

# Prior Blood Feeding Effects on Susceptibility of *Anopheles gambiae* (Diptera: Culicidae) to Infection with Cultured *Plasmodium falciparum* (Haemosporida: Plasmodiidae)

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**ABSTRACT** We examined the relative susceptibilities of *Anopheles gambiae* Giles of different physiological ages to infection with cultured *Plasmodium falciparum* (Welch). Cohorts of mosquitoes were divided into three groups; one was fed uninfected blood on day 3 after emergence (i.e., one prior blood meal); another on days 3 and 7 after emergence (i.e., two prior blood meals); and a control group was maintained on sucrose. On days 10 to 12 after emergence, mosquitoes were fed human blood containing *P. falciparum* gametocytes. Prior blood feeding accelerated digestion of the infective blood meals and subtly altered susceptibility to infection with *P. falciparum*. When gametocyte cultures were highly fertile, all experimental groups were equally susceptible to infection. However, when gametocyte fertility was low, accelerated digestion had a detrimental effect on the transition of ookinetes to oocysts. Accelerated digestion may raise the threshold density of ookinetes required for the successful conversion of ookinetes to oocysts.

**KEY WORDS** *Anopheles gambiae*, *Plasmodium falciparum*, blood meal

ANOPHELINES BEGIN THEIR adult life relatively undernourished compared with teneral culicine species, such as *Aedes aegypti* (L.) (Briegel 1990). The need for adult female anophelines (particularly smaller individuals) to accumulate protein reserves in excess of that necessary for vitellogenesis (Briegel 1990, Briegel & Horler 1993) may be the primary factor responsible for frequent blood feeding by anophelines observed in the field (Boreham & Garrett-Jones 1973, Burkot et al. 1988). Therefore, in terms of malaria transmission, *Anopheles* in the field may feed several times before contacting an infective host. Yet, it is standard practice to give mosquitoes the infective blood meal as their first (usually only) blood meal when conducting studies on anopheline susceptibility to infection with malaria parasites. The influence of prior blood feeding history on anopheline vector competence is unknown. In this study, we examined the effect of prior blood feeding on the susceptibility of *Anopheles gambiae* Giles to infection with *Plasmodium falciparum* (Welch).

## Materials and Methods

The G-3 strain of *An. gambiae* was used in all experiments. Cohorts of mosquitoes of the same chronological age were divided into three experimental groups. One group was fed on an uninfected human volunteer (J.A.V.) on day 3 after emergence (i.e., one prior blood meal); one group was similarly fed on days 3 and 7 after

emergence (i.e., two prior blood meals); and one group was maintained on sucrose (control). After each blood feeding, unengorged mosquitoes were removed from the cages. Mosquitoes were maintained at 27°C, 80% RH, 16:8 (L:D) h.

In addition to blood feeding effects, experimental groups originally were designed to test also the effect of ovipositional status of *An. gambiae* on *P. falciparum* infectivity by providing or withholding oviposition cups to individual cages. However, oviposition could not be controlled by this method. Mosquitoes in cages provided with oviposition cups often retained their eggs and did not oviposit. Likewise, gravid mosquitoes in cages with no oviposition cups often deposited eggs spontaneously on the cage floor. Because the true ovipositional status was uncertain, data were grouped only according to blood feeding history.

On days 10-12 after emergence, the three groups of mosquitoes were fed simultaneously on human blood containing *P. falciparum* (NF54 strain) gametocytes via membrane feeders (Beier et al. 1991). Unengorged mosquitoes were removed. Pools of mosquitoes were sampled from each cage at various times after feeding to obtain estimates on the absolute densities of macrogametocytes, ookinetes and oocysts per mosquito (Vaughan et al. 1992). Six to 12 mosquitoes per group were sampled for macrogametocytes immediately after feeding. Additional mosquitoes (6-12 per time interval) were sampled for ookinetes at 18, 24, 32, and 48 h after feeding.

**Table 1. Stage-specific densities (geometric means and 95% CL), mortalities (k-values) and oocyst infection rates (% and range) of *Plasmodium falciparum* parasites developing in *Anopheles gambiae* mosquitoes of differing blood feeding histories.**

Group	Macrogametocytes per mosquito	k-1	Ookinete per mosquito	k-2	Oocysts per mosquito	K	Oocyst infection rate (%)
No prior bloodmeal	9,068 (3,557-23,115)	1.23 (17-fold)	532.9 (165.2-1,717.4)	1.57 (37-fold)	14.27 (3.23-62.98)	2.80 (631-fold)	83 (74-90)
1 Prior bloodmeal	7,746 (3,108-19,299)	1.10 (12-fold)	609.2 (186.9-1,985.6)	1.66 (46-fold)	13.34 (6.34-28.06)	2.76 (575-fold)	82 (74-90)
2 Prior bloodmeals	8,041 (3,734-17,317)	1.39 (24-fold)	325.3 (155.4- 680.8)	1.52 (33-fold)	9.87 (3.11-31.32)	2.91 (813-fold)	82 (61-92)

Values represent the means of four separate experimental infections with highly fertile gametocyte cultures. k-Values—k-1 =  $\log_{10}$  (macrogametocyte) -  $\log_{10}$  (ookinete), k-2 =  $\log_{10}$  (ookinete) -  $\log_{10}$  (oocyst), K = k-1 + k-2.

Ookinete sampling over time was necessary to determine whether blood feeding histories of the mosquitoes altered the kinetics of ookinete formation. A minimum of 10 infected midguts were sampled for oocysts on days 9-12 after infection, except when oocyst infection rates were low (<40%). In such cases, oocyst dissections continued until all mosquitoes within a cage were examined.

To determine the effect of prior blood feeding on blood meal digestion, 6-12 mosquitoes were dissected at 0, 8, 12, 24, 32, and 48 h after the infective feed. Mean erythrocyte densities of the infectious blood meal for each time point were determined (Vaughan et al. 1991) and the kinetics of erythrocyte degradation were compared among groups.

### Results

In total, eight infectious feeds were conducted, each with different gametocyte cultures. Three cultures yielded negligible mosquito infection (=infertile gametocytes). Four cultures were highly infective and resulted in extremely good oocyst infection rates (73-90%). The remaining culture was of suboptimal infectivity, resulting in low to moderate oocyst infection rates (<40%). Unfortunately, the innate fertility of the cultures could not be controlled or even predicted in advance.

The effect of mosquito blood feeding history on *P. falciparum* sporogony was subtle and contingent on culture fertility. When fertility was very good, there were no differences among groups in the subsequent development of *P. falciparum* (Table 1), including; the kinetics of ookinete formation (peak = 32 hours postfeeding), mean ookinete densities (ANOVA,  $F = 1.00$ ;  $df = 2, 9$ ;  $P = 0.405$ ), mean oocyst densities (ANOVA,  $F = 0.29$ ;  $df = 2, 9$ ;  $P = 0.758$ ), or mean oocyst infection rates (multiple  $\chi^2$  tests among groups;  $\chi^2 = 1.19, 0.85, 0.85$ ;  $df = 1$ ;  $P = 0.3, 0.8, 0.8$ ).

However, when gametocyte fertility was suboptimal, there were significant differences among groups (Table 2). In this experiment, thin smears of the infectious blood were not obtained and so macrogametocytemia could not be calculated. Generally, there is negligible variation in gametocytemias among feeders within an infectious feeding (Vaughan et al. 1992) and thus, starting macrogametocyte densities for this feed probably were similar among mosquito groups. Ookinete densities peaked earlier (24 h after feeding) in the previously fed groups than in control mosquitoes (32 h). There were slight differences among groups in peak ookinete densities, but the differences were not significant (ANOVA,  $F = 2.05$ ;  $df = 2, 24$ ;  $P = 0.151$ ). However, ookinete populations in the various experimental groups experienced different mortalities (k-2) during their transition to oocysts resulting in significant

**Table 2. *Plasmodium falciparum* ookinete and oocyst densities (geometric means and 95% CL), mortalities (k-2) and oocyst infection rates (%) in *Anopheles gambiae* mosquitoes of differing blood feeding histories.**

Group	Ookinete per mosquito	k-2	Oocysts per mosquito	% Oocyst infection rate
No prior blood meal	257.9a (129.4-517.2) n = 6	2.59 (389-fold)	0.66a (0.37-1.00) n = 60	38.3a (23/60)
1 Prior blood meal	276.5a (151.2-504.8) n = 12	2.88 (758-fold)	0.36b (0.20-0.53) n = 90	25.6a (23/90)
2 Prior blood meals	95.3a (23.1-383.1) n = 9	3.28 (1,906-fold)	0.05c (0 -0.11) n = 76	3.9b (3/76)

Values represent the results of a single experimental infection with a gametocyte culture of suboptimal fertility. k-Value—k-2 =  $\log_{10}$ (ookinete) -  $\log_{10}$ (oocyst). Values within columns followed by the same letter are not significantly different at 0.05 level. Duncan's multiple range test on  $\log_{10}(x + 1)$  transformed data for means; multiple Chi square tests for infection rates.

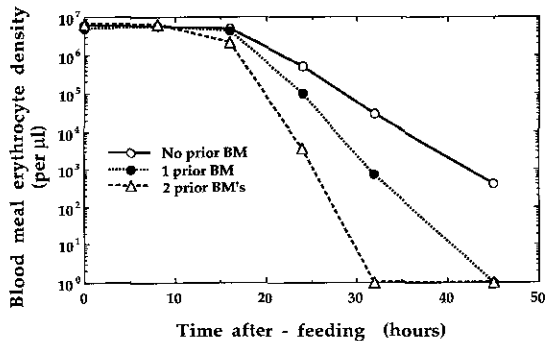


Fig. 1. Blood meal (BM) erythrocyte content (per  $\mu$ l) over time in *Anopheles gambiae* mosquitoes of different blood feeding histories and fed blood containing *Plasmodium falciparum*-infected human erythrocytes.

differences among groups in oocyst densities (ANOVA,  $F = 12.01$ ;  $df = 2, 222$ ;  $P < 0.0001$ ). Control mosquitoes produced the most oocysts (0.66 per mosquito), followed by mosquitoes with one prior blood meal (0.36 per mosquito) and lastly, mosquitoes with two prior blood meals (0.05 per mosquito). Mosquitoes with two prior blood meals had significantly lower oocyst infection rates (4%) than mosquitoes with either one prior blood meal (26%,  $\chi^2 = 14.56$ ,  $df = 1$ ,  $P < 0.001$ ) or no prior blood meals (38%,  $\chi^2 = 25.64$ ,  $df = 1$ ,  $P < 0.001$ ).

The rate at which blood meal erythrocytes were digested differed among groups (Fig. 1). Mosquitoes with a history of two prior blood meals digested their infectious blood meals the fastest, followed by mosquitoes with a history of one prior blood meal. Previously unfed mosquitoes had the slowest digestion times and intact erythrocytes were still present in the blood meal 48 h postfeeding.

### Discussion

There are two main processes in blood meal digestion known to affect early sporogony. First, mosquito digestive enzymes can damage young, retort-form ookinetes (Gass & Yeates 1979). Enzyme secretion peaks at ca. 30 h after feeding in previously unfed *Anopheles stephensi* Liston (Billingsley & Hecker 1991), a time when *P. falciparum* ookinetes have fully matured (Vaughan et al. 1992). Second, the mosquito peritrophic membrane may act as a barrier, retarding the exodus of mature ookinetes from the midgut lumen (Ponnudurai et al. 1988, Seiber et al. 1991, Miller & Lehane 1993). Coherent peritrophic membrane begins to form in previously unfed *An. gambiae* 12–15 h after feeding and is thickest at ca. 30 h (Freyvogel & Staubli 1965). Although both are separate physiological processes, enzyme secretion and peritrophic membrane formation act in synchrony during blood meal diges-

tion. Because parasite fertilization, transformation and penetration through the midgut require time, the kinetics of blood meal digestion may be a crucial factor in modulating mosquito susceptibility to infectivity (Terzian 1956, Ponnudurai et al. 1988, Feldmann et al. 1990, Billingsley & Rudin 1991). The faster the digestion, the less time afforded the parasite for fertilization/transformation/penetration.

Ingestion of prior blood meals by *An. gambiae* accelerated the digestive processes of subsequent blood meals. This is quite different from what occurs in *Ae. aegypti* where digestion of a second blood meal takes longer than digestion of the first (Edman 1970). Interestingly, oocyst production in *Ae. aegypti* was enhanced when the infective blood meal was given after complete digestion of a previous uninfected blood meal (Terzian et al. 1956, Gass 1977). Thus, slower kinetics of digestion in previously fed *Ae. aegypti* favored *P. gallinaceum* Brumpt ookinete success. Similarly, two different strains of *An. stephensi* selected for refractoriness and susceptibility to infection with *P. falciparum* were found to have correspondingly faster and slower rates of blood meal digestion (Feldmann & Ponnudurai 1989, Feldmann et al. 1990).

We found that accelerated blood meal digestion resulting from prior blood feeding in *An. gambiae* had a detrimental effect on the ability of *P. falciparum* ookinetes to penetrate the midgut. However, this effect was only operational when gametocyte fertility (hence ookinete abundance) was suboptimal. When fertility was optimal, there was no effect. The reason for this is probably related to the fact that the conversion of *P. falciparum* ookinetes to oocysts in *An. gambiae* is not linear (i.e., oocysts are not produced below a threshold density of ookinetes) (Vaughan et al. 1992). Above this density, oocyst production increases geometrically as a function of ookinete density. This threshold requirement is suggestive of a physical barrier, such as peritrophic membrane, which must be degraded enzymatically by the ookinetes to permit their passage (Huber et al. 1991, Sieber et al. 1991, Shahabuddin & Kaslow 1993). In the presence of many ookinetes (i.e., above threshold), ookinete-derived chitinases are probably plentiful and the barrier is degraded and traversed easily, even if it is at an advanced state of development as the result of accelerated digestion. However, when ookinete populations are at or near threshold density, exogenous chitinase levels are probably low, making it difficult for ookinetes to penetrate the barrier. Ookinete penetration at threshold densities becomes even more difficult when the kinetics of blood meal digestion, particularly peritrophic membrane formation, have been accelerated and the *window of opportunity* reduced. Thus, the kinetics of blood meal digestion may actually shift the threshold density of ookinetes required

for successful conversion to oocysts. Accelerated digestion may raise the threshold, whereas delayed digestion may lower the threshold.

Difficulties arise in attempting to reconcile this threshold concept (derived from cultured parasites in the laboratory) with what is thought to occur in the field. Gametocyte densities commonly used in laboratory infections (i.e., many thousands per mosquito) far exceed densities of gametocytes ingested by mosquitoes in the wild (i.e., generally less than 100 per mosquito) (James 1931, Kligler & Mer 1935, Jeffery & Eyles 1955, Muirhead-Thomson 1957, Githeko et al. 1992, Boudin et al. 1993). Likewise, ookinete densities in wild-caught *An. gambiae* from western Kenya (1–12 per mosquito; Beier et al. 1992) are orders of magnitude less than densities required for the successful conversion to oocysts in the laboratory. Yet, sporozoite rates in the same locales are extremely high (4–18%). Obviously, ookinetes in nature are capable of producing oocysts at much lower densities than is required of ookinetes in the laboratory. If so, does there even exist a threshold requirement or is conversion of ookinetes to oocysts in the wild nearly 100% efficient, irrespective of their density? Can accelerated digestion as the result of prior blood feeding alter this efficiency? These are important, yet unresolved issues in understanding how vector species of mosquitoes acquire malarial infections in nature. They are best resolved by examining the population dynamics of sporogony for naturally occurring parasites in their indigenous vectors.

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