either of these species in this study. This speed may account, in part, for the low fructose-positive prevalence seen in the field collections of Beier (1996). The most important factor affecting the rate of digestion appears to be energy reserve. Female *An. gambiae* with high reserves, built from feeding for 5 days on 50% sucrose, digested sugar at a significantly lower rate than low-reserve females fed on 1.5% sucrose for 5 days. Considering that *An. gambiae* emerge with low teneral reserves (Briegel 1990b) and that wild mosquitoes generally have substantially lower reserves than laboratory mosquitoes (Day and Van Handel 1986), low-reserve *An. gambiae* probably represent the standard in nature.

The concentration of sugar solution fed to *An. gambiae* did not have a large effect on the rate of digestion, but because the total sugar ingested in a dilute solution was less, the time to complete digestion also was less. Most adult mosquitoes probably ingest sub-maximal quantities of nectar of varied concentration in nature (Smith and Kurtz 1994). Therefore, most meals probably are quickly converted to the fuel necessary to find food, mates, and oviposition sites. Smith and Kurtz (1994) suggested this might explain why species that are known to nectar-feed extensively often test negative for fructose

(Sandholm and Price 1962, Grimstad and DeFoliart 1974, 1975, Harada et al. 1976, Magnarelli 1977, 1978, El-Akad et al. 1989). Mosquitoes in this study were confined in small lab cages, where flight activity may be reduced, and they were given maximum-size sugar meals. Therefore, digestion rates and total digestion times presented here for *An. gambiae* should be considered as overestimates of those occurring in nature.

Accordingly, it is safe to assume that sugar feeding in this species is underestimated by studies, such as Beier's (1996), which have looked only at resting and biting females.

Male *An. gambiae* took smaller sugar meals and digested them faster than females. This seems to be typical in other species as well (Smith and Kurtz 1994, Costero et al. 1998a). Sometimes males are found to be fructose positive in higher proportions than females (Edman et al. 1992), which is considered evidence that females do not feed on nectar, even when it is available. It is important to consider that males of some species (Van Handel and Lum, 1961; Van Handel 1984), but not all (Briegel 1990b) are poor at building metabolic reserves in comparison to females. If so, they must necessarily feed upon sugar more often than females. In addition, males have more of their time budget available for nectar feeding than do females, which must divide their time between two food sources. Therefore, caution should be exercised when drawing conclusions about female sugar feeding based on comparisons to males. Male *An. gambiae* die sooner than females in the absence of sugar, under similar conditions (Gary and Foster, unpublished), suggesting that they are among the species needing to feed more often.

In field studies of nectar feeding, cold-anthrone test results are generally read visually as either negative or positive (Bidleingmayer and Hem 1973, El Akad et al. 1989, Edman et al. 1992, Beier 1996). Although the test is highly sensitive (Van Handel 1972),

it is subject to reading errors when visual techniques are employed and small amounts are present, increasing the risk of false negatives (Haramis and Foster 1983), effectively shortening the detectable digestion time. Alternatively, spectrophotometry, particularly via a 96-well plate reader, offers a high degree of precision in quantifying cold-anthrone results. This technique can detect exceptionally small amounts of fructose, but it tends to overestimate fructose content when samples contain crushed or blooded mosquitoes (Gary unpublished). Costero et al. (1998a) found that that blooded negative controls tested positive for fructose by spectrophotometry. This is probably caused by small particles of mosquitoes or blood present in the supernatant, even after centrifugation at 14,000 rpm, which absorb up to 100% of the light and give an erroneously elevated optical density. This is likely the source of the false positives reported by Costero et al. (1998a) that led them to implement positive cut-offs of 3-7 μg and nullifies their claims that the test gave reliable results down to 0.6 μg of fructose. To overcome this problem, we found it necessary to filter each sample through a filter, which removed insect parts and much, but not all, of the visible blood meal. To account for the remaining blood, plates were read immediately after samples were added, and the resulting optical densities were subtracted from those of the final reading. This method proved an effective means of increasing accuracy and precision in obtaining test results.

When negative results are interpreted, it is important to consider not only the method of detection employed, but also the rate of sugar digestion in relation to the timing of nectar-feeding behavior. In the case of *An. gambiae*, Beier's (1996) interpretation is that sugar feeding is infrequent (Beier 1996). This view has a lot of support (Muirhead-Thomson 1951, Gillies 1968, McCrae 1989) and may very well be the

case. However, grouping *An. gambiae* among the anthropophagic species that do not sugar-feed may be premature. It has been shown that feeding on sugar increases survivorship in these mosquitoes (Gary and Foster 2001), and that they can locate and feed from nectar sources that are readily available around domestic habitats in Africa (Gary and Foster 2004, Impoinvil et. al., 2004). Negative anthrone tests, particularly those read visually, can be interpreted only over a very short time frame. If, for example female *An. gambiae* feed on sugar only after ovipositing and start to digest it rapidly while seeking a blood meal, it is logical to expect more anthrone tests to be positive among biting collections than resting collections. Although there have been no studies to determine if there is a cyclical component (meaning a gonotrophic-state-dependent component, not diel cyclicity) to sugar feeding in this species, there are indications that it is likely the case (Beier, 1996; Gary, Foster, Knols, unpublished). Future field research should focus more completely on elucidating periodicity of sugar feeding by collecting and testing *An. gambiae* throughout its natural diel and gonotrophic cycles and under various environmental conditions. A sugar meal taken periodically may occur less than daily, but nonetheless be important in the nutritional ecology and vectorial capacity of this important species.

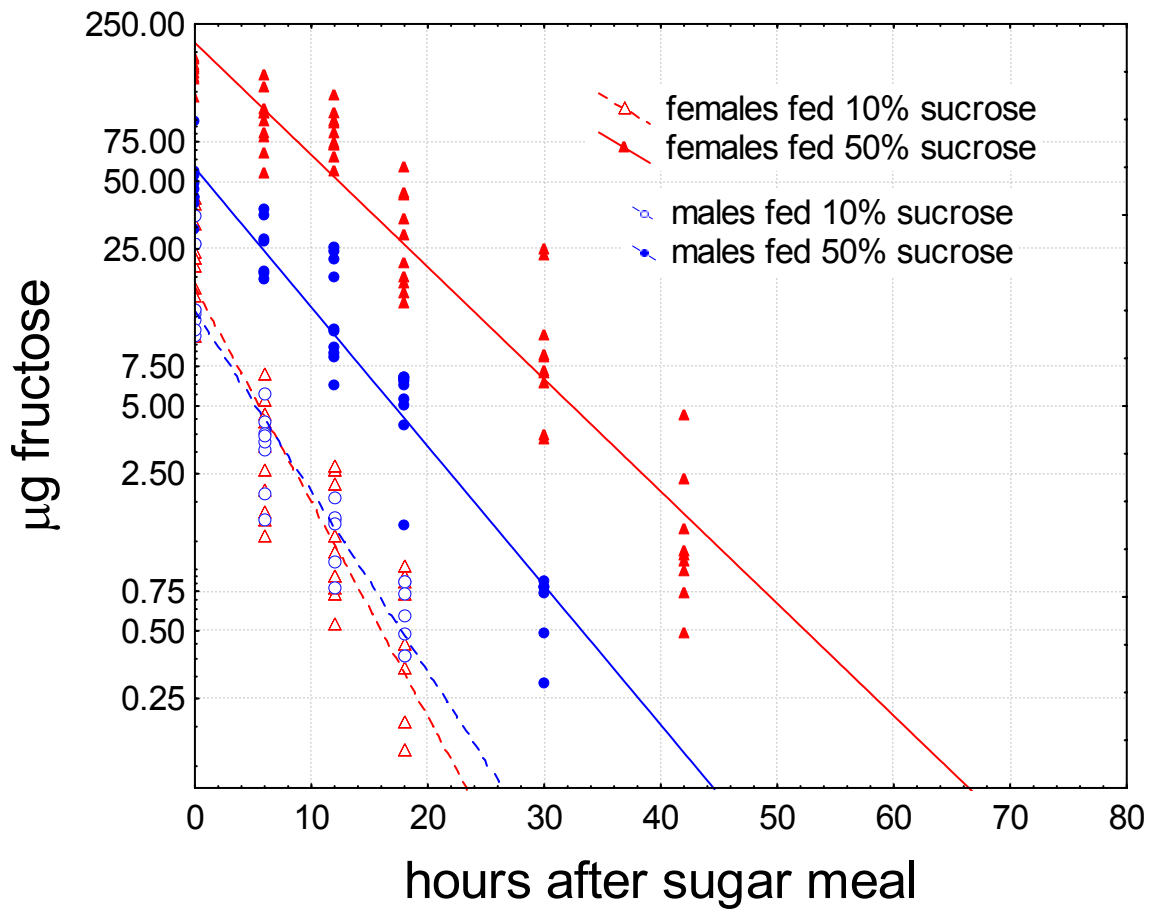


Figure 4.1. Detection of sugar in male (blue) and female (red) *An. gambiae* after being fed to repletion on 10% (dotted lines) or 50% (solid lines) sucrose.

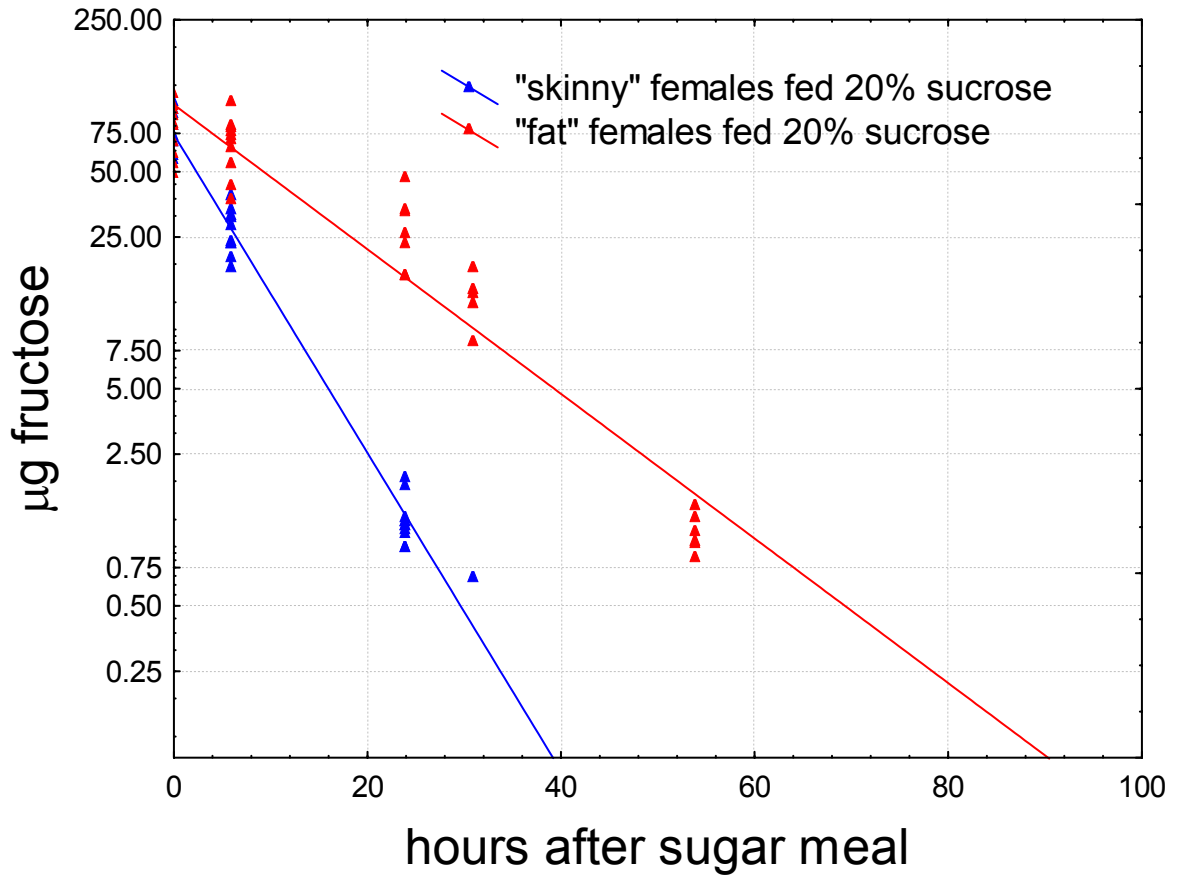


Figure 4.2. Detection of sugar in females *An. gambiae* that were maintained initially on a diet of 1.2% (blue) or 50% (red) sucrose and then fed to repletion on 20% sucrose after allowing crops to empty.

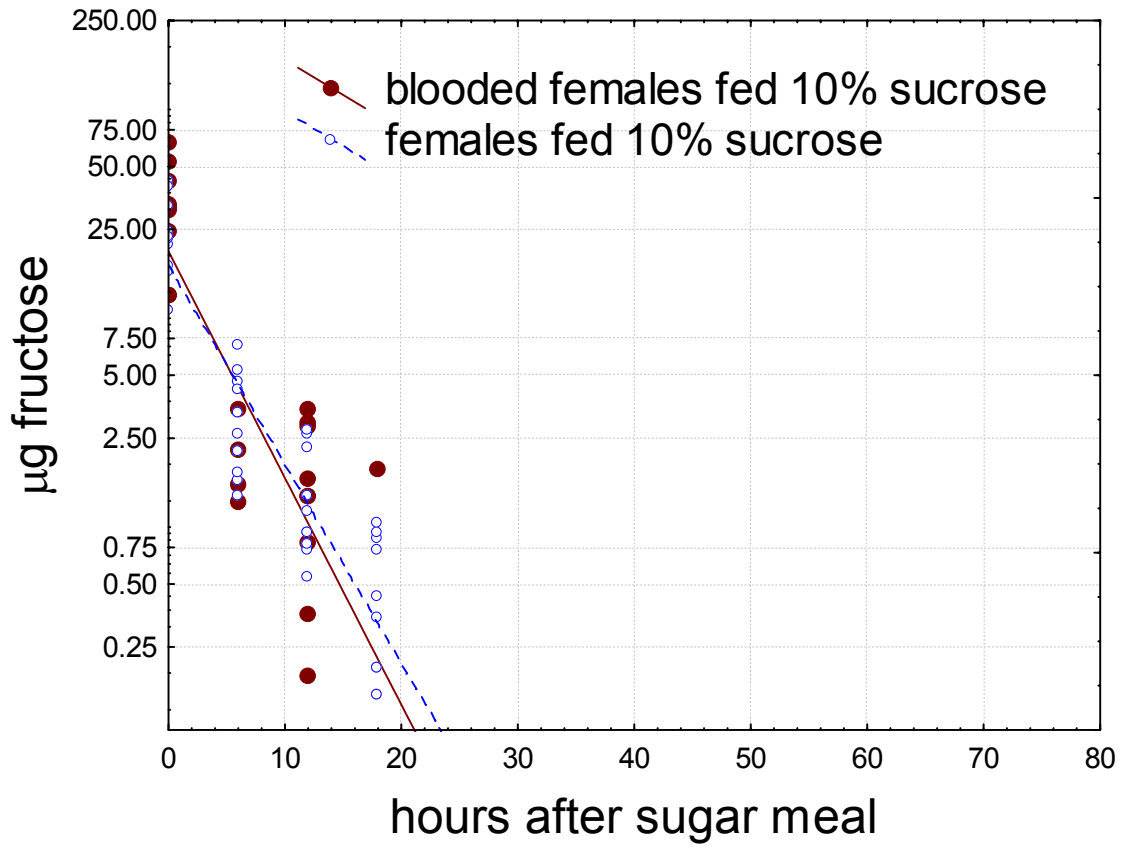


Figure 4.3. Detection of sugar in female *An. gambiae* with (solid, dark red) and without (blue dotted) a 24-hour-old blood meal at the time of sugar feeding after being fed to repletion on 10% sucrose.

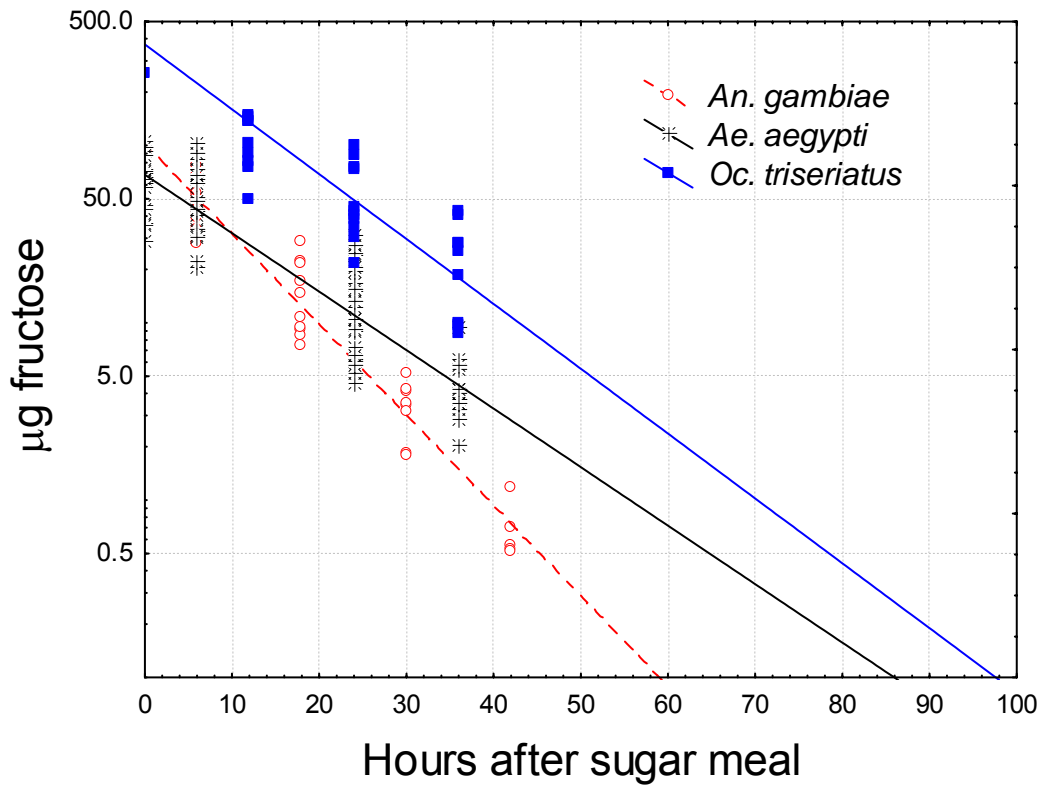


Figure 4.4. Detection of sugar in female *An. gambiae* (red), *Ae. aegypti* (black) and *Oc. triseriatus* (blue) after being fed to repletion on 20% sucrose.

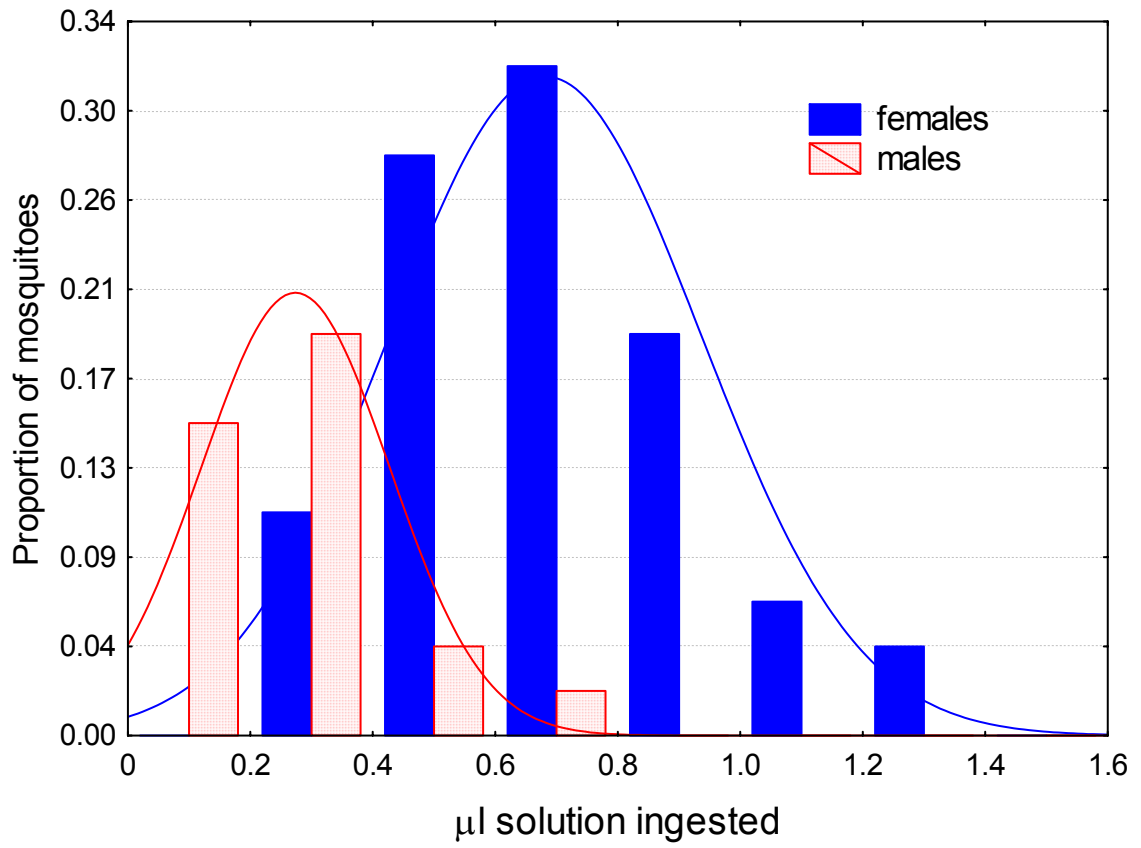


Figure 4.5. Volumes of sucrose solution ingested by *An.gambiae* males and females after being allowed to feed for 10 min.

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Chapter 5

Sugar-Dependent Egg-Batch Size and Oviposition Pattern of First Cycle Female *Anopheles gambiae* (Diptera: Culicidae) According to Body Size & Number of Blood Meals

5.1 Abstract

Anopheles gambiae females, especially smaller ones, emerge with relatively low teneral reserves, which may need to be supplemented by feeding on blood or sugar before blood meals can be utilized for fecundity. To determine the effect of supplemental meals on fecundity and oviposition pattern in the first gonotrophic cycle, *An. gambiae* were reared under normal and crowded conditions to yield two size classes of adults (small, winglength $\bar{x} = 3.07$ mm; large, winglength $\bar{x} = 2.71$ mm) and offered one or two bloodmeals with or without sugar. Sugar-fed large females developed more eggs in the first cycle than those without sugar. Most females refused supplemental blood meals until after eggs were mature (gravid). Those that fed once gravid produced a larger egg batch than did those that fed on sugar and one blood meal, but oviposition was delayed >48 hours. Small females did develop eggs on one blood meal alone, but required a second blood meal in addition to sugar feeding to increase egg batch size. Based on oviposition patterns and dissections during oogenesis, there was no evidence of overlapping gonotrophic cycles.

5.2 Introduction

In anautogenous female mosquitoes, sugar meals are used to build and maintain energy reserves while a large proportion of the blood meal is used to develop eggs. In the standard scenario, each gonotrophic cycle begins with a blood meal and ends with oviposition. In this cyclic mode of reproduction, known as gonotrophic concordance (Swellengrebel 1929), biting frequency is determined by the duration of the gonotrophic cycle. This measurement is important in models, such as vectorial capacity (Garrett-Jones 1964), that predict a vector's pathogen-transmitting potential. In some species, however, biting frequency has been found not to correspond to a gonotrophic cycle (Briegel and Hörler 1993, Scott et al. 1993). Females of these species often take supplementary blood meals, independent of egg development and oviposition, or exhibit asynchronous egg development. These models therefore can underestimate a mosquito's potential of pathogen transmission.

For most culicines, a full meal will inhibit further host seeking (Klowden and Lea 1972), and multiple blood meals therefore are associated with interrupted feedings. One well known exception is *Aedes aegypti*, which commonly takes supplementary blood meals within a gonotrophic cycle, as the eggs develop following a primary blood meal (Macfie 1915, Edman et al 1992, Scott et al. 1993). This is particularly likely to occur when there is no sugar available (Van Handel et al. 1994, Martinez-Ibarra et al 1997).

In anophelines, particularly tropical species, supplemental blood feeding appears to be more widespread (Lyimo and Takken 1993, Briegel and Hörler 1993, Beier 1996, Hogg et al. 1996, Takken et al. 1998, Amerasinghe and Amerasinghe 1999, Ramasamy et al. 2000) and probably is associated with their relatively low metabolic reserves (Briegel

1990b, Takken et al. 1998) and inefficient blood meal utilization (Briegel and Hörler 1993), especially when they develop under crowded larval conditions (Lyimo and Takken 1993, Takken et al 1998). There is even evidence that supplemental feeding leads to increased fecundity in some anophelines (Briegel and Hörler 1993, Ramasamy et al. 2000).

Supplemental blood feeding by *An. gambiae*, like other mosquitoes, occurs mostly prior to the 1st gonotrophic cycle (Gillies 1954), when the female is still gonoinactive (i.e., previtellogenic), and this behavior is associated with poor larval nutrition and small size (Takken et al. 1997). Gonoinactive females, which fail to develop eggs after their first blood meal, have been called “pre-gravid” (Gillies 1954). The first meal typically initiates the gonoactive condition, so that a subsequent meal causes egg development. Occasionally more than one meal is required. The gonoinactive state is most often associated with anophelines, but it can occur in other genera as well, when larvae are crowded and undernourished (Feinsod and Spielman 1980).

Sugar feeding after emergence can close the nutritional gap between large and small females, reducing the need for previtellogenic blood meals (Feinsod and Spielman 1980, Mer 1936). In *An. gambiae*, however, Takken et al. (1998) found that small sugar-fed females remained gonoinactive until they had taken at least one previtellogenic blood meal. In tropical Africa, field evidence based on the prevalence of undigested fructose (Beier 1996) suggests that *An. gambiae* rarely or never feed on sugar, possibly because nectar sources are too rare (McCrae 1989) or because it is an unnatural diet for them (Muirhead-Thomson 1951). If this is the case, then small females might not have the access or inclination to feed on sugar in the field. Instead, they would ingest three or more

blood meals both to survive and to become gravid, making them considerably more dangerous than larger females. Alternatively, they may simply die, lacking the nourishment to engage in host seeking (Lyimo and Takken 1993, Takken et al 1998).

Foster and Takken (2003) have found that in an experimental situation, newly emerged females tended to select nectar-associated odors over host-associated odors. Also, confined *An. gambiae* males and females will feed and survive on nectar from plants that commonly grow near human settlement (Gary and Foster 2004, Impoinvil et al. 2004). It is possible, therefore, that sugar feeding is more prevalent and important, especially in the first gonotrophic cycle, than previously believed. To determine the importance of sugar feeding to reproduction in newly emerged female *An. gambiae* s.s., we investigated the effect of sugar feeding on vitellogenesis, fecundity, and oviposition pattern of large and small females under laboratory conditions.

5.3 Materials and Methods

5.3.1 Mosquito rearing and maintenance

An. gambiae used in all experiments were of the Suakoko strain, established by M. Coluzzi in 1987 from material originating in Suakoko, Bong, Liberia. Colony adults were maintained on honey-soaked sponges, water wicks, and periodic human blood meals. Oviposition cups were placed with caged adults 2 days after each blood meal, and eggs were collected the following day. The ambient conditions for the colony and experiments were $27 \pm 1^\circ\text{C}$, $85 \pm 5\%$ RH, and 13:11 (L:D), with 75-min gradual crepuscular transitions between photophase and scotophase.

Larvae for both the colony and experiments were reared as follows: Eggs were disinfected with 0.05% sodium hypochlorite solution and hatched overnight in flat enamel-coated pans of aged tap water. To produce adults of distinctly different body-size classes, first-instar larvae were placed, 100 each (for large-bodied mosquitoes) or 1000 each (for small-bodied mosquitoes), into 22.8 x 33.0-cm aluminum pans with 450 ml of aged tap water. The larvae were fed a standard diet of finely ground TetraMin[®] flakes, following a daily schedule that provided 0.5 mg (large) or 0.01 mg (small) of food per larva per day. Pupae were collected daily and segregated in separate 41-liter cages, according to rearing regime, and supplied with water wicks.

5.3.2 Examination of adult size and reproductive development

Four groups (designated as large sugar-fed females, large water-only females, small sugar-fed females, and small water-only females) were created on day 1 (x-y hr) after emergence, by allocating each group to one of four 41-liter cages. To these cages were added sugar-fed 4-day-old males at a ~1:2 female-male ratio, to allow mating. After the mosquitoes had been allowed to feed for 10 min, by which time nearly all had engorged and ceased feeding, they were transferred to the cage containing the males and water wicks. Females that did not take this initial sugar meal were removed from the study. A 10% sucrose solution was supplied *ad lib.* from two wicks to the two groups designated as sugar-fed.

After 1 night with the males, females were offered blood from a human hand (R.E.G.) during the afternoon, 2 hr prior to crepuscular dimming and allowed to feed

until replete, indicated by the females' voluntarily withdrawal of their mouthparts. After this, large and small females that had fed on blood only (no sugar) were further divided into 1- blood-meal and 2-blood-meal groups.

A second blood meal was difficult to induce without manipulation, especially in large females. The vast majority of large females with access to sugar simply refused to take a second blood meal at any time within 72 hr of the first. To induce large females to take a second blood meal, it was necessary to withhold oviposition cups, then provide the host 72 hr after the first blood meal. Small females were more likely to take a second blood meal at 24 or 48 hr after the first, but they were treated the same as large females (i.e., prevent oviposition and provide the host a second time at 72 hr) to eliminate additional variables.

Females were frozen within 48-72 hr after their last blood meal. To establish that rearing regimens produced significantly different-sized groups of adults, one wing from each adult was removed, mounted on a slide and measured from the distal end of the alula to the wing tip to the nearest 0.0001mm using an Auto-Montage digital imaging microscope (Syncroscopy USA, Frederick, Maryland). Dry weights were not taken, because the general relationship between dry body weight and wing length is well established (Lyimo et. al. 1992, Takken et. al. 1998, but cf. Koella and Lyimo 1996).

After the wings were removed, females were dissected in 0.01 M phosphate buffered physiological saline, pH 7.2. Ovaries were removed and oocytes were examined to determine developmental stage. Fecundity was expressed as the number of mature (stage V) oocytes present (Christophers 1911).

In a similar study, there was concern that failed oogenesis was more likely in virgin *An. gambiae* (Takken et. al. 1998). In addition, Klowden and Russell (2004) determined that virgin females, fed small amounts of blood, more often failed to develop eggs than inseminated females. Because not all females in this study were likely to be mated in the short amount of time allowed, because of the dietary restriction of some groups, spermathecae were examined to determine if there was an association between vitellogenesis and insemination under the conditions of this experiment.

5.3.3 Examination of oviposition patterns

Large females were treated as above, except that they were placed in individual cages and monitored to determine the time of oviposition with or without available sugar (10% sucrose soaked cotton wick). All females were offered blood daily for 3 days and separated into groups for analysis, based on when or if they took a second blood meal. In addition, a group of females without access to sugar was denied oviposition until a 2nd blood meal was taken, as in the experiment above, to determine the effect on oviposition pattern. In this experiment, fecundity was measured as the total number of eggs laid. The duration of the gonotrophic cycle was measured to the nearest 24-hr period. Oviposition was monitored for 6 days after the last blood meal.

5.3.4 Data analysis

Analyses were performed with Statistica (StatSoft, Inc., Tulsa Ok.). Chi-square was used to analyze differences in proportion of females developing eggs. Other differences between treatment groups were analyzed with *t*-tests and Kruskal-Wallis rank tests. A Bonferroni adjustment was applied to post-hoc pairwise comparisons.

5.4 Results

5.4.1 Examination of adult size and reproductive development

Wing-length measurements for mosquitoes reared at densities of 100 per pan ($\bar{x} = 3.07$ mm) and 1000 per pan ($\bar{x} = 2.71$ mm) demonstrated that rearing procedures resulted in two significantly different body-size classes (*t*-test, $P < 0.0001$) (Fig. 5.1.). Field measurements (Lyimo and Takken 1993, Charlwood et al. 2002a, 2003) indicate that these size classes are representative of natural extremes of adult size.

An attempt was made to use inseminated females. Not all were, especially those that did not have access to sugar (25% inseminated, vs 61% inseminated when sugar available). Because males in those groups also did not have access to sugar during the night of mating, this result does not indicate that females need sugar in order to mate. Examination of the spermathecae and ovaries showed that insemination did not influence vitellogenesis ($X^2 = 0.124$, $P = 0.7245$) or fecundity ($t = -0.488$, $P = 0.6259$) (Fig. 5.2.). As figure 5.2 shows, both inseminated and virgin females were represented among those that developed eggs and those did not.

Sugar feeding and number of blood meals appeared to affect vitellogenesis (Fig. 5.3.) (Table 5.1). In small females, sugar feeding increased the proportion undergoing vitellogenesis after one blood meal (44 to 63%; $X^2 = 7.25$, $df = 1$, $P = 0.007$), and after two blood meals (59 to 83%; $X^2 = 13.99$, $df = 1$, $P < 0.001$). In large females, sugar feeding increased vitellogenesis from 77 to 93% after one blood meal ($X^2 = 10.04$, $df = 1$,

$P = 0.002$) (Fig. 5.3.) (Table 5.1). Large females would not take a 2nd blood meal even while gravid, if sugar was present. A portion of females failed to develop eggs in all of the treatment groups examined.

Fecundity was affected in both large and small females by various combinations of sugar and blood meals (Kruskal-Wallis rank test, $P < 0.001$ and $P = 0.006$ respectively) (Fig. 5.4). Post-hoc comparisons (Bonferroni adjustment of $\alpha = 0.008$ for small females and $\alpha = 0.017$ for large females) showed that large females with access to sugar developed more eggs after one blood meal than those without sugar access. Neither a sugar meal ($P = 0.998$) nor an additional blood meal ($P = 0.558$) improved fecundity for small females. However, the combination of sugar feeding and an additional blood meal did improve fecundity over one blood meal alone ($P = 0.024$).

Large females taking a 2nd blood meal 72 hours after the 1st had a substantially increased fecundity over those females that took only one blood meal, either with ($P < 0.0001$) or without ($P < 0.0001$) sugar (Fig. 5.3.) (Table 5.1). Females dissected within 24 hr after the 2nd blood meal were found to have mature follicles as well as follicles in trophic (= vitellogenic) stages of development (Fig. 5.5.). Those dissected 48 hr or longer after the second blood meal contained only mature oocytes and resting stage oocytes.

5.4.2 Examination of oviposition patterns and number of eggs laid

Only a few small females laid eggs. Examination of spermathecae showed that most small females failed to become inseminated and therefore to lay eggs. Though few small females laid eggs, the patterns of oviposition were the same as those observed in larger females. Therefore, only large females were analyzed for this experiment.

Under the laboratory conditions of this study, the gonotrophic cycle was determined typically to be two days. Sugar appeared to delay the cycle in some females, but the difference was not significant overall between those fed sugar and those not fed sugar ($P = 0.9804$) (Table 5.2). The effect that a second blood meal had on the length of the gonotrophic cycle depended on the time of the second meal. A 2nd blood meal taken within 1 day of the first meal had no effect on the time of oviposition, and the gonotrophic cycle continued to end two days after the 1st blood meal ($P = 0.1000$). However, a 2nd blood meal taken 2 ($P = 0.0062$) or 3 ($P < 0.0001$) days after the 1st blood meal had the effect of delaying oviposition until two days after the 2nd blood meal (Table 5.2). Only a few of the females offered a single blood meal laid eggs over more than 1 night. One individual laid eggs on three successive nights. None of the females that took two blood meals laid eggs over multiple nights.

As in the first experiment, a 2nd blood meal 72 hr after the 1st led to a substantially higher fecundity ($P = 0.0012$). While the trend suggested that the number of eggs increased with increasing time between the 2 blood meals, the samples were relatively small and the differences were not statistically significant between females taking only 1 blood meal and those who took a 2nd blood meal either 24 ($P = 0.4017$) or 48 ($P = 0.1966$) hours after the 1st blood meal (Table 5.2).

5.5 Discussion

Takken et al. (1998) found that, even with sugar, small females need multiple blood meals to produce eggs when fed rat blood. On the contrary, we found that, when fed human blood to repletion, more than half of the sugar-fed small females in this study

did mature eggs after one blood meal. Even without sugar feeding, 44% of the small females matured a batch of eggs. The different results between these two studies are difficult to reconcile. Rodent blood has high levels of isoleucine (Chang and Judson 1977), an amino acid necessary for mosquito egg production (Lea et al. 1958), while human blood has quite low levels (Chang and Judson 1977). It is logical to conclude that rat blood would yield more eggs and a higher rate of oogenesis. However, we have observed in our lab (Gary unpublished observations) that female *An. gambiae* appear to take shorter and smaller blood meals from rodents, even when they are restrained, than they do from humans. Humans are known to be a preferred host for *An. gambiae*, so there may be some as yet unknown quality of humans or their blood that results in females feeding more completely or deriving more benefit from human blood. We did find, especially when allowed to sugar feed, that additional blood meals increased vitellogenesis in small females, similar to the results of Takken et al. (1998), though direct comparison is difficult because they delivered blood meals by enema, which does not allow for pre-diuresis, i.e., natural concentrating of the blood meal. We found that in both large and small sugar-fed females, a single blood meal more frequently produced eggs than even two blood meals did, if sugar was not available.

Large female *An. gambiae* that sugar-fed had a significantly larger first egg batch than large females that did not sugar feed. This species is unusual in catabolizing protein for non-reproductive purposes, such as building reserves, when under nutritive stress (Briegel 1990b). Sugar feeding early in adult life, therefore, allows the females to more efficiently use a blood meal for vitellogenesis. For small females, sugar feeding alone did not increase fecundity. Only sugar feeding in combination with two blood meals

increased fecundity over one blood meal alone. Even two blood meals alone did not increase fecundity significantly. Many small females emerge with such low reserves that multiple blood meals are needed to build reserves prior to initiating oocyte maturation (Briegel and Hörler 1993, Takken et al. 1998). Although 44 % of small females in this study did develop eggs after one blood meal alone, there was still competition for nutrients between maintenance and vitellogenesis (Briegel 1990b). Fecundity was not maximized in small females until after two or more meals, demonstrating the priority of self-preservation.

Whether or not supplementary (multiple) blood meals increased fecundity was dependent on the time between the 1st and 2nd blood meal. Of the few females that took a supplementary blood meal within 48 hr, fecundity was slightly, but not significantly, increased. This has been observed by others (Briegel and Hörler 1993, Takken et al. 1998) and probably represents primarily the building of reserves.

Gravid females readily took blood meals when oviposition sites were withheld and no sugar was available. While these mosquitoes were forced to take a blood meal to prevent starvation, it represents a naturally occurring phenomenon because gravid females are known to seek blood meals in the field (Beier 1996). This second blood meal led to further egg development and a significantly larger egg batch than one blood meal. Apparently, the mechanism responsible for oostasis in most mosquitoes (Meola and Lea 1972, Readio and Meola 1985) is missing in this mosquito. Similar results were seen in *An. albimanus* by Briegel and Hörler (1993), demonstrating that this difference might be widespread in anophelines. Unlike *An. albimanus*, however, there was no evidence that oviposition could be nightly, the result of overlapping cycles of egg development.

Rather, any eggs present after the 1st blood meal were retained until the eggs resulting from the 2nd blood meal were mature. Why this should be the case is unclear.

Oviposition sites were made available at the time of the second blood meal, so egg retention was not caused by preventing oviposition. Unfortunately there is very little known about the hormonal regulation of egg development in this genus, so it is not currently possible to understand the underlying mechanisms at work here. The important implication of blood feeding by gravid female *An. gambiae* is that, while the egg batch becomes larger, oviposition is delayed, so overall fecundity is reduced. It is hard to understand a female making a choice that reduces her fecundity unless there is some greater benefit. This behavior might be expected in the field when sugar sources or, more important, oviposition sites are unavailable.

Large *An. gambiae* females did not readily take multiple blood meals in the first gonotrophic cycle. Even the smaller females did not take multiple blood meals very often. The small samples of females taking a second blood meal at 24 or 48 hr in the 2nd part of this experiment represent less than 5 % of the females offered re-feedings. This corresponds to the finding of Takken et. al. (2001) that host seeking was strongly inhibited for at least 40 hours after a blood meal. Other studies have suggested that this species will readily take a blood meal within 12 to 24 hours after an initial blood meal (Briegel and Hörler 1993, Klowden and Briegel 1994, Takken et. al. 1998). Klowden and Briegel (1994) found a strong diel rhythm of host seeking such that 70 – 80% *An. gambiae* females (Suakoko strain) reared under ideal laboratory conditions engaged in

host seeking near the end of scotophase, 24 hr after the 1st blood meal. Even during this apparent peak time of host seeking, females of our Suakoko strain did not take a second blood meal (3/69, 0/80; R.E.G. unpublished data).

This difference in results is perhaps easier to reconcile with respect to studies that utilized rodents (Takken et. al. 1998), an unnatural host, for blood meals. It is harder to reconcile our findings with those of Klowden and Briegel (1994), who used a human host, yet found virtually no inhibition to host-seeking during oogenesis. The authors suggested that, while large blood meals are known to inhibit further blood feeding due to abdominal distention (Klowden and Lea 1979), many anophelines have a relatively small gut capacity (Briegel and Rezzonico 1985), and distention-related inhibition does not last long. However, we found partial blood meals well beyond 24 hr old in females refusing a second blood meal. Possibly, mechanisms to inhibit further blood feeding during blood meal digestion and vitellogenesis do not include orientation towards host stimuli. It is important to point out that, while females rarely took multiple blood meals in this study, this does not imply that multiple blood meals are not taken in nature. Numerous field studies show that multiple blood meals are taken by *Anopheles* spp. (Senior-White 1952, Boreham and Garret-Jones 1973, Boreham et. al. 1979, Lyimo and Takken 1993, Beier 1996).

Inhibition from taking multiple blood meals seems to be related to the blood meal itself, at least on the first day after feeding, because the number of blood-fed females that failed to develop eggs far exceeded the number of females taking a 2nd blood meal. This suggests that even those females not developing eggs are not ready to re-feed until the blood meal is digested. This is the case for other mosquitoes as well (Klowden and Lea

1979). It is possible that an additional inhibition related to egg maturation occurred in those females that did develop eggs because they did not readily take a 2nd blood meal until 72 hr, at which time they would have oviposited if allowed. This is supported by activity experiments in our laboratory (see chapter on sugar feeding frequency) as well as by Jones and Gubbins (1978), who demonstrated a depressed activity during blood digestion.

Takken et al. (2001) reported that females return to host-seeking behavior at 72 hours after a blood meal, even if they had not yet oviposited. We found that gravid females will readily take a blood meal when denied sugar and oviposition sites. However, when sugar is present, females continue to refuse blood meals until oviposition is allowed. This likely has to do with the reproductive cost of taking a 2nd blood meal while gravid. A 2nd blood meal causes more eggs to be developed in gravid females, but it also delays oviposition while those eggs are being developed. Because the 2nd blood meal increases but does not double an egg batch (there was no evidence of secondary follicle development), it is also likely that total fecundity is reduced. Like *An. albimanus* (Briegel and Hörler 1993), *An. gambiae* can develop more eggs from a 2nd blood meal, but unlike *An. albimanus*, this does not lead to continuous egg laying, but rather it resets the gonotrophic cycle and delays oviposition.

Under some conditions, newly emerged females are more attracted to nectar related odors than to host related odors (Foster and Takken 2003) and are known to feed and survive on plants found peridomestically in Africa (Gary and Foster 2004, Impoinvil et al. 2004). The results of this study show the early reproductive benefits of sugar feeding to newly emerged females. While a proportion of both large and small females

can develop an egg batch with only one blood meal, sugar clearly provides energy and reserves necessary to maximize the likelihood of vitellogenesis and fecundity, no matter what a female's size. By providing a nutrient resource for gravid females that are delayed in ovipositing, sugar can also prevent a reduction in long-term fecundity.

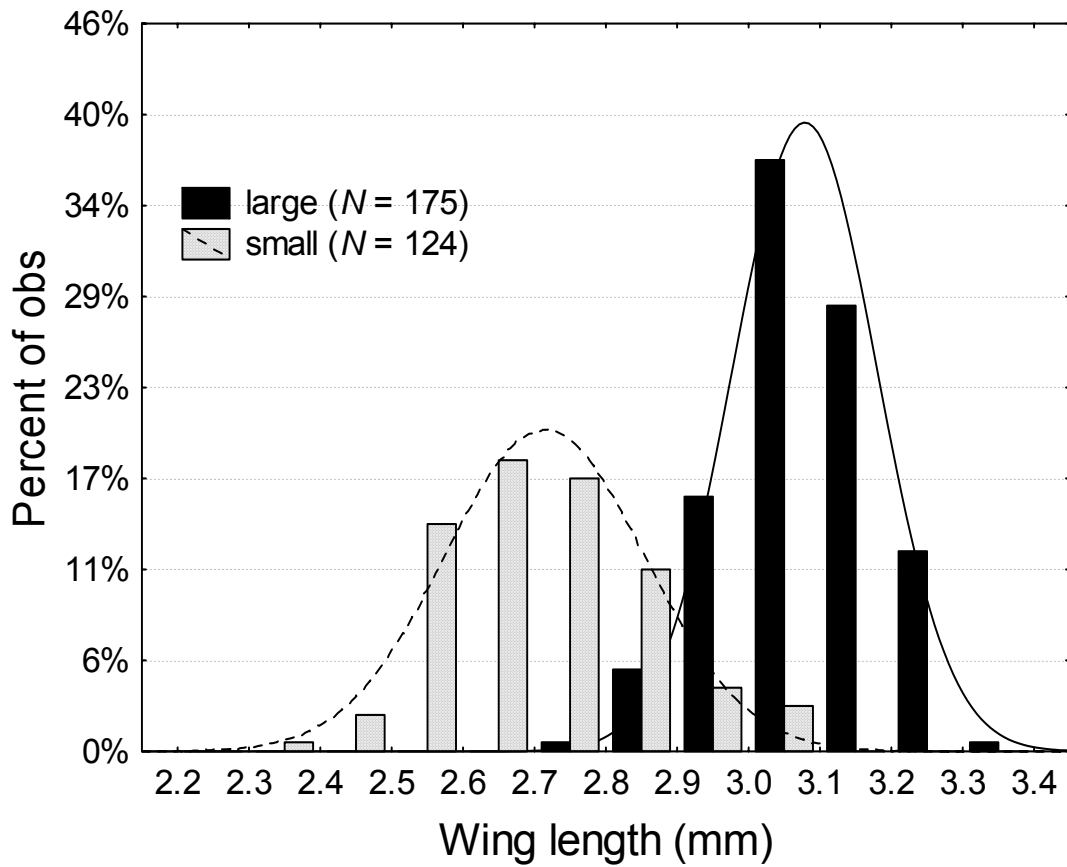


Figure 5.1. Histogram of adult female wing lengths when reared under crowded conditions (small) or uncrowded conditions (large).

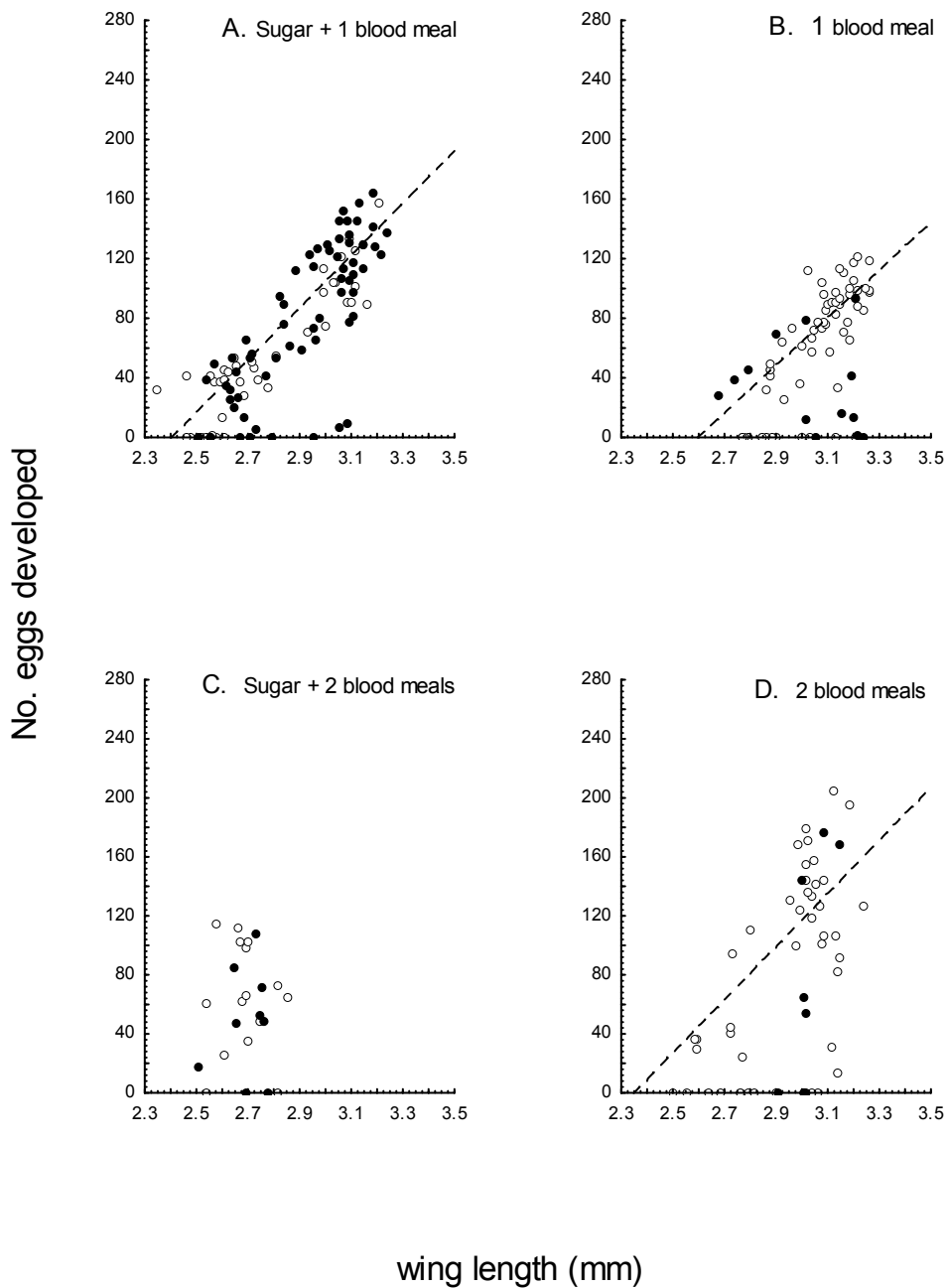


Figure 5.2. Relationship between fecundity and female size when given 1 (A,B) or 2 (C,D) blood meals with sucrose ad lib (A,C) or without (B,D). Both virgin (open circles) and inseminated (solid circles) females developed egg clutches. Dotted lines are least squares lines of eggs developed, not including failed egg batches (less than 10 eggs).

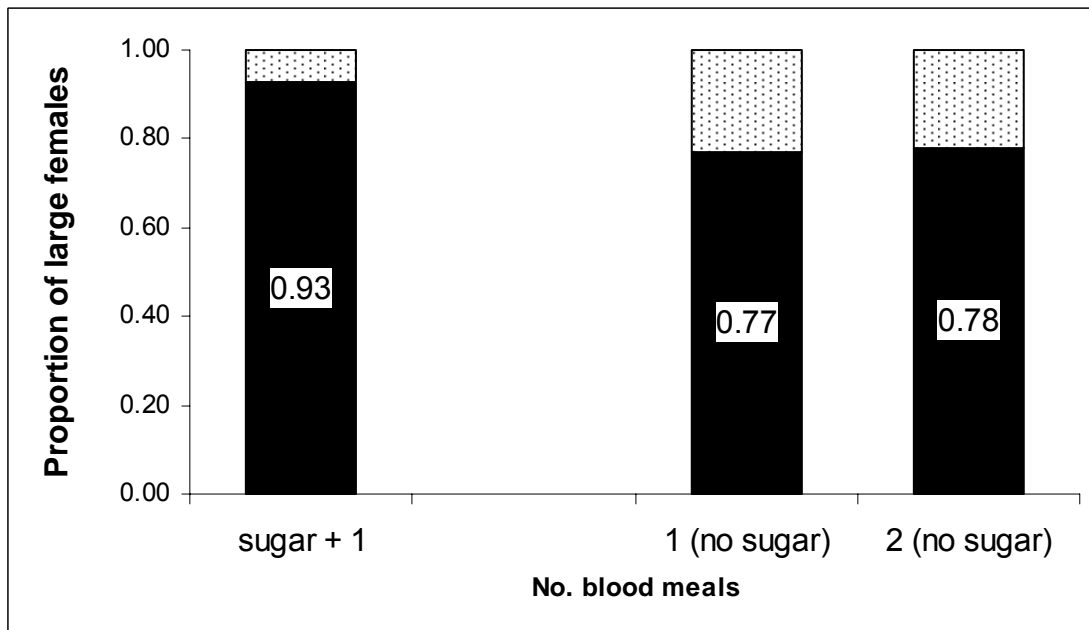
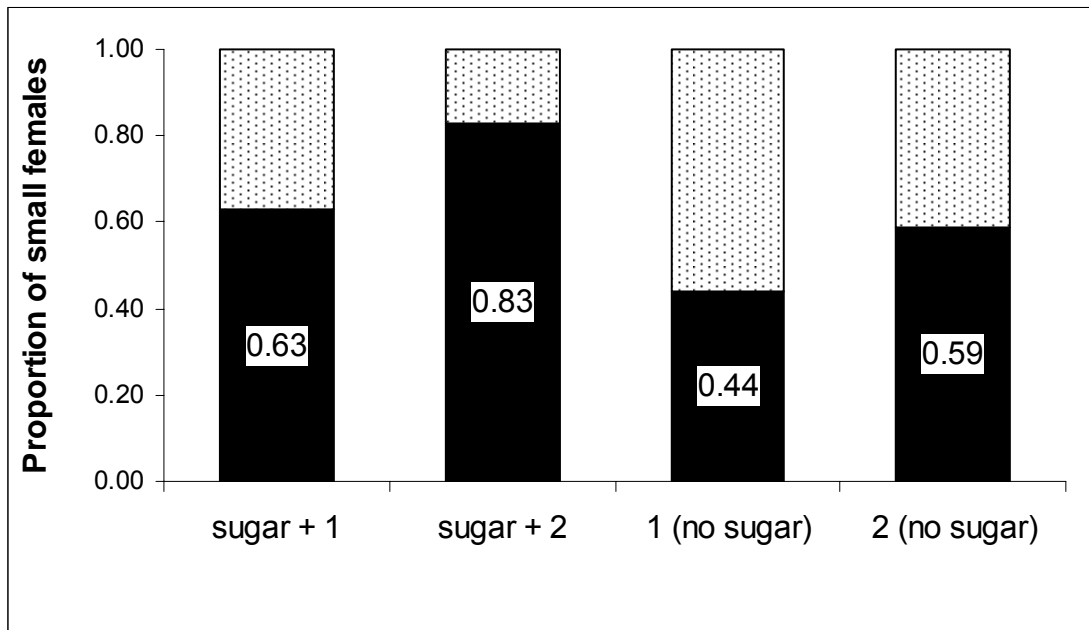


Figure 5.3. Proportion of small (top) and large (bottom) females completing oogenesis (solid) in each treatment group. Failed oogenesis (dotted) in this experiment was considered to be any less than 10 eggs produced by a female.

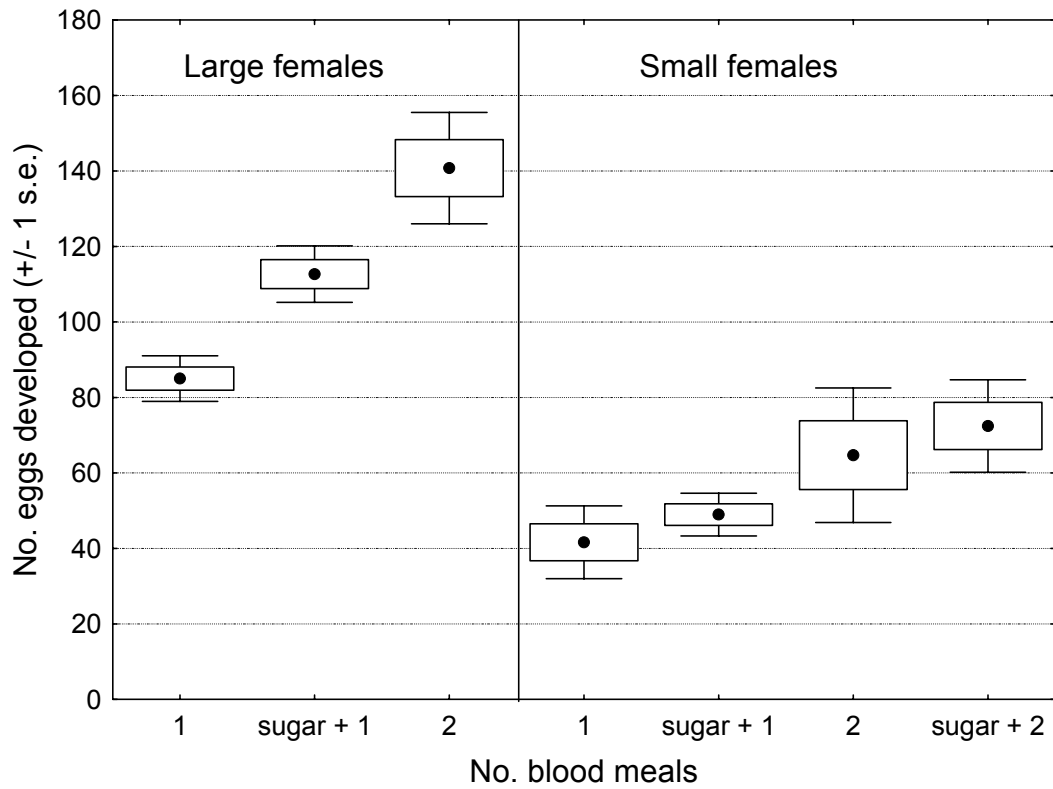


Figure 5.4. Fecundity (number of eggs developed) of large and small females after 1 or 2 blood meals, with or without sugar. Analysis includes only those females that developed 10 or more eggs. In all groups, the second blood meal was taken only after females were gravid following the first blood meal.

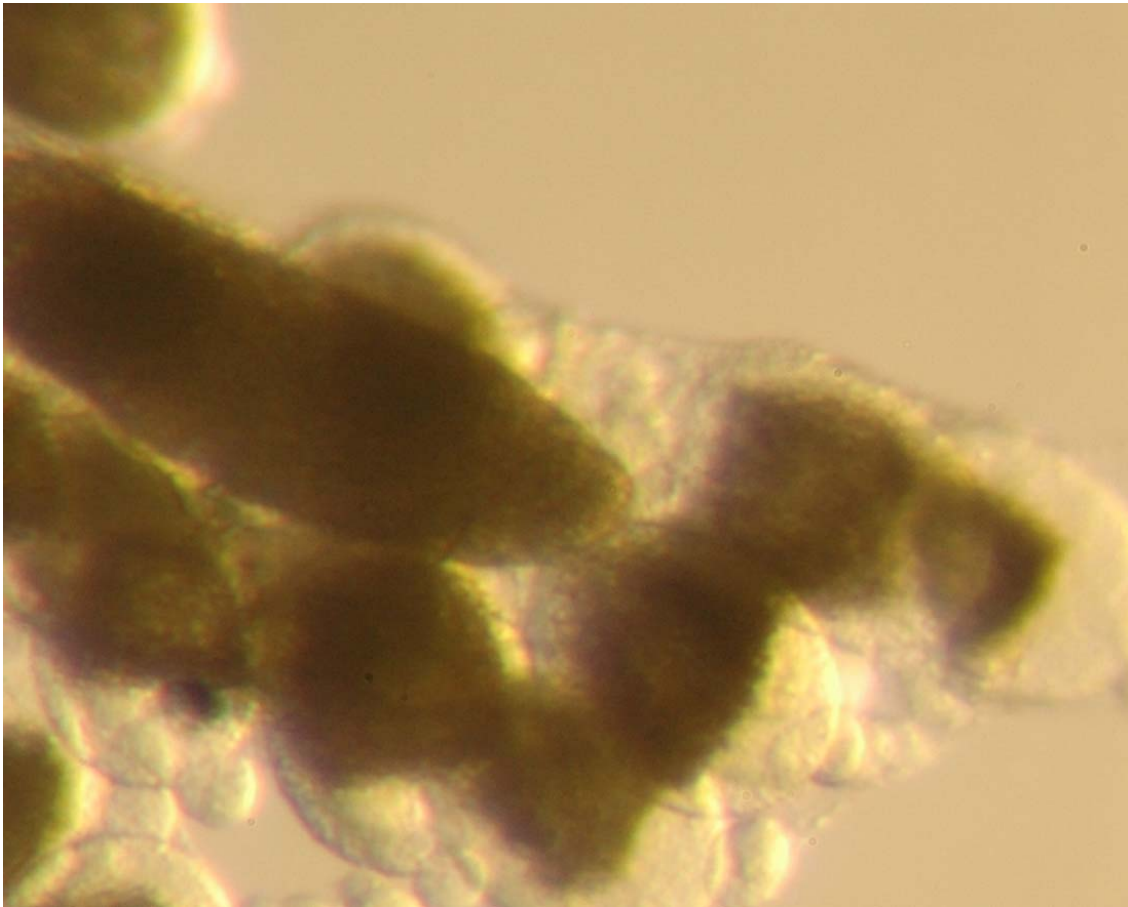


Figure 5.5. Typical ovary removed 24 hours after 2nd blood meal. In addition to the mature oocytes in the center, a large number of follicles have entered the trophic phase and can be seen in C III. Those ovaries removed 48 hours after the 2nd blood meal contained all mature oocytes.

Treatment	(N)	% with eggs	eggs / ♀ mean*± SEM
Small ♀♀			
1 blood meal (no sugar)	(18)	44	41.6 ± 4.9 _a
2 blood meals (no sugar)	(22)	59	64.7 ± 9.1 _{a,b}
Sugar + 1 blood meal	(60)	63	48.9 ± 2.9 _a
Sugar + 2 blood meals	(24)	83	72.4 ± 6.2 _b
Large ♀♀			
1 blood meal (no sugar)	(61)	77	85.0 ± 3.1 _A
2 blood meals (no sugar)	(32)	78	140.8 ± 7.5 _B
Sugar + 1 blood meal	(56)	93	112.7 ± 3.8 _C
Sugar + 2 blood meals [▲]	n/a	n/a	n/a

Table 5.1. Effect of sugar and blood meals on fecundity.

* Means with different letters are significantly different at $\alpha = 0.0083$ for small females and $\alpha = 0.0167$ for large females, Bonferroni adjustment to Kruskal-Wallis rank test.

**Second blood meal occurred 72 hr after 1st.

▲Large females with access to sugar would not take a 2nd bloodmeal.

Treatment	(N)	Length (days) of gonotrophic cycle mean* \pm SEM (median)	eggs / ♀* mean* \pm SEM
1 blood meal			
sugar	(13)	2.5 \pm 0.2 (2) _{a,b}	111.4 \pm 3.4 _{A,B}
no sugar	(20)	2.0 \pm 0.0 (2) _a	90.9 \pm 4.6 _A
2 blood meals (no sugar)			
2 nd 24 h after 1 st	(17)	2.1 \pm 0.1 (2) _a	109.6 \pm 8.9 _{A,B}
2 nd 48 h after 1 st	(9)	3.5 \pm 0.2 (4) _{b,c}	119.3 \pm 11.6 _{A,B}
2 nd 72 h after 1 st	(10)	4.6 \pm 0.2 (5) _c	147.2 \pm 13.4 _B

Table 5.2. Effect of sugar and multiple blood meals on length of gonotrophic cycle and fecundity (eggs laid) of large females.

* Means with different letters are significantly different at $\alpha = 0.005$, Bonferroni adjustment to Kruskal-Wallis rank test.

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Chapter 6

Diel Timing and Frequency of Sugar Feeding in *Anopheles gambiae* Depending on Sex and Resource Availability

6.1 Abstract

Both circadian and long term sugar-feeding activity were monitored for male and female *An. gambiae* in the laboratory. Males fed on sugar in a diel rhythm that closely approximated flight activity. Female sugar feeding patterns resembled published rhythms of host seeking. Males sugar-fed daily at a frequency that was sustained over 17 days and was higher than that of females. Females sugar-fed between gonotrophic cycles, after eggs were mature, and before the next blood meal. They did not sugar-feed during the 2 days after a blood meal. A delay in available oviposition sites or blood meals led to an increase in sugar feeding that continued each evening until the delayed resource was made available. These observations support the conclusion that sugar feeding is a normal part of the biology of *An. gambiae*.

6.2 Introduction

Sugar feeding is a fundamental characteristic of the lives of male and female mosquitoes (Foster 1995). Studies have revealed diel periodicities of sugar feeding that may or may not coincide with those of other activities (Gillett 1961, Gillett et al. 1962, Grimstad and DeFoliart 1974, McCrae et al. 1976, Yee et al. 1992a, 1992b), depending

on the species. Females of some mosquito species, for example, have almost synchronous circadian rhythms of blood feeding and sugar feeding (Yee et al. 1992, Yee and Foster 1992), but blood-feeding activity peaks slightly earlier. Knowledge of these diel rhythms, and of sugar-feeding frequency, can give a relative measure of the importance of plant-sugar sources to a species (Foster 1995).

Anopheles gambiae, the world's most important malaria vector, is thought to rely on sugar only facultatively or not at all. Proponents of this view cite the paucity of nectar sources (McCrae 1989) and the female's ability to derive maternal reserves from blood alone (Muirhead-Thomson 1951, Briegel and Hörler 1993). It is not possible to draw firm conclusions from the one field study on the topic, because collections were restricted to indoor catches made in the daytime (Beier 1996), which omit females in one portion of the gonotrophic cycle. This species digests sugar meals rapidly (see Chapter 4), and even females that regularly take sugar soon before or soon after oviposition during the early night might be missed by surveillance that fails to include females in these parts of the cycle demographic. Additionally, there are no studies addressing the differences in sugar feeding across a geographic range, where the variability of environmental stresses and resources unquestionably has an impact on a mosquito's energetic needs. Field data on sugar-feeding males, which have no dietary alternative, are altogether lacking.

Recent studies have demonstrated that male nutrition may be more important than once thought. While females can feed on blood alone without suffering a loss of reproductive success, sugar feeding does increase female survival (Straif and Beier 1996, Gary and Foster 2001) and is critical both to male mating performance and male survival (see Chapter 3). Common plants found peridomestically in tropical Africa offer the

potential for extended survival of both males and females (Gary and Foster 2004, Impoinvil et al. 2004). Additionally, under some laboratory conditions, newly emerged males and females are more attracted to nectar-related odors than host-related odors (Foster and Takken 2004), suggesting that sugar feeding is an early priority for both sexes.

Perhaps because it has been assumed to be unimportant, very little work has been done to determine the effect of plant sugar on *An. gambiae* behavior and reproduction, and this knowledge ultimately will require more extensive field studies than those conducted so far. It is not safe to assume the sugar feeding rarely occurs and is unimportant, without knowledge of sugar-digestion rates and natural timing of sugar feeding when it does occur. The first assumption was addressed in Chapter 4. This study addresses the second assumption and represents the first attempt to determine the timing and frequency of sugar feeding in this species, with emphasis on the effect of sex, gonotrophic state, and restricted availability of blood and oviposition sites. It is not meant to replace field work, but to provide a background for field studies to follow.

6.3 Materials and Methods

6.3.1 Mosquito rearing and maintenance

An. gambiae used in all experiments were of the Suakoko strain, established by M. Coluzzi in 1987 from material originating in Suakoko, Bong, Liberia. Colony adults were maintained on honey-soaked sponges, water, and periodic blood meals from a human hand and arm. Blood feeding was conducted in accordance with The Ohio State University's Biomedical protocol 2004H0193 and Biosafety protocol No. 2005R0020. Oviposition

cups (9 cm diameter, 3.5 cm deep, 200 ml) 1/3 filled with aged tap water were placed with caged adults 2 days after each blood meal, and eggs were collected the following day. The laboratory conditions for the colony were $27 \pm 1^\circ\text{C}$, $80 \pm 5\%$ RH, and 13:11 (L:D), with 75-min gradual crepuscular transitions between photophase and scotophase.

Larvae for both the colony and experiments were reared as follows: Eggs were disinfected with 0.05% sodium hypochlorite solution and hatched overnight in flat enamel-coated pans of aged tap water. First-instar larvae were placed, 100 each, in 22.8 x 33.0-cm aluminum pans with 450 ml of aged tap water. The larvae were fed a standard diet of finely ground TetraMin® fish flakes, following a daily schedule that produced a nearly synchronous pupation 7-8 days after hatching. Pupae were collected daily and placed in a 41-liter cage supplied with water-soaked cotton dental wicks. Males and females emerging overnight were either separated the following morning and placed individually into monitoring cages, or first were kept together for 3 days in the cage to achieve insemination. During the 3-day insemination period, cages were supplied with 10% sucrose-soaked dental wicks to prevent stress and maintain or build energy reserves.

6.3.2 Monitoring Diel timing and frequency of sugar-feeding

Individual mosquitoes were released each into a 41-liter clear, acrylic plastic cage which contained one water-soaked cotton dental wick and one sugar feeder. The sugar feeder was a 75-ml clear plastic cup with tight-fitting lid. In the center of the lid was a 1-cm hole covered with fine mesh, which served as a probing platform for sugar-seeking mosquitoes. The sugar solution, filling about one-fourth of the cup, consisted of 20% sucrose in deionized water. Sodium benzoate (1 g/l) was added to inhibit microbial growth, and verbanone (0.05 ml/l) was added to provide scent. Verbanone was chosen

because it is attractive to mosquitoes at low concentrations (J. Wittie, unpublished) and could be delivered in precise volumes to achieve a consistent effect. A cotton dental wick soaked with the sugar solution was then placed in the center of the cup, standing on end, so that the top end of the wick was positioned just below the mesh opening in the lid, to facilitate observations of sugar probing (Fig 1.).

Observations were made using infra-red-sensitive video cameras, fitted with 75-mm short telephoto lenses and attached close-up diopters and aimed at the probing screen of sugar feeders. A 15-watt red ($\geq 650\text{nm}$) light remained on to facilitate video recording. Cameras were connected to a time-lapse video recorder via an 8-channel sequential switcher. Recording from each camera was set to occur at 1 frame per second. Data were obtained by freeze-framing video playback when mosquitoes were observed probing for sugar. No distinction was made between probing for and imbibing sugar, and each independent appearance of a probing/feeding mosquito was considered to be one sugar visit. Clock time was recorded for each sugar visit. Deaths were recorded daily to provide current data for calculating probing rates. When a dead mosquito was found, it was presumed to have died during the midpoint of the previous 24-hr period.

In all treatments, mosquitoes had access continuously to both water and to the 20% sucrose solution. Water was changed every 4-5 days, and sucrose solution was changed once per week. The constant availability of water reduced the likelihood that the mosquitoes would visit and imbibe the sugar solution to obtain water. Ambient temperature was the same as that described above for rearing and maintenance. Relative humidity was slightly higher, due to confinement of the water feeder and sugar feeder within the plastic cage.

Individual males and females were observed under the following conditions: Immediately after taking an initial blood meal 3-4 hours prior to crepuscular dimming, virgin females were observed for 3 days following eclosion with and without daily access to a blood host; inseminated females were observed for 20 days following insemination with daily access to a blood host and oviposition site, daily access to blood host but with oviposition site delayed 5 days, or with daily access to oviposition site but with blood host access delayed 5 days; males were observed for 3 weeks following eclosion.

Virgin females were placed in observation cages from the emergence cage in the afternoon of their first day post emergence. Those that were blood-fed received their initial blood meal that evening after the beginning of scotophase. Most would not blood-feed before this time. They were then offered blood during each crepuscular dimming period for 3 days.

Due to the time constraints, blood feeding took place each evening at the beginning of crepuscular dimming. If a female bit within that time, she was allowed to feed until repletion. A human hand and forearm was offered for 10 min from one of two people.

6.3.3 Monitoring flight activity

General flight activity of newly emerged males and females, as well as blood-fed inseminated females, was recorded in order to relate the timing of sugar-feeding to other activities. Individual mosquitoes were placed separately in 1-liter acrylic recording chambers 2-3 hr prior to the onset of crepuscular dimming and were recorded for 4-5 days.

Two recording chambers were monitored simultaneously within a Faraday cage. Each recording chamber was supplied with one 5% sucrose-soaked cotton wick and one wick of water only. It was positioned in an array of 16 parallel light beams from 16 infra-red LEDs aimed at 16 photoreceptors on the opposite side of the chamber. An impulse was generated, amplified, and recorded by computer each time a mosquito interrupted one of the beams by either crawling or flying through it. For analysis and interpretation, data were totaled at consecutive 30-min intervals.

6.3.4 Data analysis

Data from diel rhythms of sugar feeding and flight recordings were grouped into 0.5 hour intervals over each 24-hr period. Each mean \pm SEM of each time interval was based on the combined data from multiple females and multiple diel periods for graphic presentation. For statistical analysis, the independent datum was either a single sugar visit, where the mosquito was observed probing for sugar (in the case of diel sugar feeding rhythms), or a single IR-beam interruption (in the case of diel flight rhythms). Each replicate consisted of data that were grouped into the same 0.5 hour interval on different days or with different mosquitoes. Differences in sugar feeding between treatment groups were analyzed by Mann-Whitney *U* test for single comparisons or by Kruskal-Wallis test with post-hoc analysis (Siegel and Castellan 1988) when multiple comparisons were needed. All analyses were performed using Statistica (StatSoft, Inc).

6.4 Results

6.4.1 Frequency of sugar feeding

Males fed on sugar an average of twice daily, significantly more often than virgin ($P < 0.0001$) or inseminated females ($P < 0.0001$), even when blood ($P = 0.0386$ virgins and $P < 0.0001$ inseminated) or oviposition ($P < 0.0001$) were withheld (Table 6.1, Fig 6.2). Males continued to sugar feed at a nearly constant daily frequency during the first 2 weeks of adult life ($P = 0.1861$) (Fig. 6.3a). Females also continued to sugar feed at nearly constant frequency through the study, whether or not blood was limited ($P = 0.7406$ and $P = 1.0$) (Fig. 6.3b). On average, females with daily access to both blood and an oviposition site fed on sugar only about once every 4 days. In delayed-blood and delayed-oviposition regimes, sugar visits averaged more frequent than once per night. Females whose oviposition was delayed by withholding the oviposition cup appeared to increase their sugar feeding substantially in the 2nd gonotrophic cycle, but the difference was not significant ($P = 0.06$, U test).

Newly emerged virgin females sugar fed almost daily in the absence of a blood meal during the 3 days of observation (Table 6.1). However, when newly emerged females were given a blood meal prior to sugar access, sugar feeding frequency was dramatically lower ($P < 0.0001$). Because blood was offered daily to the latter group, many individuals never fed on sugar.

During these *ad lib.* sugar experiments, daily biting frequency was reduced significantly by delaying access to blood after oviposition ($P = 0.0451$), but not by delaying access to an oviposition site ($P = 0.9245$) (Table 6.2) after egg maturation. However, delaying either blood or oviposition increased sugar feeding (Table 6.1). In

other words, lengthening the gonotrophic cycle duration increased both blood feeding and sugar feeding per gonotrophic cycle. A closer look at females with delayed oviposition revealed that this was usually the case, but a few individual showed a strong preference for blood feeding over sugar feeding (7 blood meals and 2 sugar meals in 17 evenings at one extreme) or vice versa (3 blood meals and 46 sugar visits in 17 evenings at the other extreme)

6.4.2 Time of sugar feeding

Male flight activity was nocturnal, with a large peak of activity during the late crepuscular period prior to scotophase and a much smaller peak of activity early in the crepuscular period following scotophase. Sugar feeding occurred in a diel pattern that approximated general flight activity (Fig. 6.4).

For at least 48 hr following a blood meal, inseminated females ceased flight activity (Fig. 6.5) and did not feed on sugar (Fig 6.6). On the 3rd evening following a blood meal, there was a striking onset of activity that slowly declined and ended prior to photophase (Fig. 6.5).

Figure 6.7 shows the diel flight activity pattern of inseminated females that had not yet fed on blood and of gravid females during the night of oviposition. Both were characterized by an initial peak of activity at the interface between crepuscular dimming and early scotophase. In the former, this was followed by a second increase in activity late in scotophase. In the latter, activity continued to decline after the initial peak (see also Fig. 6.5). Females with daily access to blood and oviposition sugar-fed infrequently during late scotophase (Fig. 6.8a) of the night of oviposition (Fig. 6.6a). When blood was limited to once every 5 days, sugar feeding began 2 nights after the blood meal and

continued nightly until the next blood meal (Fig 6.6b). Sugar-feeding activity appeared to increase as scotophase progressed (Fig. 6.8b) and was similar to the flight-activity pattern of inseminated blood-deprived females (Fig 6.7). When oviposition was limited, sugar feeding began 2 nights after the vitellogenic blood meal, presumably once eggs were fully developed, and it continued nightly until eggs were laid and a subsequent blood meal was taken. The sugar-feeding activity was highest early in scotophase and declined through scotophase (Fig 6.8c), similar to the flight activity pattern of gravid females the night of oviposition (Fig 6.7).

6.5 Discussion

Sugar feeding in both male and female *An. gambiae* followed a crepuscular and nocturnal diel rhythm similar to that of flight and other activities, as observed previously in the laboratory or field (Hocking and MacInnes 1948, Muirhead-Thomson 1948, Mattingly 1949, Haddow and Ssenkubuge 1962, Jones and Gubbins 1977, 1978, Charlwood and Jones 1980, Marchand 1984, Charlwood et. al. 2002). This is typical of other species investigated (McCrae et al. 1976, Yee and Foster 1992, Yee et al. 1992). It is beyond the scope of this study to determine whether or not sugar feeding in females has a unique endogenous rhythm separate from blood feeding. Based on studies with other species, it seems likely that appetitive activity periods are non-specific and food choice is related to strength of stimuli, food availability, and nutritional state rather than separate rhythms for sugar and blood (Yee et al. 1992). This was demonstrated in the current study when blood access was limited. Sugar feeding increased daily until blood was offered, and it closely approximated published diel host-seeking patterns (Mattingly

1949). That the increase in general flight activity increased the chance of contact with sugar source, and thereby increased sugar-feeder visits, cannot be ruled out. This might explain why females deprived of oviposition sites sugar-fed at a higher frequency, similar to the higher flight activity of gravid females the night of oviposition. An alternative explanation is that increasing energy demands associated with searching for oviposition sites, coupled with decreased blood feeding, increased the females' need for sugar early in the period of activity.

When females were not deprived of blood or oviposition sites, sugar feeding occurred at a low frequency during late scotophase. Sugar feeding probably followed oviposition, which primarily takes place in early scotophase in the laboratory (Haddow and Ssenkubuge 1962, McCrae 1983, Sumba et al. 2004). It is during this time, while the female is outdoors, that she is most likely to be sugar feeding in this otherwise endophilic species (Muirhead Thomson 1948, Hocking and MacInnes 1948).

Males sugar-fed more frequently than females and continued to feed at a sustained higher frequency over the 2 week period of observation. This is typical of the males of other species (Foster 1995) and is explained by their relatively rapid sugar digestion and lack of any alternative food (see Chapt. 4), resulting in poor performance and high mortality when sugar is absent (see Chapt 3). *An. gambiae* males' need for frequent sugar feeding implies that nectar sources must be available and used frequently where this species thrives.

Female sugar-feeding activity dropped to very low levels after blood feeding, but it returned to a higher frequency once eggs were developed and remained high until the

next blood meal. Thus, sugar feeding in females took place almost exclusively while a female was gravid and after oviposition but before the next blood meal.

It is plausible that post-oviposition sugar feeding will not occur in the field if hosts are readily available. However, evidence suggests that sugar feeding does occur between gonotrophic cycles, because the number of host-seeking females that test positive for fructose is relatively high (Beier 1996, Gary, Foster, and Knols unpublished). When hosts are not readily available (as would occur in areas with common bednet use), sugar feeding probably increases markedly after oviposition and is a valuable method for obtaining enough energy to survive until the next blood meal.

It is possible that during dry conditions or when oviposition sites are some distance from human dwellings, oviposition also might be delayed. Under these circumstances, females deprived of oviposition sites may feed on sugar, blood, or both, for sustenance starting the second night after the blood meal, when females are gravid. It appears that the mechanism that inhibits further blood feeding during vitellogenesis (Klowden and Lea 1979) also inhibits sugar feeding. It should be noted here that there are conflicting reports on the degree of inhibition to subsequent host seeking after a blood meal. Some studies have found almost no inhibition to further host seeking (Klowden and Breigel 1994), while others have found that females were inhibited from feeding until after eggs had matured (~ 72 hours) (Takken et al. 2001; Chapter 5). Furthermore, the inhibition of both feeding behaviors ends at egg maturation and is not tied to oviposition. When females took supplemental blood meals, sugar feeding was still suppressed for 2 days after the supplemental meal. Any suppression of blood feeding caused by sugar feeding was not detected in this study.

The results of this study are not to be meant to be a substitute for field work, because the conditions in the laboratory were such that temperatures were held stable, flight demands were very small, and sugar was readily available. Sugar-derived energy presumably would be utilized much more rapidly in the field than under these conditions. Conclusions from this study that should be supported by field findings are these: 1) males feed on sugar more frequently than females, 2) females feed on sugar between and not during gonotrophic cycles, and 3) under certain stressful environmental conditions such as drought or host unavailability, females increase their frequency of sugar feeding. The fact that females took advantage of sugar sources so quickly when denied blood or a chance to oviposit, and occasionally fed on sugar even when blood and oviposition sites were readily available, demonstrates the potential importance of this resource in the natural history of this species.



Figure 6.1. Sugar-feeders used to monitor sugar-feeding frequency

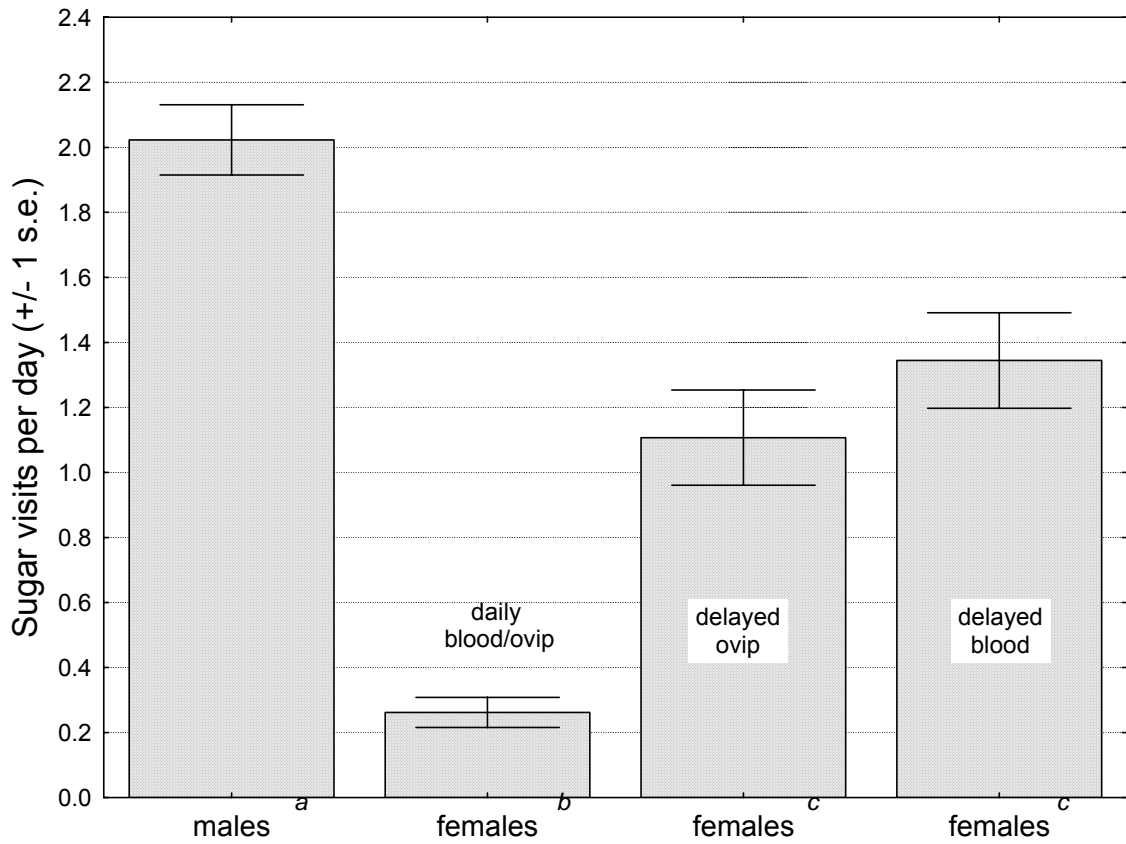


Figure 6.2. Average number of sugar visits per day over 20 days. Error bars are \pm SEM. Treatments with different letters are significantly different (Kruskal-Wallis test with post hoc analysis, $P < 0.05$).

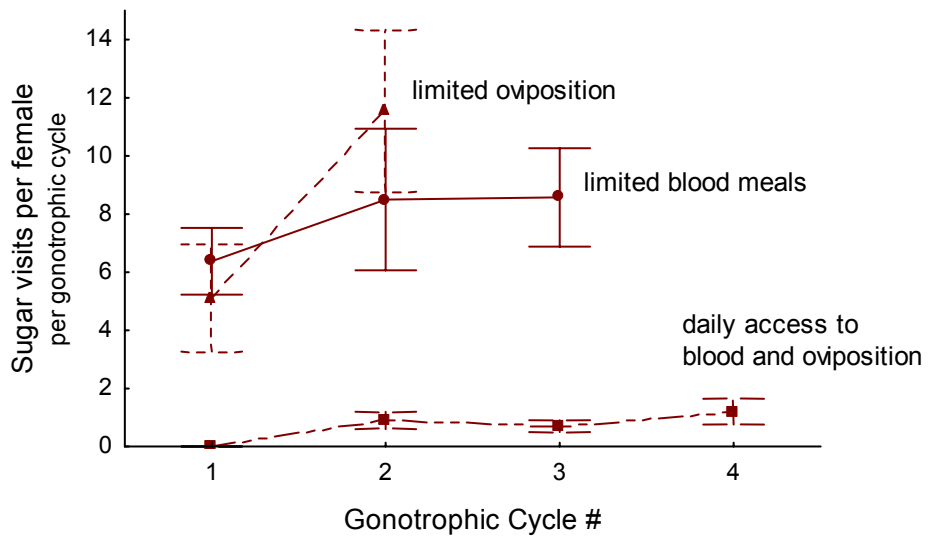
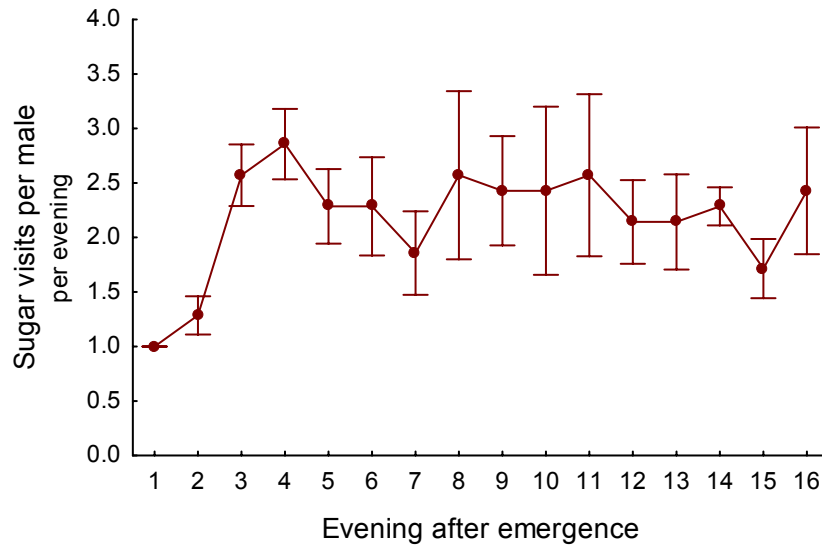


Figure 6.3. Frequency of sugar feeding by males over 16 days (top) and by females over the first 2 to 4 gonotrophic cycles (bottom). Error bars are \pm SEM. Limited = oviposition or blood was offered 5 days after previous oviposition or blood feeding.

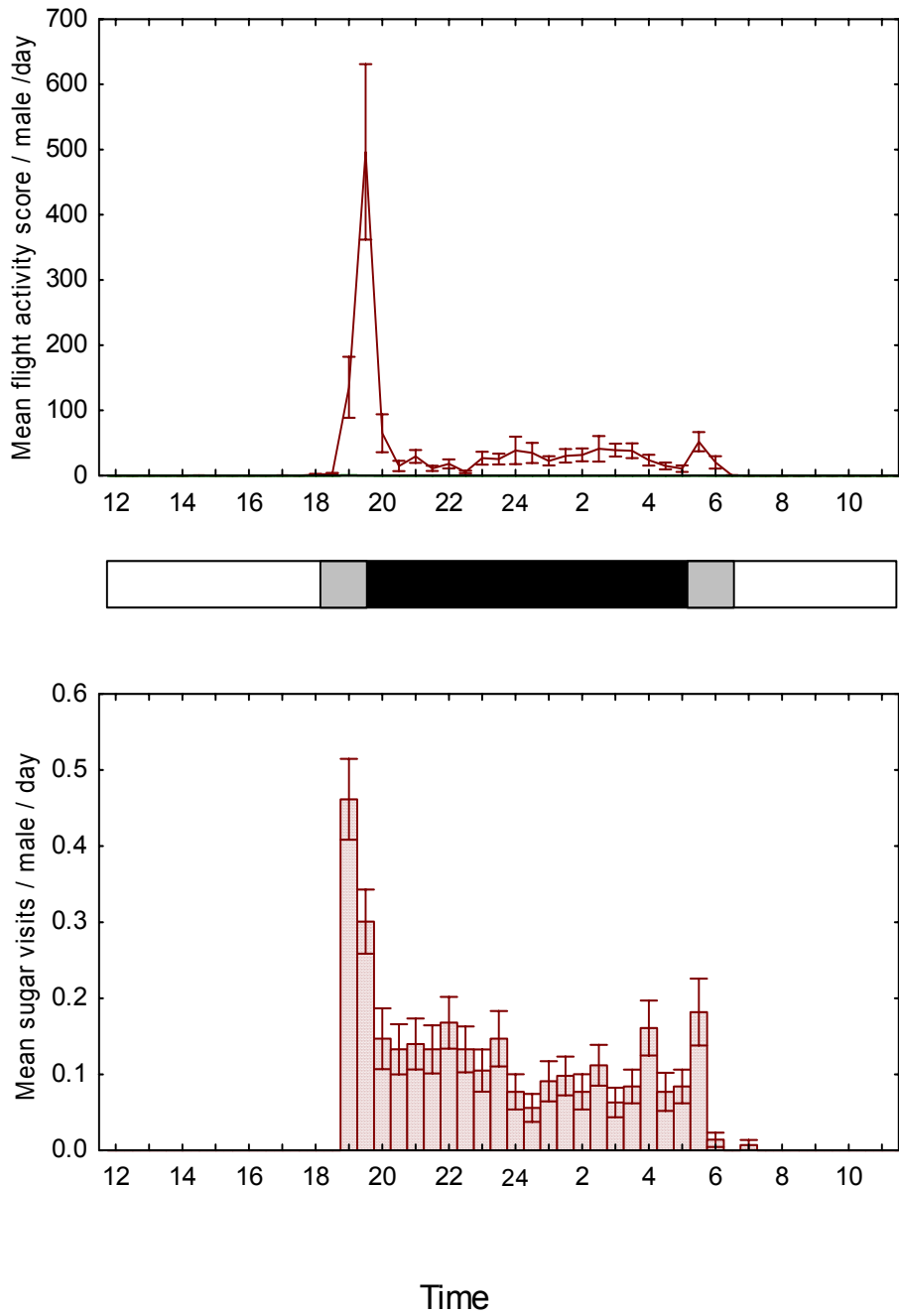


Figure 6.4. Diel timing of flight activity (top) and sugar feeding (bottom) of male *An. gambiae*. Error bars are \pm SEM.

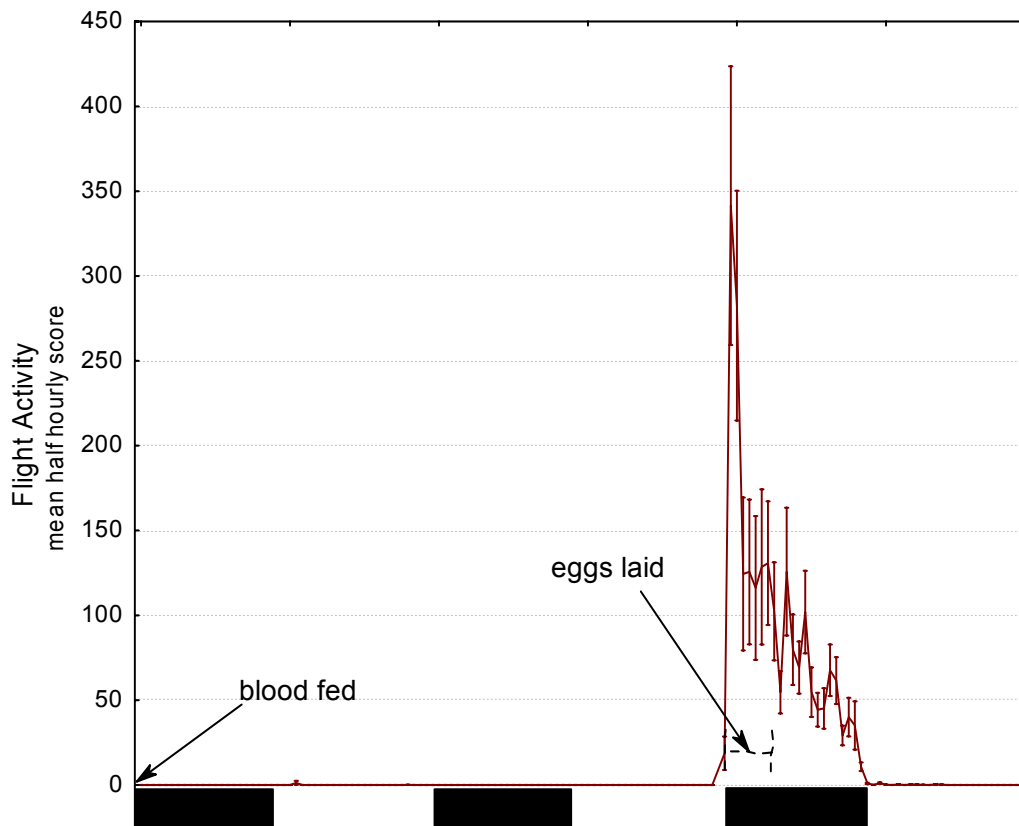


Figure 6.5. Flight activity of isolated inseminated females during 1 gonotrophic cycle when oviposition sites and sugar are available. Black bars on x-axis indicate scotophase. There were 75 minute crepuscular transitions between scotophase and photophase. Blood meals were given just prior to the first scotophase and, based on other lab studies, eggs are laid early during the 3rd scotophase. Error bars are \pm SEM. Mean values are derived from 10 females during 10 gonotrophic cycles.

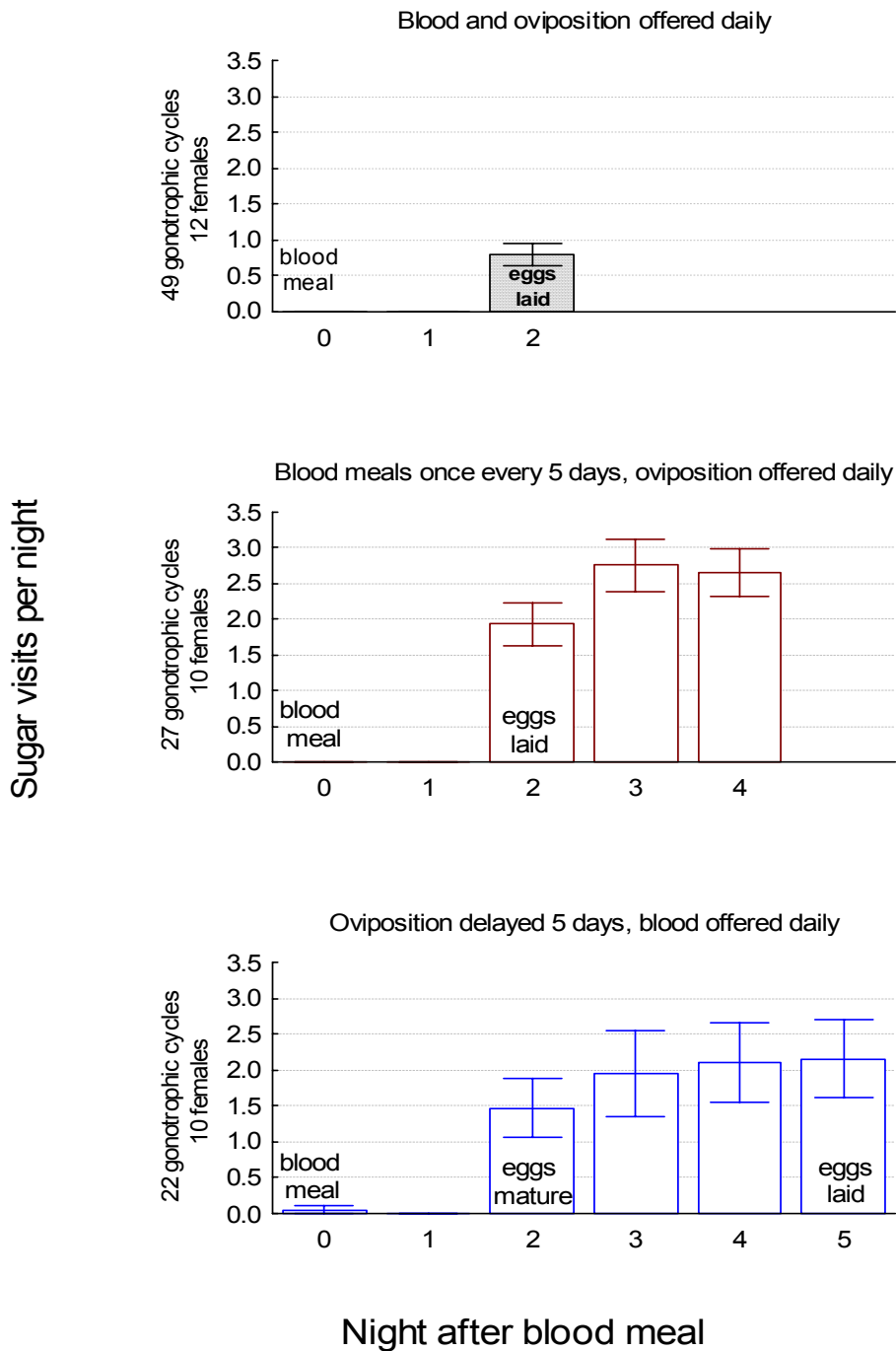


Figure 6.6. Sugar visits per night during a gonotrophic cycle for females with blood and oviposition sites offered daily (top), blood delayed for 5 days (middle) and with oviposition sites delayed for 5 days (bottom). Error bars are \pm SEM.

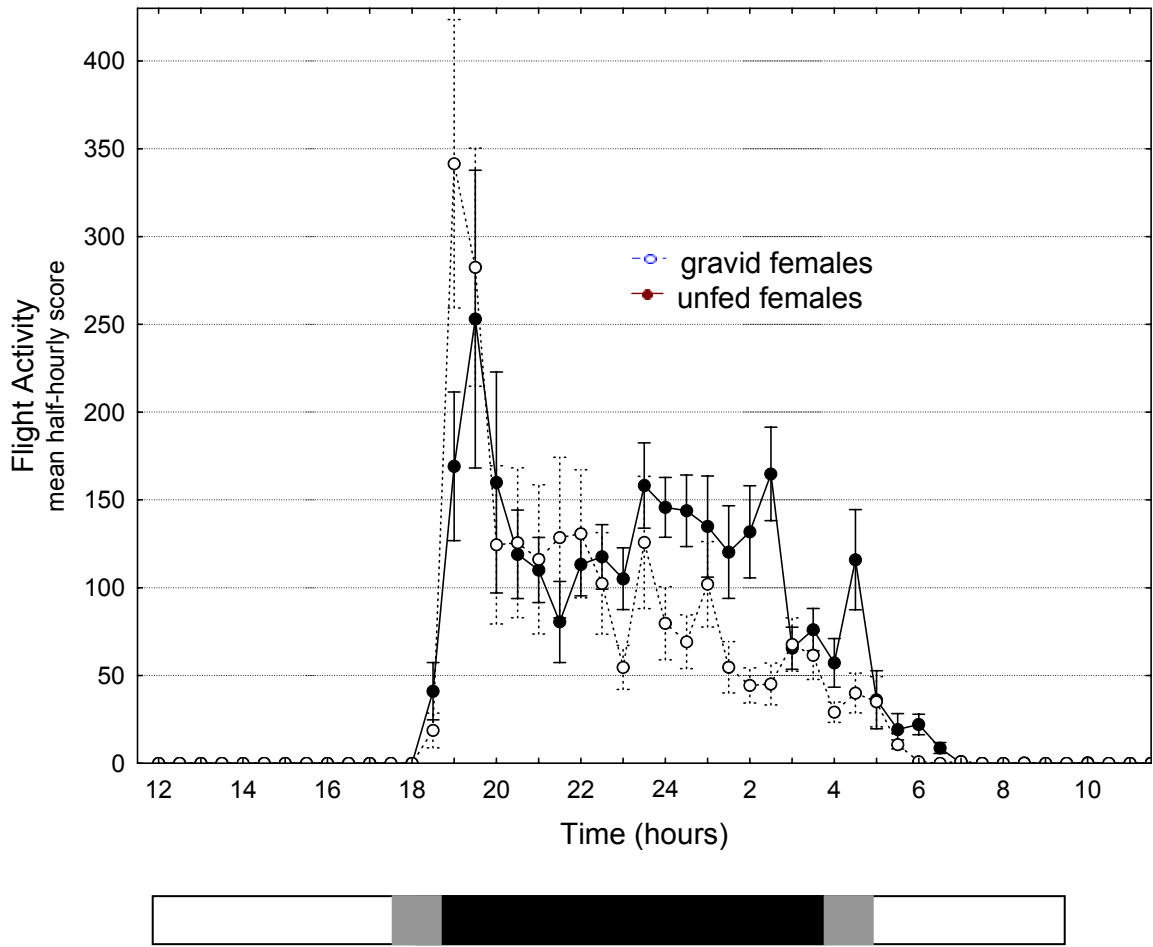


Figure 6.7. Flight activity of non-blood-fed inseminated females (solid circles) and gravid inseminated females the night of oviposition (open circles). Error bars are \pm SEM. Mean values were derived from 10 females (gravid) over 10 gonotrophic cycles and 6 females (inseminated) over 12 days.

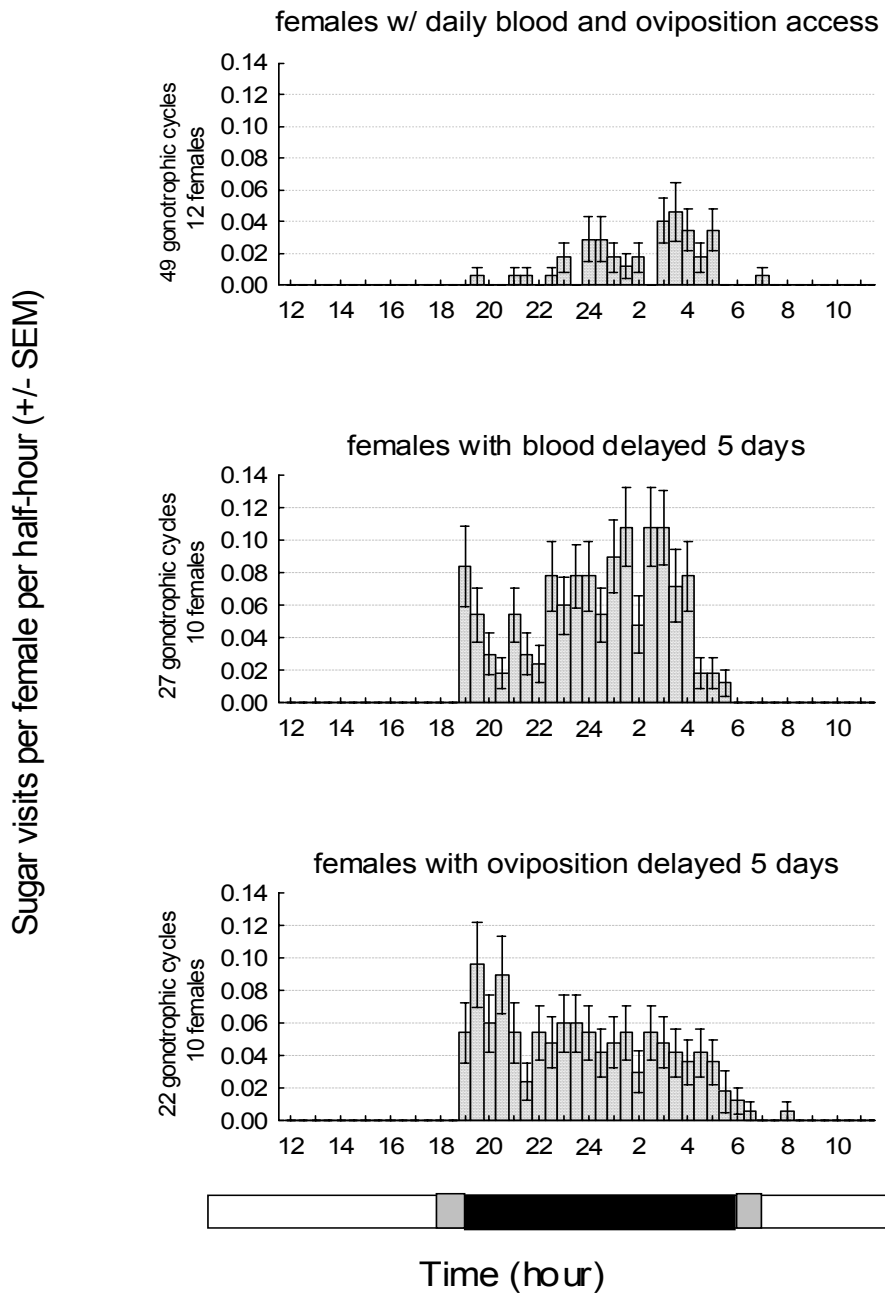


Figure 6.8. Diel timing of sugar feeding by females with daily access to blood and oviposition (top), with blood meals delayed 5 days (middle) and with oviposition delayed 5 days (bottom). Horizontal bar at the bottom represents scotophase (black), photophase (white) and crepuscular transition (grey) in relation to the x-axis. Error bars are \pm SEM.

Treatment (N)	Total No. nights (No. GTC)	<u>Mean No. Sugar meals</u>	
		per day \pm 1 SEM*	per GTC \pm 1 SEM*
Males (15)	130	2.02 \pm 0.11 _a	n/a
<u>Virgin females</u>			
Initial blood meal (10)	29	0.20 \pm 0.08 _{b,d}	n/a
No blood (10)	30	0.87 \pm 0.11 _c	n/a
<u>Inseminated females</u>			
Daily blood/ovip (12)	177 (49)	0.26 \pm 0.05 _b	0.69 \pm 0.11 _a
Limited ovip** (10)	167 (22)	1.11 \pm 0.15 _{d,e}	6.85 \pm 1.43 _b
Limited blood** (10)	148 (27)	1.34 \pm 0.15 _{c,e}	8.04 \pm 1.98 _b

Table 6.1. Average number of sugar meals per day and per gonotrophic cycle (GTC).

* Means within the column with different letters are significantly different (Kruskal-Wallis rank test with post-hoc analysis, $P < 0.05$).

**Limited ovip and Limited blood indicate that oviposition site or blood was offered 5 days after oviposition or after blood feeding, respectively.

Treatment (N)	Total No. nights (No. GTC)	<u>Mean No. Blood meals</u>	
		per day \pm 1 SEM. *	per GTC \pm 1 SEM*.
Daily blood/ovip (12)	177 (49)	0.34 \pm 0.04 _a	1.14 \pm 0.06 _a
Limited ovip** (10)	167 (22)	0.28 \pm 0.03 _{a,b}	1.68 \pm 0.15 _b
Limited blood** (10)	148 (27)	0.18 \pm 0.03 _b	1.04 \pm 0.04 _a

Table 6.2. Average number of blood meals per day and per gonotrophic cycle (GTC).

* Means within the column with different letters are significantly different (Kruskal-Wallis rank test with post hoc analysis, $P < 0.05$).

**Limited ovip and Limited blood indicate that oviposition site or blood was offered 5 days after oviposition or after blood feeding, respectively.

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Chapter 7

The Effect of Restricted Blood and Oviposition-Site Access on Sugar-Dependence for Egg Production and Survival of *Anopheles gambiae* (Diptera: Culicidae) Females

7.1 Abstract

Female *Anopheles gambiae* were placed individually in cages to evaluate the effect of 10% sucrose on fecundity, survivorship and biting (blood feeding) frequency when oviposition sites were delayed for 6 days after blood feeding or when blood meals were delayed for 6 days after blood feeding. When blood feeding and oviposition were allowed daily, females survived a few days longer when sugar was available, but they fed on blood more often and laid more eggs when deprived of sugar. When blood was delayed, sugar feeding was critical in sustaining females until the next blood meal. Females died within 2-3 days after ovipositing if deprived of sugar or blood. When oviposition was delayed, females that were offered blood daily survived as long on a diet of blood alone as they did on sugar and blood. There also was no difference in fecundity. However those that were offered blood alone took it more frequently than did those with access to sugar. Results reveal that in the absence of sugar, females can utilize blood alone to improve their reproductive success, while at the same time increasing vectorial capacity and malaria

transmission. Because they will feed on sugar when it is available, even though fecundity is reduced, it may be possible to use plants to impede malaria transmission through lowered biting frequency and reproductive output.

7.2 Introduction

For most mosquitoes, sugar can be a limiting resource, because it can alter survival and reproduction. For males of all species and females of a few, early and frequent sugar feeding is critical to survival and performance. Females of the remaining species rely on sugar when metabolic reserves are low or other resources are unavailable (Foster 1995). Females of a few medically important anthrophagic species have apparently adapted to living with little or no sugar (Macfie 1915, Muirhead-Thomson 1951, McCrae 1989, Edman et al. 1992, Scott et al. 1997). This is likely to be due to the easy availability of blood meals rather than to a lack of accessible sugar (Martinez-Ibarra et al. 1997), because males of these species still must feed on sugar to survive and are not known to fly great distances in search of sugar or females (Charlwood and Jones 1980, Marchand 1984, Edman et al. 1992, Charlwood et al. 2002b). The questions still remain whether or not and under what conditions sugar might be a limiting resource for these species.

In the case of *Anopheles gambiae*, the world's most important malaria vector, females suffer a slight reduction in survivorship when sugar-deprived (Straif and Beier 1996, Gary and Foster 2001). However, there is no apparent cost to reproductive success as long as other needed resources (e.g. blood hosts, oviposition sites) remain immediately available (Gary and Foster 2001). In the field, nectar-producing plants are available in their habitat, can improve survival (Gary and Foster 2004), and certainly are of importance to

males (see Chapter 3), but are thought not be of importance to females (McCrae 1989). This position is tenuous, because potential sampling biases neglect the outdoor population and, considering the rapid rate of digestion of this species (see Chapter 4), risk overlooking what might be an important and even limiting part of the female's biology. Females feed on nectar only before or between gonotrophic cycles, often either while the female remains gravid—in the case of delayed oviposition—or soon after the eggs are laid (see Chapter 6). It is during this time that they are most likely to encounter nectar sources. In a previous study (Chapter 6), females were found to feed on sugar periodically, even when blood meals and oviposition sites were readily available. However, sugar feeding markedly increased when either resource was delayed. A variety of conditions might lead to these or other physiological stressors in the field. Blood meals might be delayed when, through the use of bednets or other personal protection measures by humans, females are effectively prevented from biting (Lindsay et al. 1991, Seyoum et al. 2002a, b). Oviposition might be delayed in the field during the dry season or dry spells (Omer 1970), when oviposition sites are far from human habitation (Gillies 1988) or when the number of breeding sites is reduced through cultural practices. Under these circumstances, sugar may be a limiting resource for females, even if it is not one under more ideal conditions. The present study was conducted to test the hypothesis that a delay in either oviposition or blood feeding increases a female's sugar dependence, so that sugar availability has a measurable effect on her survival and reproductive success.

7.3 Materials and Methods

7.3.1 Mosquito rearing and maintenance

An. gambiae used in all experiments were of the Suakoko strain, established by M. Coluzzi in 1987 from material originating in Suakoko, Bong, Liberia. Colony adults were maintained on honey-soaked sponges, water, and periodic human blood meals. Blood feeding was conducted in accordance with The Ohio State University's Biomedical protocol No. 2004H0193 and Biosafety protocol No. 2005R0020. Oviposition cups were placed with caged adults 2 days after each blood meal, and eggs were collected the following day. The laboratory conditions for the colony were $27 \pm 1^\circ\text{C}$, $80 \pm 5\%$ RH, and 13:11 (L:D), with 75-min gradual crepuscular transitions between photophase and scotophase. Full scotophase started at 2125 hours.

Larvae for both the colony and experiments were reared as follows: Eggs were disinfected with 0.05% sodium hypochlorite solution and hatched overnight in flat enamel-coated pans of aged tap water. First-instar larvae were placed, 100 each, in 22.8 x 33.0-cm aluminum pans with 450 ml of aged tap water. The larvae were fed a standard diet of finely ground TetraMin® fish flakes, following a daily schedule that produced a nearly synchronous pupation 7-8 days after hatching. Pupae were collected daily and placed in a 41-liter cage supplied with water-soaked cotton dental wicks. Three-day-old sugar-fed males were placed with newly emerged females (2:1, M:F) overnight and replaced daily for up to 3 days to achieve insemination. On the second day, females were offered 10% sucrose or blood, depending on treatment group. Inseminated females were then placed

individually in small (15 x 21 x 27 cm) acrylic plastic cages supplied either with two water-soaked cotton wicks (water-only group) or with one water wick and one 10% sucrose-soaked cotton wick (water + sugar group).

7.3.2 Survivorship, fecundity, and biting frequency

Each water-only and each water + sugar cage was assigned to one of three treatments: daily offering of blood and one oviposition cup, blood delayed 1 week after previous blood meal, or oviposition cup delayed 1 week after previous egg batch (or blood meal in the case of the first cycle). The oviposition cups were clear plastic cups (5.5 cm diam, 3.5 cm deep), half-filled with aged tap water. The cup from each cage was replaced daily to maintain water surface tension and to remove eggs, which were counted in this experiment. Water and sucrose were replaced every 5 d. Thirteen females were observed for each treatment. To blood-feed each female, the hand and forearm of a human host was offered for 10 min, late in the afternoon (1800-2000 hours). If a female bit during that time, she was allowed to feed to repletion. Survivorship and fecundity were recorded daily before blood feeding. Bites were also recorded daily. Females that failed to lay eggs were removed from the study and later invariably were found to be uniseminated.

7.3.3 Reproductive success calculations

A life-table analysis of the adult portion of the life cycle was performed (Price 1997, Service 1993) to provide a crude estimate of relative reproductive success of females in each treatment. For the sake of simplicity, this estimate overlooks the potential influence of sugar feeding on the prospects for survival and developmental rate of offspring under natural conditions, and therefore on its contribution to the next adult

generation. The experimental mean values for daily survival (l_x) and daily fecundity ($m_x = \frac{1}{2}$ eggs laid or female offspring, assuming 1:1 sex ratio) were used to calculate basic reproductive rate (or net replacement rate) ($R_o = \sum l_x m_x$), which is the mean number of daughters born to a female during her lifetime and represents reproductive success.

7.3.4 Data analysis

Survival data were analyzed with the Kaplan-Meier test (StatSoft 1999) to obtain survivorship curves. Mean survival times were compared between groups using the Gehan modification of the Wilcoxon test (StatSoft 1999). A Mann-Whitney U test (Sokal and Rolf 1981) was used to compare means of fecundity and lifetime biting frequency. Fecundity was measured as the number of eggs laid in the lifetime of a female. Biting frequency was measured as the number of bites per female per day. All data were analyzed with Statistica software (StatSoft 1999).

7.4 Results

7.4.1 Survivorship, fecundity, and reproductive success

Females offered sugar and oviposition sites *ad lib.*, and blood daily, survived longer than the other treatments (Fig. 7.1). However, after Bonferroni adjustment ($\alpha = 0.003$) to the Log Rank test for purposes of multiple comparison, they were found to live insignificantly longer than the other groups ($P > 0.003$), except for those sugar-deprived females with delayed access to blood ($P < 0.001$) (Table 7.1). When oviposition was delayed, but blood was not, sugar feeding had no effect on survival ($P = 0.902$). On the contrary, sugar was critical to survival when blood was limited ($P = 0.0001$).

Sugar-deprived females with daily access to blood and oviposition sites laid substantially more eggs than females with access to sugar ($\bar{x} = 1469.7$ vs. 807.9) ($P = 0.001$). When blood meals were delayed, females with sugar available laid significantly more eggs than those that did not ($\bar{x} = 326.7$ vs. 56.8) ($P = 0.001$). However, when oviposition was delayed, sugar availability had no significant effect on fecundity ($\bar{x} = 347.2$ vs. 407.4) ($P = 0.496$). Differences in fecundity were not caused by delays in blood feeding alone; there were also differences in egg-clutch size (Fig. 7.3).

The life-table analysis showed that when blood and oviposition sites were available daily, sugar-deprived females had a higher basic reproductive rate than sugar-fed females ($R_0 = 706.3$ vs. 393.6) (Fig. 7.4). When blood was limited to once weekly, $R_0 = 163.4$, when sugar was available. When females were deprived of sugar and blood was delayed, females did not survive beyond the first gonotrophic cycle. When the availability of the oviposition site was delayed, the basic reproductive rate for females deprived of sugar was slightly higher than for females with access to sugar ($R_0 = 173.6$ vs. 166.2).

7.4.2 Biting Frequency

To display the differences in biting frequency between treatments clearly, Figure 5 shows the average of the cumulative number of blood meals over time. Females bit more frequently when they were deprived of sugar, whether or not oviposition sites were delayed ($P = 0.0002$ and $P = 0.0001$, respectively). By contrast, there was no difference in biting frequency caused by delayed oviposition, whether sugar was available or not ($P = 0.1124$ and $P = 0.5453$, respectively) (Table 7.1; Fig. 7.5).

7.5 Discussion

When human hosts and oviposition sites are readily available, but sugar is not, the increase in reproductive success experienced by *Anopheles gambiae* females appears to offset the slight survival advantage provided by available sugar. This finding is supported by previous studies (Straif and Beier 1996, Gary and Foster 2001) and would not be surprising to some *An. gambiae* biologists (Muirhead-Thomson 1951, Gillies 1968, Beier 1996). Yet it is unusual, because other mosquitoes allowed only blood have reduced fecundity and survivorship and may even starve to death when blood is available daily (Nayar and Sauerman 1971, 1975, Briegel and Kaiser 1973, Nayar and Pierce 1980, Foster et al. 1995). Only one other species, *Aedes aegypti*, is known to gain a reproductive advantage from feeding on blood alone (Scott et al. 1997, Costero et al. 1998b, Harrington et al. 2001). While not closely related, these two species are both anthropophilic and endophilic. Human blood is unusual in being deficient in isoleucine, so that a large proportion of each blood meal is allocated to energy reserves (Briegel 1985, 1990a,b). In an environment where human hosts are more available than nectar sources, natural selection might explain this epidemiologically significant feeding strategy. However, it is important to consider that in this study, as well as those cited above, experimental females were confined to cages where flight demands were small and therefore where energy derived from sugar feeding likely would have been utilized more slowly than in the field. Thus, it is possible that the advantage of sugar feeding is underestimated by these studies. In the ideal scenario, a gonotactically active female with free access to resources is likely to take at least an occasional sugar meal either right before or

right after oviposition, to boost her energy before seeking another blood meal, because she has just gone 2 days without feeding. This is supported by the resurgence of sugar feeding at this time under laboratory conditions (Chapter 6).

When blood meals are delayed, sugar feeding can play an essential role in survival. Female *An. gambiae* in this study were relatively large (i.e. higher reserves; see Chapter 5), but starved to death within 2-3 days after ovipositing, if deprived of both sugar and more blood. In the field, where females are likely to be metabolically stressed, blood-meal delays are possible in a few circumstances. For example, the use of treated bednets and repellents for personal protection effectively reduces contact between *An. gambiae* females and human hosts (Lindsay et al. 1991, Seyoum et al. 2002a, b). From the perspective of the mosquito, this represents blood-meal deprivation. If the location of a host is delayed too long, the female likely will die from energy depletion, because she already will have lived for at least 2 days without food, unless she had taken a supplementary blood meal during digestion of the previous vitellogenic blood meal. A repelled female may locate an alternative human host quickly after being repelled from the first. If not, sugar feeding is among her options for surviving until she finds an unprotected host.

Dry season biology of *An. gambiae* is not well represented in the literature. There are conflicting studies that suggest that some females experience a reproductive diapause (Omer 1968, 1970), while others breed through the dry season (Alemayehu et al. 1998, Minakawa et al. 2001, Carlson et al. 2004). Females that continue to breed during the dry season or during dry spells are likely to be confronted with delayed oviposition, either because of quickly drying sources or because of the need to fly greater distances between

blood hosts and more durable oviposition sites. When oviposition is delayed, sugar feeding sustains a gravid female until she can oviposit. However, she will readily feed on blood for sustenance if sugar is not available. This study demonstrates that blood and sugar are exchangeable sources of energy for gravid females, and survival and fecundity are not affected by diet when oviposition is delayed. In Chapter 6, gravid females denied oviposition fell into 3 behavioral groups: those that fed on sugar frequently and occasionally took a supplemental blood meal while gravid, those that fed on sugar frequently and refused blood meals while gravid, and those that never fed on sugar and took frequent supplemental blood meals prior to laying eggs. Most were in the first group. In the field, a successful strategy involves feeding on the most readily available food source or, if both blood and sugar are available, feeding where the risk of death or lost time is smallest and reproductive gains are highest. This could favor any of the 3 groups described above, depending on the circumstances. Nectar feeding is obviously associated with the risk of predation by insects or spiders. However, blood feeding couples the risk of host defensive behavior with the risk of predation once the female is engorged and sluggish (Service 1973, Roitberg et al. 2003).

When oviposition was delayed, fecundity was not increased if females were denied sugar, even though they often took extra blood meals. In contrast, the results of Chapter 5 revealed that gravid females taking a blood meal while awaiting oviposition ultimately laid a larger batch of eggs. However, the two studies are not directly comparable, because Chapter 5 results included only the first egg batch. Females in the present study also had a larger first batch of eggs when they took multiple blood meals. However the lifetime fecundity of females with and without access to sugar was not

significantly different when oviposition was delayed. According to Briegel (1990b), female reserves are prioritized over egg development after a blood meal, especially if the general reserves are low. This may explain why the average size of the first egg clutch in Figure 7.3 is smaller than the second egg clutch, in five of the six environmental regimes. The exception was the case of sugar-deprived females subjected to delayed oviposition, when two blood meals were taken prior to the first egg clutch.

Perhaps the most epidemiologically relevant finding of this study is that blood feeding alone, even when females are prevented from laying eggs, promotes a high survival, fecundity, and biting frequency that maximize not only reproductive success, but also vectorial capacity of this species. Sugar feeding does not greatly improve survival unless blood feeding is delayed, but it does significantly reduce fecundity and biting frequency when blood is abundant. Because females are attracted to (Foster and Takken 2004) and readily feed on sugar when it is available, despite these effects, in localities where blood is easily accessible the potential exists to utilize nectar producing plants to reduce lifetime reproductive success and vectorial capacity, thus interfering with malaria transmission.

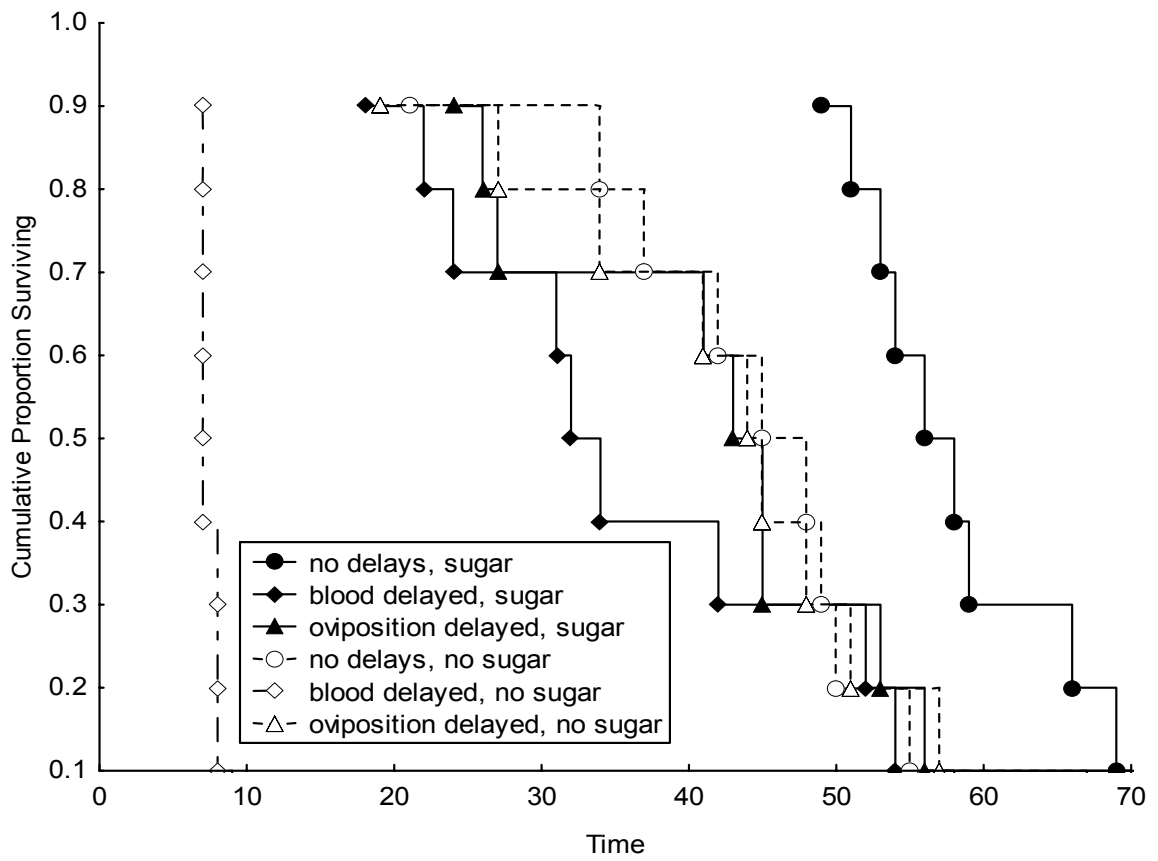


Figure 7.1. Effect of sugar availability on age-specific survivorship (l_x) of *An. gambiae* females when either blood or oviposition are delayed.

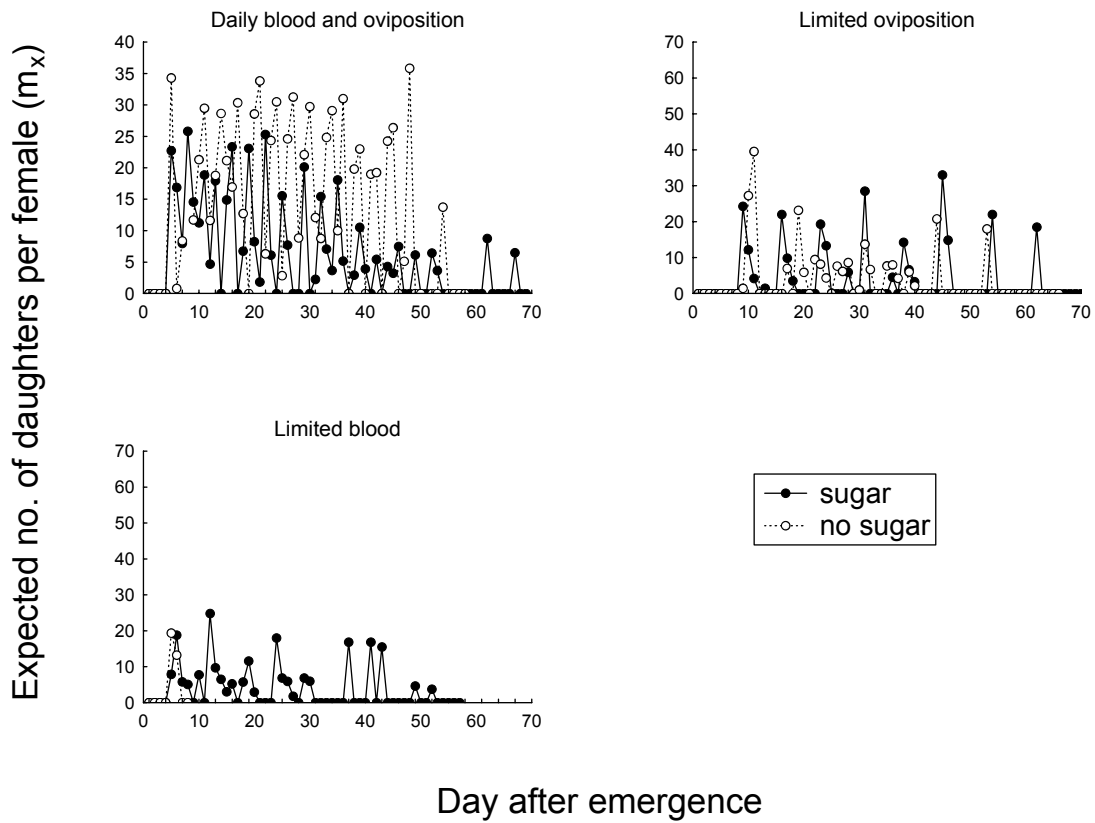


Figure 7.2. Effect of sugar availability on daily expected number of female offspring (assuming a 1:1 sex ratio) per surviving *An. gambiae* female (m_x) when either blood or oviposition are delayed.

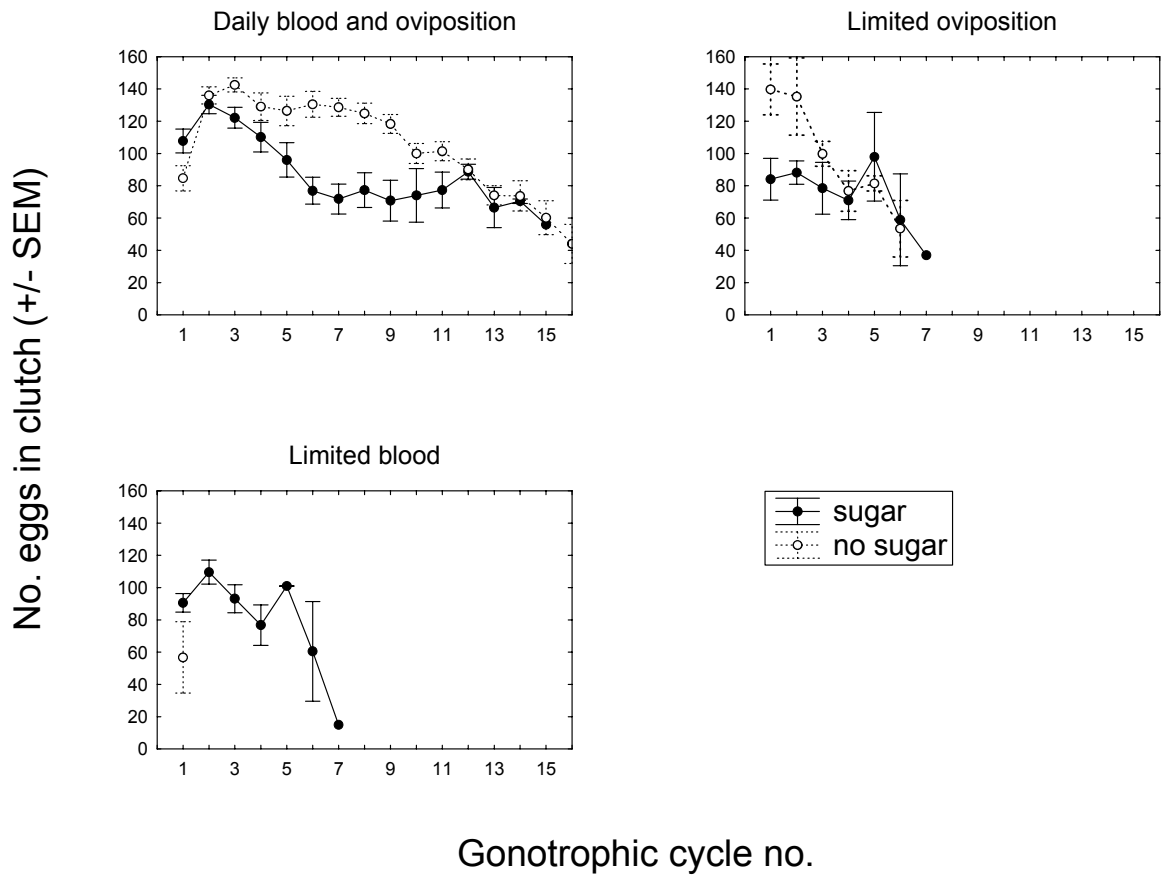


Figure 7.3. Effect of sugar availability on egg clutch size when blood and oviposition sites are available daily and when either blood or oviposition are limited.

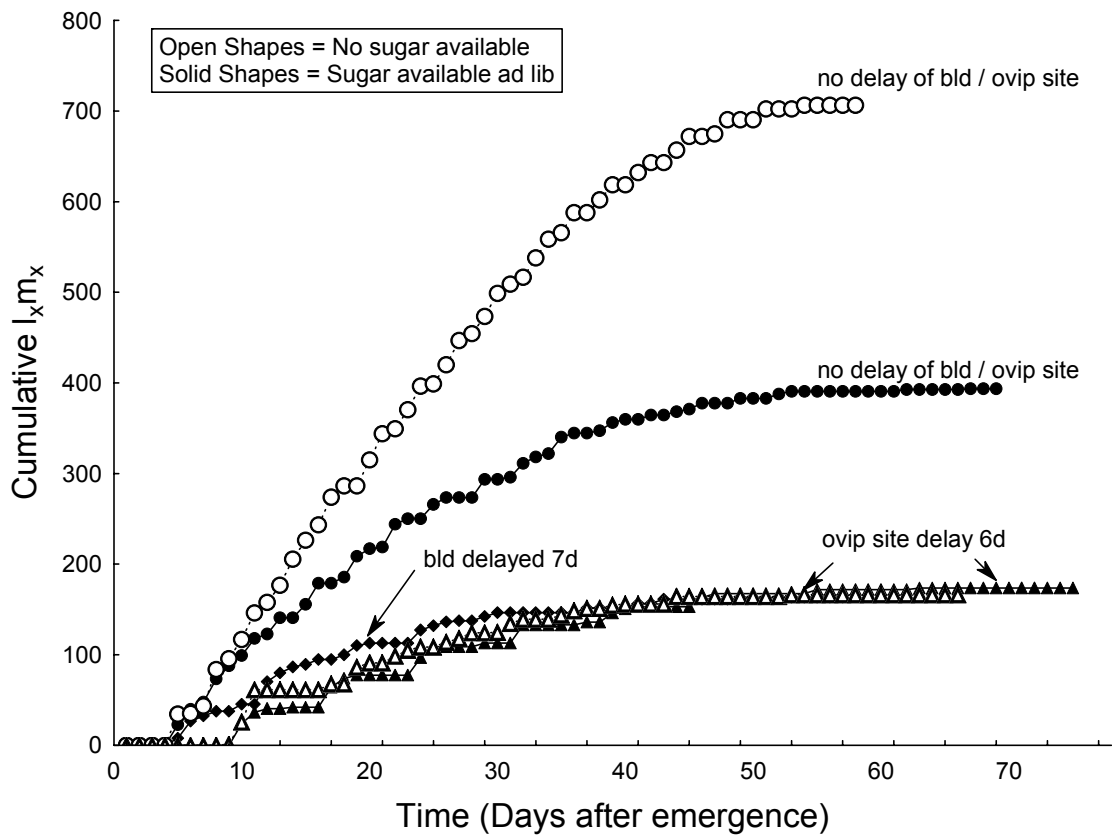


Figure 7.4. Effect of sugar availability on cumulative daily female offspring per original *An. gambiae* female (R_0 = basic reproduction rate) when blood and oviposition is available daily and when either blood or oviposition is delayed.

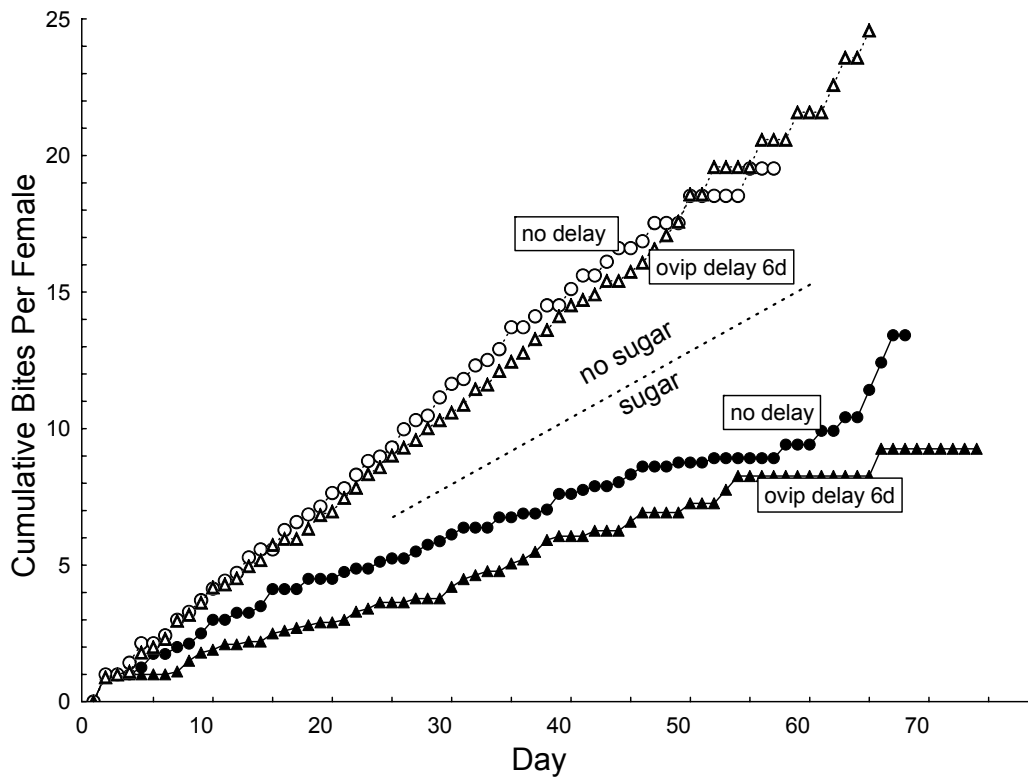


Figure 7.5. Effect of sugar availability and delayed oviposition on biting frequency.

Treatment	days survived¹ ± SEM	Total eggs² ± SEM	Bites per day² ± SEM
No delay			
Sugar (10)	58.5 ± 2.36 _a	807.9 ± 117.7 _a	0.16 ± 0.02 _a
No sugar (10)	44.0 ± 3.51 _a	1469.7 ± 144.9 _b	0.35 ± 0.01 _b
Limited Blood			
Sugar(10)	36.7 ± 4.48 _a	326.7 ± 47.1 _c	0.11 ± 0.01 _a
No sugar (10)	7.5 ± 0.22 _b	56.8 ± 9.8 _d	n/a
Limited Oviposition			
Sugar (10)	43.6 ± 5.02 _a	347.2 ± 68.9 _c	0.14 ± 0.01 _a
No sugar (10)	43.3 ± 4.45 _a	407.4 ± 73.2 _c	0.34 ± 0.02 _b

Table 7.1. Effect of sugar access on mean survival times, fecundity and biting frequency of female *An. gambiae* when access to either blood meal or oviposition is delayed.

¹ Means with different letters are significantly different at $\alpha = 0.003$, Bonferroni adjustment to Log Rank test.

² Means with different letters are significantly different at $\alpha = 0.003$, Bonferroni adjustment to Mann-Whitney *U* test.

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