Concentration of Human Erythrocytes by Anopheline Mosquitoes (Diptera: Culicidae) During Feeding

JEFFERSON A. VAUGHAN, BRUCE H. NODEN, AND JOHN C. BEIER

Department of Immunology and Infectious Diseases,
School of Hygiene and Public Health,
Johns Hopkins University, 615 N. Wolfe Street, Baltimore, Maryland 21205

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ABSTRACT Erythrocyte densities in the blood meals of six *Anopheles* mosquito species were compared with those of human host erythrocyte densities. During engorgement, *An. gambiae* Giles and *An. stephensi* Liston concentrated erythrocytes by factors of 1.8 and 1.7, respectively; *An. freeborni* Aitken did not concentrate; and *An. arabiensis* Patton and *An. dirus* Peyton & Harrison demonstrated an intermediate level of erythrocyte concentration (1.4 and 1.2, respectively). *An. albimanus* concentrated host hemoglobin, but hemolysis during engorgement decreased bloodmeal erythrocyte density below that of host blood. The degree to which anopheline species concentrated erythrocytes was related to the frequency and time spent undergoing prediuresis (anal excretion of fluid during feeding), suggesting that prediuresis is responsible for erythrocyte concentration and that the fluid produced represents efflux from the filtration of ingested blood. Differences observed in erythrocyte concentration by different anopheline species are consistent with species-specific patterns of host selection.

KEY WORDS Insecta, *Anopheles*, erythrocyte concentration, prediuresis

MALARIA REMAINS THE FOREMOST vector-borne disease of humans worldwide. The transmission cycle depends on the successful sporogonic development of the causative organism, *Plasmodium* sp., in mosquitoes of the genus *Anopheles*. Sporogony begins when a mosquito ingests mature gametocytes infecting the erythrocytes of an infected host. The early developmental events that occur within the blood meal (e.g., fertilization) may depend upon physiological processes occurring within the midgut lumen (Gass 1977, Feldemann et al. 1990). Yet little has been published describing the digestion of human erythrocytes by anophelines (Briegel 1990).

During engorgement, many anopheline species excrete fluid from the anus. This process has been termed prediuresis and is thought to be involved in the concentration of host blood proteins during feeding. Prediuresis is distinct from diuresis, a process under hormonal control that occurs after feeding has terminated (Boorman 1960, Nijhout & Carrow 1978). Briegel & Rezzonico (1985) compared the total nitrogen content of blood meals with that of host blood (guinea pig) and calculated concentration factors of 2.3, 1.9, and 2.2 for *Anopheles stephensi* Liston, *An. albimanus* Weidemann, and *An. quadrimaculatus* Say, respectively. Concentration of host blood nitrogen and hemoglobin by *An. stephensi* during "natural" blood feeding led to increased fecundity when compared with blood meals given by enemas (i.e., no concentration). Thus, bloodmeal concentration has important implications in the nitrogen budget of anophelines during ovarian development (Briegel 1990).

Our study expands upon that of Briegel & Rezzonico (1985), approaching bloodmeal concentration from the viewpoint of sporogonic development of human malaria. In their studies, no distinction was made between intact erythrocytes and proteins derived from either serum or blood cells. This distinction is important with regard to plasmodial sporogony for several reasons. First, malaria parasites ingested in an infectious blood meal are located within the erythrocytes. Therefore, estimates of initial parasite densities require estimates of the erythrocyte densities within mosquito midguts. Second, many mosquito species hemolyze erythrocytes upon ingestion via cibarial armatures (Coluzzi et al. 1982); thus, bloodmeal hemoglobin titers may overestimate erythrocyte densities. Third, previous studies used rodents as hosts. The size and density of erythrocytes in rodent blood differ from those of human blood (Wintrobe 1933). Such differences may affect the ability of a mosquito to concentrate the blood of different host species.

In our study, human erythrocyte densities were measured in the blood meals of six species of *Anopheles*, all of which are competent vectors of human malaria. We also examined the gross morphology of the pylorus as a possible site of erythrocyte concentration and examined the kinetics of engorgement to provide clues as to why some species concentrate erythrocytes but others do not.

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Materials and Methods

Mosquitoes. Six species of anopheline mosquitoes were examined: *Anopheles gambiae* Giles (G-3 strain), *An. arabiensis* Patton (G-MAL strain), *An. dirus* Peyton & Harrison (no strain designation), *An. freeborni* Aitken (no strain designation), *An. albimanus* (Panama 2 strain), and *An. stephensi* (Pakistan and Dutch strains). All were laboratory colonies obtained from the following sources: the Johns Hopkins University (*An. gambiae*); the National Institutes of Health (*An. arabiensis*, *An. dirus*, and *An. freeborni*); Walter Reed Army Institute of Research (*An. albimanus*, *An. stephensi* Pakistan strain); and University of Maryland Vaccine Development Center (*An. stephensi* Dutch strain). All test mosquitoes were previously nonblood fed and ranged in age from 7 to 22 d after eclosion, with *An. dirus* being the oldest (12–22 d). Mosquitoes were fed through screen mesh on a human forearm (J.A.V.) either individually in glass vials or in pools within 450 ml ice-cream containers. Mosquitoes were deprived of sugar 12–18 h before blood feeding, except *An. arabiensis*, which did not blood feed readily when deprived of sugar for >4 h. For all tests, mosquitoes were blood fed at room temperature (22–24°C) and immobilized by chilling (4°C) for handling.

Erythrocyte Determinations. Mosquitoes were fed in four to five pools of 10–20 mosquitoes each. Pools were weighed before blood feeding, and fully engorged mosquitoes were weighed immediately afterward to obtain estimates on the amount of blood retained in the gut of fully engorged mosquitoes. Care was taken to chill and weigh mosquitoes immediately after engorgement because diuresis occurs soon afterward and can markedly reduce the weight of ingested blood, thereby leading to an underestimation of bloodmeal size (Boorman 1960). This was particularly true of *An. freeborni*, which tended to undergo diuresis almost immediately after feeding. Replete midguts were excised, and blood meals were diluted 1:200 in physiological saline based upon their estimated volume in microliters (i.e., weight gain in milligrams divided by the specific gravity of blood, 1.05). Erythrocyte counts were performed with a hemacytometer (Oppenheim 1972). Erythrocyte counts also were performed on host blood (fingerprick) at the time of each feeding. The degree of erythrocyte concentration for each species was defined as the mean ratio of erythrocyte density of a blood meal to that of the host.

Hemoglobin Content. Hemoglobin contents of individual blood meals were quantified using the hemoglobincyanide method described by Briegel et al. (1979). Briefly, each mosquito was weighed on a microbalance before and immediately after blood feeding. The difference in prefed and postfed weight was then divided by the specific gravity of blood (1.05) to obtain a gravimetric estimate of blood meal volume (μl). Blood meals then were excised and homogenized individually in 250 μl Drabkins solution. Host blood also was collected at each feeding trial, and a standard curve was constructed by adding measured amounts (0.5, 1, 2, 3 μl) to 250 μl Drabkins solution. After a 20-min incubation, 100 μl of each sample were placed in the well of a microtiter plate and the absorbance was read at 550 nm. Hemoglobin volume (in microliters) within each blood meal was calculated using the regression equation of the standard curve. The hemoglobinometric estimate of bloodmeal volume for each mosquito then was plotted against the corresponding gravimetric estimate to describe the relationship between the two measurements. Measurements were taken over a range of bloodmeal sizes by including partially engorged as well as fully replete mosquitoes. This was done based upon the premise that bloodmeal concentration increases during feeding (Briegel & Rezzonico 1985); i.e., concentration is negligible during the initial phase of engorgement and becomes more substantial as the gut distends. Including the data points from partially fed mosquitoes resulted in regression lines that all had similar y intercepts (the ideal y intercept being zero). The resulting regression slopes provided a more accurate description of the rates of hemoglobin concentration during blood consumption than did regression slopes based only on replete mosquitoes.

Kinetics of Engorgement. Mosquitoes were fed individually on the forearm, and feeding times were recorded with stopwatches. Mosquitoes were allowed to probe into the skin, and a stopwatch was activated at the first signs of engorgement (red color and abdominal swelling). A second stopwatch was activated at the first sign of prediuresis (anal excretion). Both watches were stopped when feeding terminated (withdrawal of proboscis). Thus, total time feeding was divided into a gut-filling phase and a prediuresis phase.

Pyloric Armature. The pyloric region of the hindgut was examined as a possible site of blood filtration. Alimentary tracts were dissected, cleared in Essig's aphid fluid (20 parts lactic acid/2 parts phenol/4 parts acetic acid/1 part water) and examined under phase contrast microscopy at various magnifications (100×, 400×, 1,000×-oil). Spines and spicules were measured with an ocular micrometer, and spicule length, width, spacing, and overall arrangement were noted.

Results

Erythrocyte Determinations. There were significant differences among anopheline species in their ability to concentrate erythrocytes ($F = 37.5$; df = 5, 229; $P < 0.0001$) (Table 1). *An. gambiae* and *An. stephensi* concentrated erythrocytes by factors of 1.8 and 1.7, respectively. *An. arabiensis* and *An. dirus* demonstrated an intermediate level of erythrocyte concentration (1.4 and 1.2, respectively). *An. freeborni* did not concentrate eryth-

Table 1. Erythrocyte densities[a] in the blood meals of anopheline mosquitoes after feeding to repletion on a human host

Species of *Anopheles*	Blood meal	Host	Concentration factor[b]
An. gambiae	1,042 ± 349 (n = 44)	564 ± 40 (n = 7)	1.85 ± 0.62a
An. stephensi Dutch strain	1,015 ± 217 (n = 29)	583 ± 14 (n = 4)	1.74 ± 0.37a
An. arabiensis	794 ± 191 (n = 47)	570 ± 59 (n = 23)	1.39 ± 0.33b
An. dirus	727 ± 273 (n = 36)	592 ± 54 (n = 4)	1.23 ± 0.46bc
An. freeborni	644 ± 163 (n = 29)	606 ± 96 (n = 4)	1.06 ± 0.27c
An. albimanus	442 ± 161 (n = 50)	539 ± 72 (n = 4)	0.82 ± 0.30d

[a] Number of erythrocytes × 10^4/mm^3 ($\bar{x}$ ± SE).
[b] Blood meal/host ratio. Means (±SD) followed by the same letter are not significantly different (P = 0.05, Duncan's multiple range test).

rocytes. *An. albimanus* had bloodmeal erythrocyte densities below that of host cell density (concentration factor, 0.8).

Prediuresis. Patterns of prediuretic excretion differed among species as visualized by feeding ≈40 mosquitoes of each species over filter paper disks (Fig. 1). The prediuretic fluid excreted by *An. stephensi* was bright red and was produced in copius amounts. The prediuretic fluid excreted by *An. gambiae*, *An. dirus*, *An. arabiensis*, and *An. albimanus* was in smaller quantities and was more serous than that of *An. stephensi*, although there was often a definite reddish tinge to the fluid. The prediuretic fluid produced by *An. freeborni* was clear and resembled the fluid produced following blood feeding during diuresis as described by Nijhout & Carrow (1978). When mosquitoes were fed individually, prediuresis appeared to be obligatory for *An. gambiae* and *An. stephensi*. In contrast, prediuresis appeared to be facultative for *An. arabiensis* and *An. dirus* because not every mosquito feeding to repletion ejected fluid. The erythrocyte densities in blood meals of *An. dirus* not undergoing prediuresis (530 ± 83 cells × $10^4/\mu$l) were essentially the same as those in host blood (533 ± 15 cell × $10^4/\mu$l), indicating that without prediuresis, there was no erythrocyte concentration. Microscopic examination of the prediuretic fluid revealed substantial numbers of intact erythrocytes in the prediuretic fluid of *An. stephensi*, much less in that of *An. gambiae*, and none in that of *An. freeborni*. In addition to prediuresis, all species exhibited diuresis.

Hemoglobin Content. The relationships between gravimetric and hemoglobinometric estimates of bloodmeal volume for each species are described as regression equations in Table 2. The slope (i.e., rate of hemoglobin concentration) is the important parameter to consider. The closer a slope is to 1.00, the closer the blood meal hemoglobin content approximates that of host blood throughout the process of engorgement (i.e., no concentration of hemoglobin). Analysis of covariance indicated heterogeneity of slopes (F = 2.76; df = 5, 177; P < 0.02). As might be predicted from the erythrocyte determinations, *An. gambiae* and *An. stephensi* had the largest regression slopes (b = 1.38 and 1.24, respectively) indicating a hemoglobin concentration effect. *An. dirus* had an intermediate slope (b = 1.15), and *An. freeborni* had a slope of 1.02. *An. albimanus* had a larger regression slope (b = 1.14) and *An. arabiensis* had a smaller slope (b = 1.07) than would be predicted based upon erythrocyte determinations (Table 1).

Kinetics of Engorgement. Significant differences among species were observed for gut filling times (F = 11.1; df = 5, 128; P < 0.0001), prediuresis times (F = 58.5; df = 5; P < 0.0001), and total feeding times (F = 50.5; df = 5; P < 0.0001) (Fig. 2). Duncan's multiple range test (P = 0.05) was used to compare means within each category. Gut-filling times were statistically similar for *An. stephensi* (84 s), *An. gambiae* (81 s), *An. dirus* (97 s), and *An. freeborni* (76 s). The species having the largest gut capacity (i.e., *An. arabiensis*, 2.6 ± 0.5 mg blood retained) had the longest gut-filling time at 165 s, and the species having the smallest gut capacity (i.e., *An. albimanus*, 1.6 ± 0.2 mg blood retained) had the shortest gut-filling time (52 s). *An. stephensi* spent significantly longer time in prediuresis (215 s) than did *An. gambiae* (150 s). Prediuresis times were statistically identical for *An. arabiensis* (50 s), *An. dirus* (26 s), and *An. albimanus* (30 s). *An. freeborni* had the significantly (P < 0.05) shortest prediuresis time (4 s). All species exhibited prediuresis, but the proportion of individuals that did so within a species varied. Most *An. stephensi*, *An. gambiae*, and *An. albimanus* exhibited prediuresis (95, 100, and 87%, respectively), whereas only about half of the *An. arabiensis*, *An. dirus*, and *An. freeborni* displayed detectable prediuresis (55, 44, and 32%, respectively).

Pyloric Armature. There were differences among species with respect to pyloric armatures. *An. stephensi* was unique among the six species examined

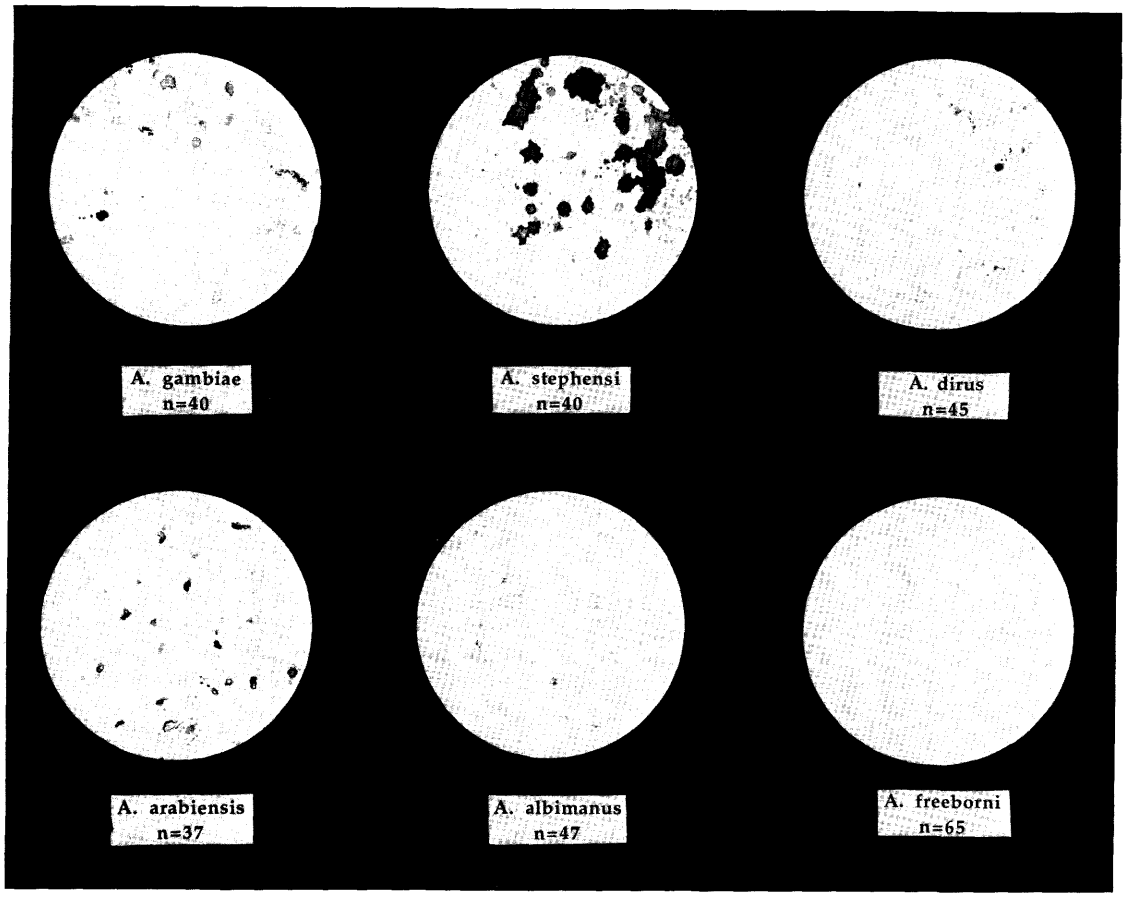

Fig. 1. Pattern of prediuresis for six species of anopheline mosquitoes when fed to repletion on human host over filter paper (Whatman No. 1); *n* is number feeding to repletion.

in having relatively large (14–18 μm), bladelike spines irregularly arranged. The armatures of *An. gambiae*, *An. arabiensis*, and *An. dirus* were similar in gross morphology and consisted of small (3–9 μm), diamond-shaped spicules arranged in rosettes, becoming clustered, sometimes fused (palmate) posteriorly. The armatures of *An. freeborni* and *An. albimanus* consisted of small (3–7 μm), simple spicules arranged in either an alternating rosette pattern (*An. freeborni*) or in distinct rows (*An. albimanus*).

Discussion

Four of the six anopheline species concentrated erythrocytes. The degree to which a species concentrated erythrocytes was related to its propensity to undergo prediuresis. There was no concentration effect in instances where prediuresis was negligible or absent (e.g., *An. freeborni* and non-prediuresing *An. dirus*). This indicated that prediuresis was directly responsible for erythrocyte concentration and, because concentration of cells necessitates the re-

Table 2. Regression equations of bloodmeal volumes determined gravimetrically (x) and from hemoglobin content (y) in replete and partially fed anopheline mosquitoes fed on a human host

Species of *Anopheles*	*n*	Regression equation	*R*	Regression slopes with 95% confidence intervals
An. gambiae	28	$y = 1.38x + 0.19$	0.81	1.38 (0.98–1.78)
An. stephensi Pakistan strain	49	$y = 1.24x + 0.03$	0.96	1.24 (1.14–1.34)
An. arabiensis	27	$y = 1.07x + 0.10$	0.98	1.07 (0.99–1.14)
An. dirus	27	$y = 1.15x + 0.07$	0.95	1.15 (0.99–1.31)
An. freeborni	27	$y = 1.02x + 0.24$	0.96	1.02 (0.87–1.17)
An. albimanus	31	$y = 1.14x + 0.06$	0.92	1.14 (1.02–1.26)

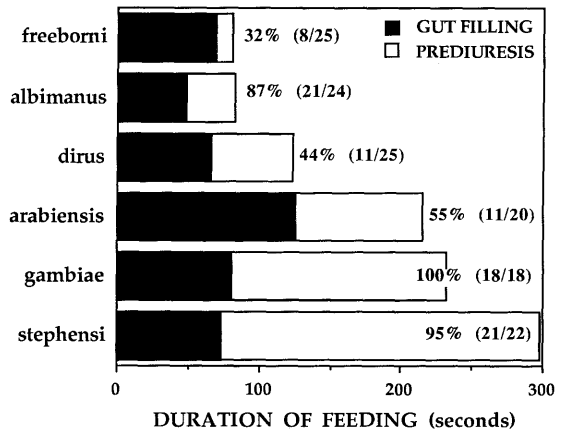

Fig. 2. Kinetics of engorgement for six species of anopheline mosquitoes when fed to repletion on human host. Percentages represent the proportion of the population that exhibited prediuresis.

moval of fluid, the fluid produced during prediuresis represented efflux from the concentration process.

In general, erythrocyte concentration as measured by bloodmeal erythrocyte counts was corroborated by rates of hemoglobin concentration, with the notable exceptions of *An. arabiensis* and *An. albimanus*. *An. albimanus* had a larger regression slope (b = 1.14) and *An. arabiensis* had a smaller slope (b = 1.07) than would be predicted based upon erythrocyte determinations. The incongruence of *An. arabiensis* is difficult to reconcile because erythrocyte concentration should have resulted in a concomitant increase in hemoglobin content. Frequency of prediuresis was not recorded during these trials, but subsequent studies indicated that *An. arabiensis* was heterogeneous in its propensity to undergo prediuresis. Perhaps a low frequency of prediuresis occurred in these trials, hence a low erythrocyte (i.e., hemoglobin) concentration.

The reverse situation observed for *An. albimanus* (i.e., low erythrocyte counts but elevated hemoglobin content) may have resulted from the physical shearing of erythrocytes as ingested blood was drawn through the cibarial armature en route to the midgut (see Barraud & Covell [1928] and Sinton & Covell [1928] for morphological details). Hemolysis during engorgement by *An. albimanus* also is supported strongly by the fact that replete *An. albimanus* had bloodmeal erythrocyte densities less than those of the host, an otherwise physical impossibility. Bloodmeal hemolysis by cibarial armature has been reported for several species of mosquitoes, including *An. albimanus*, *An. gambiae*, and *An. stephensi* (2.3, 12.8, and 21.9% hemolysis, respectively) (Coluzzi et al. 1982). Partial hemolysis of the blood meal may explain why the concentration factors for total dietary nitrogen in *An. albimanus* and *An. stephensi* (1.9 and 2.3, respectively) (Briegel & Rezzonico 1985) exceed the values for erythrocyte concentration in the same species (0.9 and 1.7, respectively) (Table 1).

The general mechanism of erythrocyte concentration may operate in two ways. First, erythrocyte concentration may be achieved by physical filtration of the blood. In this case, the anatomical site of filtration almost certainly would reside posterior to the midgut in either the pylorus, illeum, rectum, or anus. Microscopic examination of the alimentary tract of female mosquitoes revealed that, in addition to cibarial armature of the foregut, an armature of spicules and spines also exists in the pyloric region of the hindgut (Trembley 1951). There were noticeable differences among species. Presumably, the pyloric armature acts in concert with peristaltic contractions of the pyloris to trap erythrocytes as blood is passed through the hindgut and expelled from the anus. Species-specific morphology of the armature and its integration with pyloric peristalsis may dictate the filtration efficiency. For example, there was substantial passage of intact erythrocytes from the anus of *An. stephensi*. This may reflect the relative inefficiency of its large bladelike pyloric spines to retain human erythrocytes compared with the increased retention efficiency of the smaller, more closely-spaced spicules of *An. gambiae*.

Erythrocyte concentration also may be augmented by "metabolic" filtration involving active transport in a manner analogous to diuresis. Here, blood-derived fluids would pass through the distended midgut into the hemolymph and be actively excreted via the Malpighian tubules. Erythrocytes, being too large, would concentrate in the midgut lumen. In such a process, one would expect to see both an increase in hemolymph volume during engorgement and the appearance in the hemolymph of host serum components (e.g., sugars and proteins). Recent studies have demonstrated that both occur during engorgement in *An. stephensi* (Vaughan et al. 1990). Furthermore, passage of host IgG into the hemolymph is reported to occur only in those species that also concentrate erythrocytes or hemoglobin or both (i.e., *An. gambiae*, *An. stephensi*, *An. albimanus*) but not in the hemolymph of species that do not concentrate erythrocytes or hemoglobin (i.e., *An. freeborni*) (Vaughan & Azad 1988). Such observations support the hypothesis that metabolic filtration has a role in augmenting erythrocyte concentration.

Why do some species concentrate erythrocytes and others do not? This is a rather natural biological question to ask, yet difficult to answer empirically. We propose that species differences in erythrocyte concentration may be explained in terms of host preference and optimal foraging strategies. First, the ability to concentrate erythrocytes during engorgement affords a mosquito the benefit of an increase in nutrition and fecundity (see Briegel & Rezzonico 1985) without a concurrent increase in wingloading. However, this benefit carries with it the risk of having to spend more time on the host

to filter the blood. The longer the time spent on the host, the greater the likelihood that a mosquito will incur injury or death resulting from the host's defensive behavior (see Edman & Scott 1987). Second, the net outcome of this benefit/risk ratio may depend on host selection patterns. For example, *An. stephensi* feeds primarily at night on bovids (Bruce-Chawatt et al. 1966, Garrett-Jones et al. 1980). The host defensive behavior of a sleeping water buffalo may be rather minimal; thus, for *An. stephensi*, the benefit outweighs the risk. For other species such as *An. freeborni* (frequent host is rabbit [Washino & Tempelis 1967]), the host defensive behavior may be such that the risk outweighs the benefit. As a consequence, such species may have evolved a feeding strategy such that they engorge to repletion and leave the host immediately, forfeiting the benefit of a concentrated blood meal in lieu of staying alive.

Another way in which a species may balance the benefit/risk ratio is through filtration efficiency. For instance, both *An. stephensi* and *An. gambiae* concentrate human erythrocytes equally, but the filtration mechanism of *An. gambiae* is more efficient as demonstrated by the passage of fewer red cells and shorter time spent in prediuresis. Perhaps humans, the preferred host for *An. gambiae* (Garrett-Jones et al. 1980), are more sensitive to mosquito bites and their defensive behaviors are more easily aroused and effective than that of bovids, the preferred host for *An. stephensi*. If so, natural selection in *An. gambiae* would favor phenotypes possessing a fast, efficient filtration system. Selection for such a trait would be of less importance in species such as *An. stephensi* that feed on large, relatively passive hosts (i.e., low risk).

An alternate foraging strategy may involve phenotypic heterogeneity. For example, in *An. arabiensis* and *A. dirus*, only half of the test populations underwent prediuresis. This is reflected also in the intermediate values obtained for erythrocyte concentration. Reported host records (Bruce-Chawatt et al. 1966, Garrett-Jones et al. 1980) indicate that both of these species are more indiscriminate in their feeding preferences than is *An. gambiae* or *An. stephensi*. For species exhibiting a broad host range, phenotypic heterogeneity may be advantageous and may serve to balance the benefit/risk ratio among the entire population. Thus, even if host species composition (and hence blood feeding patterns) within a deme changes, more versatile species will always maintain at least a segment of their population that is able to maximize the benefit/risk ratio.

Further refinement of this theory will require blood feedings of wild *Anopheles* on their preferred hosts (i.e., *An. stephensi* on cattle) because different vertebrate species have different erythrocyte sizes and densities (Wintrobe 1933). Presumably, erythrocyte filtration efficiency for a species is optimal when feeding on its preferred host. Indeed, we have found that *An. stephensi* fed on anesthetized mice undergo copius prediuresis but fail to concentrate mouse erythrocytes (concentration factor, 0.92 ± 0.32, $n = 21$), even though *An. stephensi* concentrate human erythrocytes quite well. The smaller size of mouse erythrocytes (corpuscular volume, 49 μm^3) versus that of human erythrocytes (87 μm^3) suggests that the filtration mechanism of *An. stephensi* is adapted for larger-sized erythrocytes, such as that of cows (59 μm^3). Detailed studies on host selection patterns of wild anophelines and the bloodmeal "processing" they undergo while on the host will greatly increase our understanding of anopheline foraging strategies.

Erythrocyte concentration by anopheline mosquitoes is also of considerable interest to the field of malariology because the sexual cycle of malaria within the mosquito begins with the ingestion of gametocyte-infected erythrocytes. The degree to which an anopheline species concentrates erythrocytes (thereby increasing the number of gametocytes) may play a critical role in parasite fertilization. Increased numbers of gametocytes within the blood meal could lead to an increase in the total number of fertilization events, resulting in greater overall parasite success. Alternatively, the densely packed confines of a concentrated blood meal may physically impede the motility of male gametes which, upon exflagellation, must locate and "swim" to the relatively immobile female gamete for fertilization to occur. Such results have been reported in dilution experiments with *Plasmodium berghei* ookinete formation in vitro (Janse et al. 1985). It has yet to be determined what effect erythrocyte concentration (or partial hemolysis) of the infectious blood meal by anopheline mosquitoes may have on the sporogony of human *Plasmodium* species in vivo.

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