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Longitudinal Studies of *Aedes aegypti* (Diptera: Culicidae) in Thailand and Puerto Rico: Blood Feeding Frequency

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ABSTRACT We used a histologic technique to study multiple blood feeding in a single gonotrophic cycle by engorged *Aedes aegypti* (L.) that were collected weekly for 2 yr from houses in a rural village in Thailand (n = 1,891) and a residential section of San Juan, Puerto Rico (n = 1,675). Overall, mosquitoes from Thailand contained significantly more multiple meals (n = 1,300, 42% double meals, 5% triple meals) than mosquitoes collected in Puerto Rico (n = 1,156, 32% double meals, 2% triple meals). The portion of specimens for which frequency of feeding could not be determined was 31% at both sites. We estimated that on average *Ae. aegypti* take 0.76 and 0.63 human blood meals per day in Thailand and Puerto Rico, respectively. However, frequency of multiple feeding varied among houses and, in Puerto Rico, the neighborhoods from which mosquitoes were collected. In Thailand 65% of the mosquitoes fed twice on the same day, whereas in Puerto Rico 57% took multiple meals separated by $\geq$ 1 d. At both sites, the majority of engorged specimens were collected inside houses (Thailand 86%, Puerto Rico 95%). The number of blood meals detected was independent of where mosquitoes were collected (inside versus outside of the house) at both sites and the time of day collections were made in Puerto Rico. Feeding rates were slightly higher for mosquitoes collected in the afternoon in Thailand. Temperatures were significantly higher and mosquitoes significantly smaller in Thailand than in Puerto Rico. At both sites female size was negatively associated with temperature. Rates of multiple feeding were associated positively with temperature and negatively with mosquito size in Thailand, but not in Puerto Rico. Multiple feeding during a single gonotrophic cycle is a regular part of *Ae. aegypti* biology, can vary geographically and under different climate conditions, and may be associated with variation in patterns of dengue virus transmission.

KEY WORDS *Aedes aegypti*, multiple blood feeding, histology, Thailand, Puerto Rico

FEEDING BEHAVIOR OF adult female *Aedes aegypti* (L.) is unusual compared with most other mosquitoes (Clements 1992), because they frequently take >1 blood meal during each gonotrophic cycle (reviewed in Scott et al. 1993a, b)—a behavior we refer to as multiple feeding. Moreover, they seldom feed on plant sugar compared with male *Ae. aegypti* and females of most other mosquito species (Edman et al. 1992, Van Handel et al. 1994, Martinez-Ibarra et al. 1997, Costero et al. 1999). When closely associated with humans, which is most often the case for the domestic form of this species (Christophers 1960), females feed almost exclusively on human blood (Scott et al. 1993b) and appear to use blood as their sole source of nutrients for reproduction and synthesis of energy reserves (Van Handel et al. 1994; Naksathit et al. 1998a, b; L. C. Harrington, unpublished data). Multiple meals within a single gonotrophic cycle are necessary to avoid starvation (Costero et al. 1999). Results from studies that used life table parameters to examine fitness effects of diet demonstrated that cohorts of female *Ae. aegypti* fed only human blood had higher net replacement rates and intrinsic rate of growth than cohorts fed human blood plus sugar (Scott et al. 1997, Costero et al. 1998a, Naksathit and Scott 1998, Morrison et al. 1999). There appears to be a selective advantage for females that feed only on human blood. Restricting their diet to human blood increases human contact (Canyon et al. 1999; L. C. Harrington, unpublished data) and, therefore, the potential for spread of dengue and yellow fever viruses.

To examine more closely the phenomenon of multiple feeding, we applied a histologic technique developed by Romoser et al. (1989) to *Ae. aegypti* (Scott et al. 1993a). Laboratory standardization experiments and examination of field-caught specimens demonstrated that the technique can be used for longitudinal, community-wide surveys of multiple feeding by this species. Limitations of the procedure are that it de-

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tects 80% of multiple blood meals only if they are separated by 1 to ≤24 h and specimens are fixed ≤12 h after the last meal, and that it does not detect host contacts when blood is not imbibed.

Because the gonotrophic cycle of *Ae. aegypti* lasts ≥3 d (Christophers 1960) and multiple meals could be imbibed at intervals beyond the limits of effective histologic detection, values associated with this technique probably underestimate blood-feeding frequencies per gonotrophic cycle. However, it does provide greater resolution of multiple feeding than alternative methods such as antibody-based bloodmeal identification (Boreham and Garrett-Jones 1973, Burkot et al. 1988, Scott et al. 1993b), ABO blood group analyses (Bryan and Smalley 1978), and serum protein haptoglobin assays (Boreham and Lenahan 1976, Boreham et al. 1979). Those techniques cannot differentiate meals taken from different individuals of the same species, with the same blood type or with the same serum proteins, respectively. Nor can they be used to estimate the time interval between multiple meals.

This is the 2nd article on longitudinal studies of *Ae. aegypti* that we conducted in Thailand and Puerto Rico. Our goal in the current study was to determine if rates of multiple feeding vary seasonally and geographically. We used the histologic technique to estimate blood-feeding frequencies over a 2-yr period for engorged females collected from natural resting sites at 2 geographically and ecologically distinct locations. We then determined how blood-feeding frequency was associated with different times of the year, weather, and geographic locations. In the 1st study (Scott et al. 2000), we examined adult *Ae. aegypti* abundance, age structure, gonotrophic status, and host species from which blood meals were taken and determined how those population attributes are associated with changes in weather and study location.

Materials and Methods

We collected adult *Ae. aegypti* weekly by aspiration for 2 yr from inside, under, and around houses in rural Village 6, Hua Sam Rong (13° 38′ N, 101° 18′ E) in south central Thailand (June 1990–June 1992) and inside and around houses in the urbanizations (neighborhoods) of Reparto Metropolitano (RM) and Puerto Nuevo (PN), Rio Piedras (18° 23′ N, 66° 3′ W), San Juan, Puerto Rico (January 1991–January 1993). Descriptions of the study areas, methods for collecting and processing mosquitoes, collection of weather data, and procedures for measuring mosquito wing length are presented in Scott et al. 2000. For this study, engorged females were selected from half of the collection houses each week, using a table of random numbers, and processed for histologic detection of multiple blood feeding. Methods and results associated with all other *Ae. aegypti* collected are presented by Scott et al. (2000).

Histologic Procedure. The procedures used for histologic examination of engorged mosquitoes were modified from Romoser et al. (1989) and described in detail by Scott et al. (1993a). Briefly, cold sedated mosquitoes were fixed in Smith's modified Alcoholic Bouin's solution. After dehydration clearing steps, specimens were infiltrated with paraplast, embedded, sectioned (5–8 μm) with a rotary microtome, mounted onto glass slides, stained using a modified Azan trichrome technique (Hubschman 1962), and then examined with bright-field microscopy. Histologic parameters we used to detect multiple feeding included the peritrophic membrane, peritrophic plug, heme or zone of digested blood, fully digested blood meal, physical separation between meals, and development of ovaries in relation to bloodmeal digestion (Romoser et al. 1989, Scott et al. 1993a). We define multiple feeding, as a mosquito that imbibed >1 blood meal during a single gonotrophic cycle. The times from capture to fixation and the intervals between blood meals were estimated based on the detection of histologic parameters, degree of bloodmeal digestion, and the extent of ovarian development identified during laboratory time series studies with mosquitoes that took multiple blood meals at known time intervals and were fixed at known times after their 2nd meal (Scott et al. 1993a).

Data Analysis. The proportion of females that could be scored histologically and the proportion of females imbibing multiple blood meals were compared between the 2 study sites (Thailand and Puerto Rico), as well as location of capture (indoor versus outdoor), urbanization (Puerto Rico only), collection time (0800–1000, 1000–1200, 1200–1400, 1400–1800 hours), histologic parameters observed, and time intervals between blood meals. We classified specimens in 2 groups histologically (scored, not scored). A specimen that could not be scored contained a meal(s) that was digested beyond the limits of histologic detection. We fixed and examined mosquitoes with any amount of blood in their abdomen, ranging from those that were fully engorged to specimens with what appeared to be only a tiny remnant of a dark, digested meal.

To compare rates of multiple feeding, we eliminated specimens not scored from the analysis and compared the relative proportions of single, double, and triple meals. In many cases it was necessary to combine double and triple meals into 1 (multiple meals) group so that expected values in chi-square analyses were ≥5. All chi-square analyses were carried out by using the frequency procedure in SAS (SAS Institute 1990).

Analysis of variance (ANOVA) was used to characterize seasonal (monthly) variation in weather patterns in Thailand and Puerto Rico (SAS Institute 1990). We defined seasonal categories for monthly temperatures (hot, moderate, or cool) and rainfall (dry, wet) as described by Scott et al. (2000). Proportions of specimens imbibing multiple blood meals were compared among seasons by chi-square analysis.

Linear regression analysis (SAS Institute 1990) was used to examine the effect of mosquito size and temperature on blood-feeding behavior. We regressed at weekly intervals and weighted by the number of specimens examined each week the average number of blood meals imbibed by average wing length, average

Table 1. Frequency of blood feeding by *Ae. aegypti* in Thailand (June 1990–June 1992) and Puerto Rico (January 1991–January 1993)

Site	No. of blood meals (%)			Total
	1	2	3	
Thailand	699 (54)	541* (42)	60* (5)	1,300
Puerto Rico	770* (67)	369 (32)	17 (2)	1,156

* Indicates that within a column the higher proportion is statistically significant ($P < 0.001$), where significance after Bonferoni correction is $P = 0.0167$.

number of blood meals by daily temperature, and average wing length by average daily temperature. Separate analyses were carried out for each study site.

Results

In total, 1,675 and 1,891 engorged *Ae. aegypti* were examined histologically from field collections in Puerto Rico and Thailand, respectively (Table 1). The proportion of unclassified specimens was identical for the 2 study areas (31%; $\chi^2 = 0.03$, df = 1, $P = 0.863$). Among scored specimens, the proportion of multiple blood meals was highest in Thailand ($\chi^2 = 52$, df = 2, $P = 0.001$), where 42% imbibed double meals and 5% triple meals were detected ($n = 1,300$) compared with 32% double meals and 2% triple meals in Puerto Rico ($n = 1,156$).

Effects of Collection Site and Time. *Household Differences.* The abundance of engorged *Ae. aegypti* varied among houses in Thailand (Scott et al. 2000). Interhouse variation in combination with the random assignment of half of the engorged specimens to enzyme-linked immunosorbent assay (ELISA) bloodmeal identification studies resulted in a wide range of 7–175 total histologic specimens examined per house. Among houses, the proportion of specimens that could not be scored ranged from 22 to 38%. The rate of multiple feeding ranged from 20 to 67% for each house (Fig. 1A).

In Puerto Rico, there were 22 houses from which only 1–5 specimens were histologically examined over the entire study period. Ten houses accounted for a total of 6–15 specimens each. Because of the small sample sizes from those houses, we pooled the data into 3 groups of houses with 1–5, 6–10, and 11–15 specimens. Houses with >15 specimens were analyzed individually. After pooling, the number of histologically examined specimens in each group or individual house ranged from 1 to 249. The percentage of specimens that could not be scored in each house or group of houses ranged from 15 to 39%. The rate of multiple feeding ranged from 16 to 65% per household (Fig. 1B).

Indoor Versus Outdoor Collections. Of the 1,891 histologically examined specimens from Thailand, 1,633 (86%) were collected inside houses. Of these, 31% could not be scored, 37% contained a single blood meal, 29% double blood meals, and 3% triple meals. Of the 255 specimens collected outdoors, 30% could not be scored, and 40, 27, and 3% imbibed single, double, or triple meals, respectively. Overall, both detection and number of blood meals were independent of the location (indoor versus outdoor) where mosquitoes were captured ($\chi^2 = 1.48$, df = 3, $P = 0.686$).

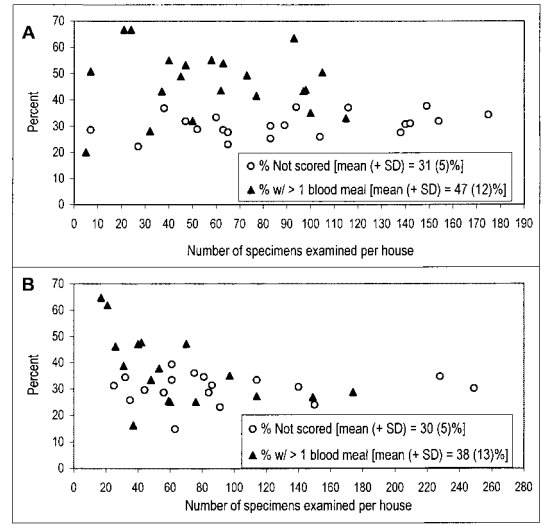

Fig. 1. Number of specimens examined histologically, percentage of specimens that could not be scored, and percentage of specimens that imbibed >1 blood meal per household in Thailand (A) and Puerto Rico (B).

In Puerto Rico an even larger portion of engorged females (95%; $n = 1,675$) was captured indoors than in Thailand. We were unable to score 31% ($n = 1,593$) of the indoor captures and 26% ($n = 81$) of the specimens collected outdoors. Single meals were observed in 46 and 56% of the females collected indoors and outdoors, respectively. Multiple meals were detected in 23% of the females collected indoors and 19% of those collected outdoors. As for specimens collected in Thailand, detection of meals and the number of meals detected were not significantly different between the indoor and outdoor collections ($\chi^2 = 3.14$, df = 2, $P = 0.208$).

Urbanization. At both Puerto Rican urbanizations, we were unable to score 31% ($n = 1,103$ in PN; $n = 572$ in RM) of the specimens. Although more houses were sampled in RM (30) than PN (19), 66% ($n = 1,675$) of the specimens from Puerto Rico were collected in PN. The proportion of observed double (39%) and triple (3%) meals in RM ($n = 393$) was higher than double (28%) and triple meals (1%) in PN ($n = 763$; $\chi^2 = 18.7$, df = 2, $P = 0.001$).

Collection Time. We attempted to sample houses as early in the day as possible in both Thailand and Puerto Rico, and the order of collection from houses was randomized each week. However, the hour of collection often was influenced by the activities of human occupants. To determine if there were any differences in multiple feeding rates associated with the time of day that collections were carried out,

Table 2. Blood-feeding frequency of female *Ae. aegypti* in Thailand and Puerto Rico by time of collection

Collection hour	No. of blood meals (%)							
	Thailand				Puerto Rico			
	NS	Single	Double	Triple	NS	Single	Double	Triple
0800–1000	109	134	93	9	122	201	87	9
	(32)	(39)	(27)	(3)	(29)	(48)	(21)	(2)
1000–1200	183	217	145	12	280	428	216	7
	(33)	(39)	(26)	(2)	(30)	(46)	(23)	(1)
1200–1400	221	271	231	23	69	81	41	1
	(30)	(36)	(31)	(3)	(36)	(42)	(21)	(1)
1400–1800	78	75	72	15	48	60	25	0
	(33)	(31)	(30)	(6)	(36)	(45)	(19)	(0)

NS, not scored histologically.

blood-feeding frequencies were compared over 2-h intervals (Table 2). At both sites, the proportion of specimens collected that we were unable to score within the 4 time intervals was consistent, ranging from 30 to 31% in Thailand ($\chi^2 = 1.8$, df = 3, $P = 0.621$) and 29 to 36% in Puerto Rico ($\chi^2 = 4.9$, df = 3, $P = 0.182$). Blood-feeding frequency was independent of collection time in Puerto Rico ($\chi^2 = 13.6$, df = 9, $P = 0.136$). In Thailand the proportion of multiple meals increased slightly during the afternoon (Table 2; $\chi^2 = 17.1$, df = 9, $P = 0.048$). If only the scored Thai specimens are considered and the 2 morning and 2 afternoon collections are pooled, significantly more multiple feeding was detected in the afternoon collections (44% double meals, 6% triple meals) than in the morning (39% double, 3% triple; $\chi^2 = 8.2$, df = 2, $P = 0.017$). Among the scored specimens from Puerto Rico, rates of blood feeding were nearly identical for each collection time ($\chi^2 = 1.0$, df = 3, $P = 0.799$). In contrast, the proportion of unscored specimens was higher in the afternoon (36%) than morning (30%) collections ($\chi^2 = 4.7$, df = 1, $P = 0.029$; Table 2).

Histologic Parameters. Heme was the most common histologic parameter detected, followed by the peritrophic membrane (Table 3). With only 1 exception (line of demarcation), all of the histologic parameters studied were observed more commonly in specimens from Thailand ($P < 0.0002$). The line of demarcation was a more useful parameter for mosquitoes collected in Puerto Rico than in Thailand for identification of the number of blood meals imbibed ($\chi^2 = 29.6$, df = 1, $P < 0.0001$; Table 3); 19% for specimens in Puerto Rico compared with 11% in Thailand.

Time Interval Between Blood Meals. Because mosquitoes were chilled on wet ice immediately after capture and kept cold until processed, the time of fixation was considered to be the same as when the mosquitoes were captured. In Thailand, 46% of 1,300 took their last blood meal on the day of capture, 49% fed the day before their capture, and 6% took their last meal >2 d before capture (Table 4). The percentage of females that had fed last on the day of capture in Puerto Rico (48%; $n = 1,156$) was nearly the same as observed in Thailand. In contrast, the percentage last feeding the day before their capture (26%) was significantly lower and the percentage feeding >2 d before capture (26%) was significantly greater than observed in Thailand ($\chi^2 = 250$, df = 2, $P = 0.001$; Table 4).

For mosquitoes that imbibed a double meal, the time interval between the 1st and 2nd meals was <24

Table 3. Frequency of detection of histologic parameters in blood fed *Ae. aegypti* in Thailand and Puerto Rico

Histologic parameter	No. of blood meals (%)							
	Thailand				Puerto Rico			
	Single $n = 699$	Double $n = 541$	Triple $n = 60$	Total $n = 1,300$	Single $n = 770$	Double $n = 369$	Triple $n = 17$	Total $n = 1,156$
Peritrophic membrane	451	374	52	877	403	217	11	631
	(66)	(69)	(87)	(67)	(52)	(59)	(65)	(55)
Heme	533	501	57	1,091	567	322	12	901
	(76)	(93)	(95)	(84)	(74)	(87)	(71)	(78)
Fully digested meal	8	157	19	184	6	87	4	97
	(1)	(29)	(32)	(14)	(1)	(24)	(24)	(8)
Physical separation	0	280	49	329	1	146	8	155
	(0)	(52)	(82)	(25)	(0.1)	(40)	(47)	(13)
Line of demarcation	2	123	17	142	51	156	9	216
	(0.3)	(23)	(28)	(11)	(7)	(43)	(53)	(19)
Pupal castoff	92	42	8	142	36	10	0	46
	(13)	(8)	(13)	(11)	(5)	(3)	(0)	(4)
Ovarian development	304	335	43	682	399	98	5	502
	(43)	(62)	(72)	(52)	(52)	(27)	(29)	(43)

Parameters as defined by Romoser et al. (1989) and Scott et al. (1993a).

Table 4. Estimated time interval between the most recent blood meal and capture for *Ae. aegypti* in Thailand and Puerto Rico

Site	No. blood meals detected	Interval between last blood meal and capture (%)			
		Same day	1 d	≥2 d	Total
Thailand	Single	255 (36)	416 (60)	26 (4)	699
	Double	310 (57)	193 (36)	38 (7)	541
	Triple	30 (50)	23 (38)	7 (12)	60
	Total	595 (46)	634 (49)	71 (6)	1,300
Puerto Rico	Single	293 (38)	216 (28)	258 (34)	767
	Double	244 (66)	83 (22)	42 (11)	369
	Triple	11 (65)	6 (35)	0 (0)	17
	Total	548 (48)	305 (26)	300 (26)	1,156

h for 65% ($n = 358$) of the specimens from Thailand but only 43% ($n = 268$) for mosquitoes from Puerto Rico ($\chi^2 = 41.8$, df = 2, $P = 0.001$; Table 5). In Puerto Rico, most (48%; $n = 268$) double meals were separated by ≥2 d; the remaining 9% ($n = 268$) were separated by 1 d. Among the mosquitoes in which a triple meal was detected, the time interval between most blood meals was <24 h at both sites. Moreover, there were no significant differences in the proportions of females feeding at different time intervals between the 2 sites for either the 1st ($\chi^2 = 2.2$, df = 2, $P = 0.329$) or 2nd meal ($\chi^2 = 4.2$, df = 2, $P = 0.124$; Table 5).

We estimated daily rates of imbibing human blood for *Ae. aegypti* in Thailand and Puerto Rico based on the portion of engorged females that fed on a human (Scott et al. 2000), estimates of blood meals taken on the day of capture (Table 4; mosquitoes imbibing blood per day), portion of detected double and triple blood meals (Table 1), and portion of 2nd and 3rd meals imbibed on the same day as a preceding meal (Table 5). Our calculations were as follows: human blood meals per day = percent engorged mosquitoes that fed on a human (percent mosquitoes imbibing a blood meal on the day of capture + percent mosquitoes that took 2 meals and fed twice on the same day + percent of mosquitoes that took 3 meals and imbibed their first 2 meals on the same day + percent of mosquitoes that took 3 meals and imbibed their second 2 meals on the same day). Thailand = $0.76 = 0.95 \times (0.46 + [0.42 \times 0.65] + [0.05 \times 0.61] + [0.05 \times 0.78])$. Puerto Rico = $0.63 = 0.97 \times (0.48 + [0.32 \times 0.43] + [0.02 \times 0.63] + [0.02 \times 0.82])$.

Seasonal Patterns of Blood Feeding. *Thailand.* Between June 1990 and June 1992, average (±SD) maximum and minimum temperatures were 33.1 ± 2.4°C and 23.2 ± 2.5°C. Average monthly temperatures ranged from 24.8°C in December 1990 to 31.5°C in April 1992 (Fig. 2A). ANOVA confirmed significant seasonal temperature (month; $F = 113$; df = 11, 12; $P < 0.0001$) and rainfall (month; $F = 8$; df = 11, 23; $P < 0.0001$) fluctuations. Months of the study fell into 3 temperature groups: high temperatures were observed from March to May each year, cool temperatures from November to January, and moderate temperatures during June–October and February (determined by Tukey honestly significant difference [HSD]). Rainfall patterns fell into a distinct wet (March–October) and dry (November–February) seasons (Tukey HSD; see Fig. 2A).

The number of specimens examined histologically each month ranged from 14 to 188 (Fig. 2B). The percentages of specimens that we could not score, because meals were digested beyond the limits of detection, during the hot, moderate, and cool months were 23, 34, and 33%, respectively; the lower rate observed during the hotter months was statistically significant ($\chi^2 = 22.8$, df = 2, $P = 0.001$; Fig. 2B).

The monthly percentage of specimens containing multiple blood meals ranged from 28 to 80% (Fig. 2C). When data were grouped by hot, moderate, and cool months, no statistically significant differences in multiple feeding rates were observed ($\chi^2 = 1.5$, df = 2, $P = 0.473$).

Puerto Rico. Between January 1991 and January 1993, the average maximum and minimum temperatures were 30.3 ± 1.7°C and 21.2 ± 2.1°C. Average monthly temperatures ranged from 23.1°C in January 1992 to 27.8°C in June 1992 (Fig. 3A). ANOVA confirmed seasonal temperature (month; $F = 146$; df = 11, 23; $P < 0.0001$) fluctuations. There was a distinct cool (November–May) and hot (June–October) season

Table 5. Estimated time between multiple blood meals imbibed by *Ae. aegypti* in Thailand and Puerto Rico

Site	No. blood meals detected	Interval between blood meals (%)			
		Same day	1 d	≥2 d	Total
Thailand	Double	232 (65)	42 (12)	84 (23)	358
	Triple				
	1st–2nd meal	34 (61)	20 (36)	2 (4)	56
	2nd–3rd meal	43 (78)	12 (22)	0 (0)	55
Puerto Rico	Double	115 (43)	24 (1)	129 (48)	268
	Triple				
	1st–2nd meal	10 (63)	4 (25)	2 (13)	16
	2nd–3rd meal	14 (82)	2 (12)	1 (6)	17

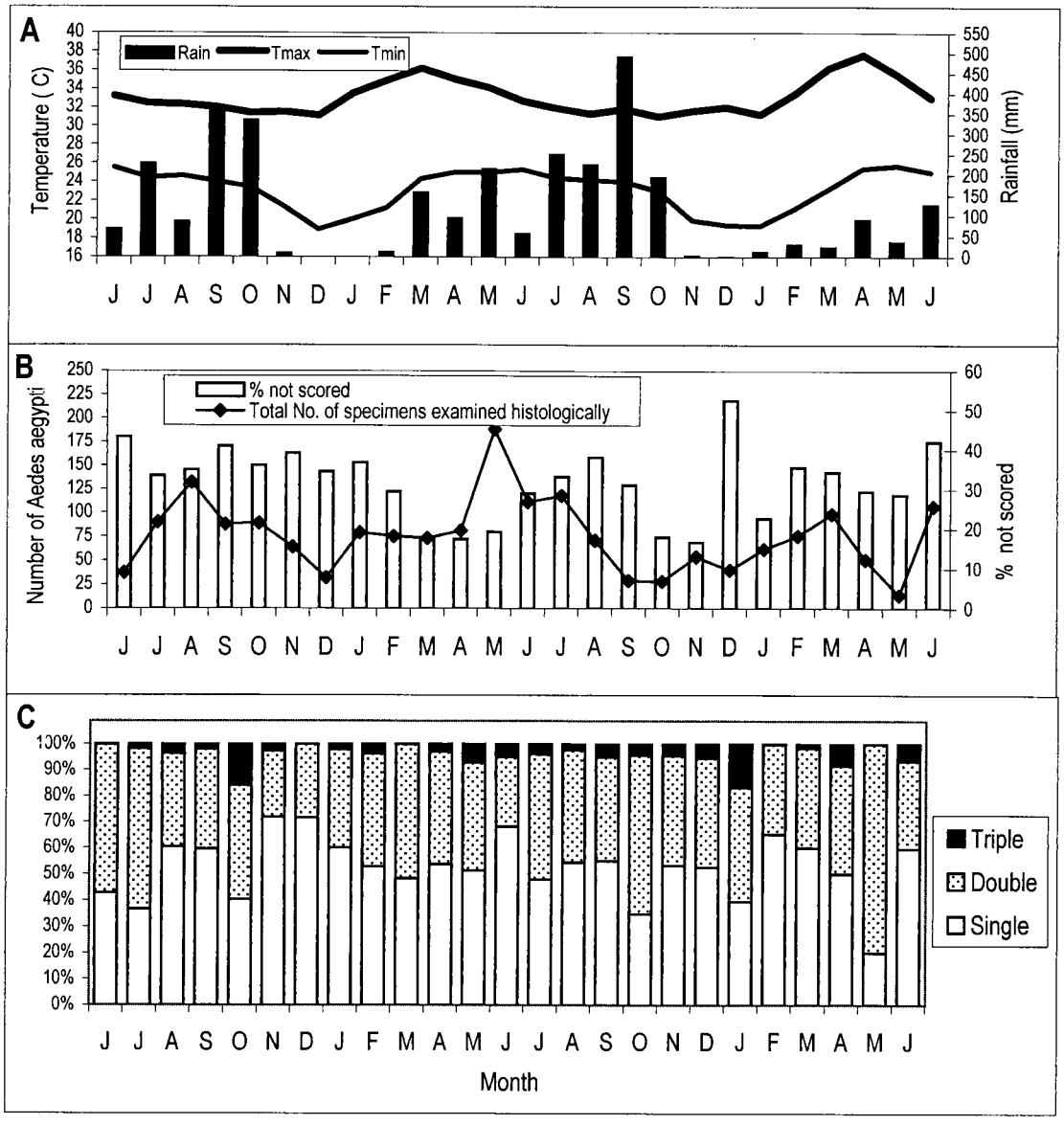

Fig. 2. Seasonal patterns (1990–1992) of temperature and rainfall (A), number of *Ae. aegypti* examined histologically (B), and number of blood meals imbibed by *Ae. aegypti* in Thailand (C).

(determined by Tukey HSD, Fig. 3A). Although rainfall varied from month to month, no distinct seasonal patterns were detected (Fig. 3A).

Neither the proportion of specimens we were unable to score ($\chi^2 = 1.2$, df = 1, $P = 0.266$) nor the proportion of females with multiple blood meals ($\chi^2 = 0.24$, df = 1, $P = 0.627$) differed between the cool and hot seasons (Fig. 3 B and C).

Effect of Body Size and Temperature on Frequency of Blood Feeding. The average wing length (millimeters) of females (±SE) captured per week in Thailand (2.46 mm ± 0.009) was significantly smaller than in Puerto Rico (2.64 mm ± 0.008; *t*-test = 6.56, df = 145, $P = 0.0001$). Linear regressions of the mean wing length and number of blood meals observed for each week of collection indicated that in Thailand there was a negative association between size and feeding frequency. As mosquitoes from Thailand decreased in size (indicated by decreasing wing length [Nasci 1986]), their frequency of blood feeding increased ($P = 0.0018$, Fig. 4). Wing length explained 18% (R^2) of the variation in the number of observed blood meals. In contrast, in Puerto Rico, where *Ae. aegypti* are on average larger than those in Thailand, the slope of the regression line was not significant ($P = 0.11$).

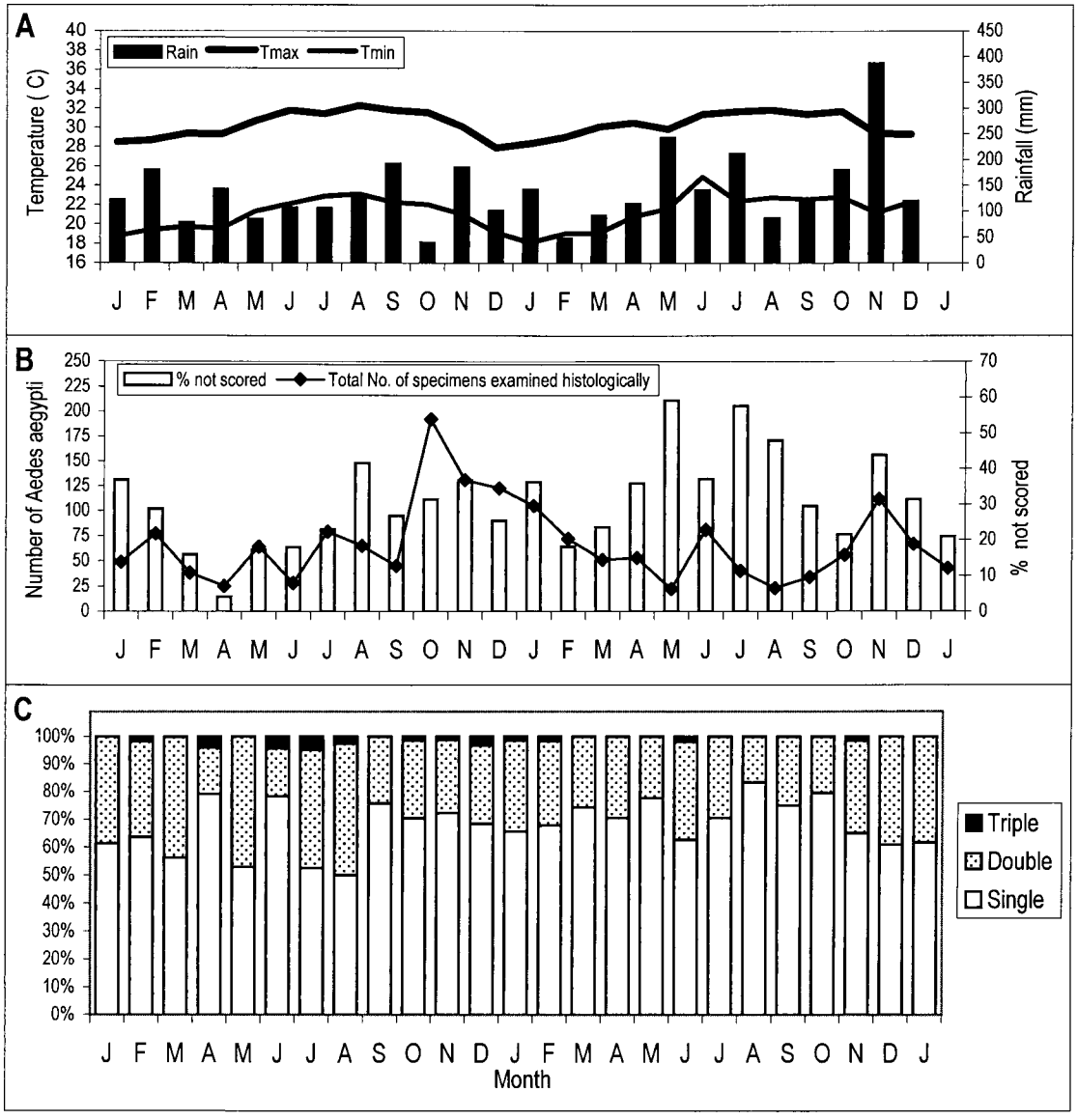

Fig. 3. Seasonal patterns (1991–1993) of temperature and rainfall (A), number of *Ae. aegypti* examined histologically (B), and number of blood meals imbibed by *Ae. aegypti* in Puerto Rico (C).

Regression analysis of average daily temperature and the average number of blood meals imbibed each week (Σ meals taken/mosquitoes examined) showed that blood feeding increased significantly with warmer temperatures ($P = 0.05$) in Thailand, but not in Puerto Rico ($P = 0.37$), where temperatures were on average lower than those in Thailand (Fig. 5). Average temperature only explained 8 and 0.8% of the variation in blood feeding frequency in Thailand and Puerto Rico, respectively.

Because *Ae. aegypti* size can be influenced by temperature (Rueda et al. 1990; Focks et al. 1993a, 1995; Fig. 6), we also carried out regressions of average weekly temperature and wing length (Fig. 6). A strong and statistically significant negative relationship between temperature and body size was observed in both Thailand ($P = 0.0001$) and Puerto Rico ($P = 0.0006$), explaining 34% and 11% of the variation in size, respectively. As temperature increased, average body size of female *Ae. aegypti* decreased.

Discussion

Results from our study extend earlier reports that wild *Ae. aegypti* regularly imbibe multiple blood meals during a single gonotrophic cycle (reviewed in Scott

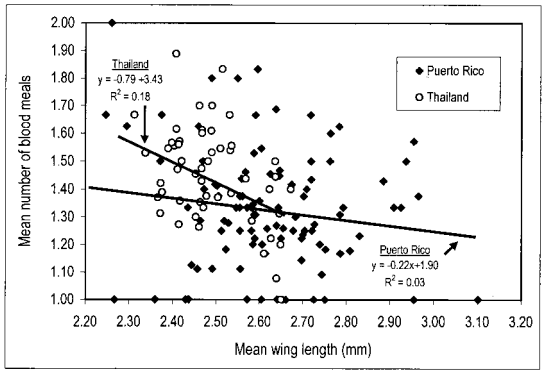

Fig. 4. Relationship between wing length and blood-feeding frequency of female *Ae. aegypti* collected weekly in Thailand (1990–1992) and Puerto Rico (1991–1993). Linear regression lines and equations for each site are included.

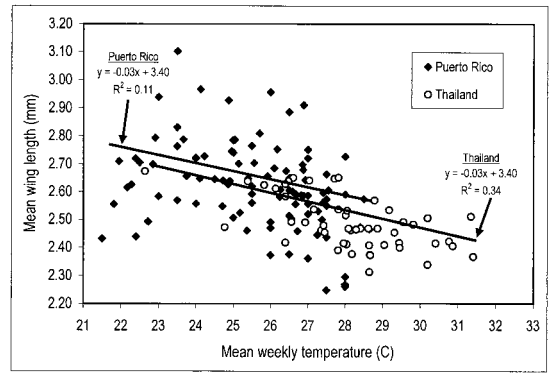

Fig. 6. Relationship between temperature and wing length of female *Ae. aegypti* collected weekly in Thailand (1990–1992) and Puerto Rico (1991–1993). Linear regression lines and equations for each site are included.

et al. 1993a). Multiple feeding occurred throughout the year at a relatively high rate—40% of all scored specimens from both study areas—even though there were significant seasonal and geographic differences in blood-feeding frequency. Preliminary results from studies using a DNA fingerprinting procedure that identifies the exact person from whom a blood meal was taken indicated that many multiple meals were taken from different people (Chow-Schaffer 1997; J. A. DeBenedictis and T.W.S., unpublished data). Epidemiologically, these results imply that as frequency of feeding during each gonotrophic cycle increases so will the risk of dengue virus transmission (Dye 1992). Frequent human-host contact by *Ae. aegypti* may explain the important role of this mosquito in dengue virus transmission even though it is relatively insensitive to virus infection compared with many other *Aedes* spp. (Monath 1994, Rodhain and Rosen 1997); sustained dengue virus transmission when *Ae. aegypti* population densities are low (Kuno 1995, 1997); clusters of dengue patients in the same household (Halstead et al. 1969a; Waterman et al. 1985; Kuno 1995, 1997; Morrison et al. 1998); and the rapid and often explosive spread of dengue epidemics (Gubler 1989, Monath 1994).

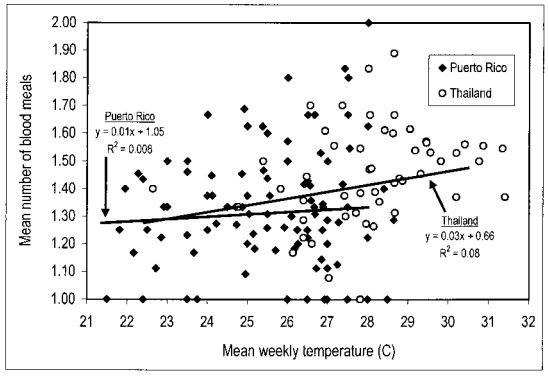

Fig. 5. Relationship between temperature and blood-feeding frequency of female *Ae. aegypti* collected weekly in Thailand (1990–1992) and Puerto Rico (1991–1993). Linear regression lines and equations for each site are included.

We do not mean to imply that frequency of blood feeding is the only factor influencing the dynamics of pathogen transmission. Our assertion is one of relative effects (Dye 1992). If other factors remain the same, including those related to human hosts, an increase in the biting rate of *Ae. aegypti* should increase the force of dengue virus transmission. Because of the nonlinear relationship between the human biting rate and the potential for pathogen transmission by an arthropod vector (Garrett-Jones and Shidrawi 1969, Dye 1992), small changes in the biting rate are expected to result in relatively large increases in the potential for the spread of disease within a community.

Details of the *Ae. aegypti*-dengue virus interaction support the idea that the biting rate is a sensitive component of this mosquito's role in pathogen transmission. For example, *Ae. aegypti* remain infected for life and their ability to transmit virus is not reduced after imbibing multiple blood meals (Siler et al. 1926) or probing a host as many as 20 consecutive times (Putnam and Scott 1995a). Some parasites modify host behavior in ways that enhance their transmission (Moore 1984, Molyneux and Jefferies 1986). Whether dengue virus interferes with the location of blood meals and, therefore, increases the number of hosts contacted has not been resolved. In one study with small sample sizes and high variance, virus infection increased probing time and total time to obtain a blood meal (Platt et al. 1997). However, in a different study, with larger sample sizes and better measures of within treatment variance, there were no significant differences in times to locate or imbibe blood for infected versus uninfected *Ae. aegypti*, indicating that virus infection did not impair or enhance blood-feeding efficiency (Putnam and Scott 1995b).

Multiple feeding might have a dampening effect on virus transmission if a mosquito imbibed blood from a person immune to dengue virus before taking a 2nd

meal from a person viremic with a homologous serotype. If meals are separated by ≤6 h, virus in the 2nd meal may be neutralized by antibodies in the 1st (Chen and Chen 1990). Data from our histologic study indicate that if this does occur it may be more important in Thailand, where the frequency of feeding twice on the same day is higher than in Puerto Rico, where most meals are separated by ≥1 d.

The high multiple feeding rates observed in our study are significant because the histologic procedure we used to monitor blood-feeding behavior probably underestimates the actual number of multiple blood meals imbibed. The histologic procedure favors detection of meals separated by 1–24 h and fixed within 12 h of the 2nd meal (Scott et al. 1993a). Despite this limitation and because of our extensive (>10 houses per week) standardized sampling design, results from application of the histologic technique are appropriate for estimating relative differences in multiple feeding rates between geographic locations and over time.

Blood-engorged females from all our weekly collections were split, half for histology and the other half for ELISA-based bloodmeal identification. Results from our bloodmeal identification studies indicated that the vast majority of multiple meals were taken from humans and, therefore, were epidemiologically important. Of the 1,230 females collected between May 1990 to June 1991 in Thailand, 88% had only fed on human blood and an additional 7% had mixed blood meals containing human blood (Scott et al. 1993b). Similarly, from February 1991 to February 1993 in Puerto Rico, 95% of the females collected were positive for human blood and 2% contained mixed meals between humans and a different host species (Scott et al. 2000).

Fluctuations in the proportion of specimens that were scored histologically may bias our interpretation of the longitudinal data because the probability of scoring a specimen decreased as the time interval increased between blood meals and from the last meal to fixation. The proportion of specimens that could not be scored was surprisingly consistent between Thailand and Puerto Rico (31%) and among houses (31 ± 5% in Thailand, 30 ± 5% in Puerto Rico) and was independent of whether the females were collected resting indoors or outdoors, urbanization (Puerto Rico only) or the time of day they were collected. The only significant difference observed was a decrease in the proportion of unscored specimens from 33 to 34% during the cool and moderate months to 23% during the hot months in Thailand. Presumably, this reflects a decrease in the average feeding interval caused by increased metabolism by small mosquitoes that fed more frequently or more rapid digestion of blood meals during warmer months. Therefore, differences in multiple feeding rates appeared to reflect real behavioral differences rather than artifacts caused by sensitivity of the histologic procedure.

There was considerable variation in multiple feeding rates among mosquitoes collected from different houses. In Puerto Rico, houses with the lowest total number of specimens collected—including those that were pooled—had the most extreme estimates of multiple feeding. In Thailand, variation in multiple feeding rates was not associated with the number of specimens examined per house. Results from our analysis of interhouse variation indicated that multiple feeding rates can vary at a microgeographic scale and care should be taken when extrapolating results from a single site to a larger or different geographic area.

Multiple feeding rates were not significantly different between specimens collected indoors versus outdoors in either Thailand or Puerto Rico, even though >86% of the engorged females were collected indoors. In fact, when considering the total number of adult females we collected at both sites, the vast majority came from inside houses (77% in Thailand, 97% in Puerto Rico).

The time of day that specimens were collected appeared to increase the likelihood of detecting a multiple meal by 8% during afternoon collections in Thailand, when 53% of the specimens were collected. A similar increase was not observed during the afternoon in Puerto Rico when only 19% were collected. Differences between the 2 sites could be caused by differences in feeding behavior, the more complicated internal structure of houses in Puerto Rico or differences in availability of human hosts at the 2 study areas.

Not only did *Ae. aegypti* in Thailand feed more often per gonotrophic cycle than in Puerto Rico, the time interval between their multiple meals was shorter. Of the specimens from Thailand, 13% more imbibed 2 or more blood meals than those collected in Puerto Rico. Because there were more mosquitoes collected in the afternoon in Thailand, we compared multiple feeding rates between the 2 study areas for mosquitoes collected only in the morning. Seven percent more of the specimens collected in the morning in Thailand took 2 or more meals. Moreover, the time intervals between capture and the last blood meal imbibed and between multiple meals were consistently shorter during the morning in Thailand than in Puerto Rico.

We estimate that on average *Ae. aegypti* imbibe 0.76 and 0.63 human blood meals per day in Thailand and Puerto Rico, respectively. Feeding rates may vary as follows: (1) per unit of time by season—rather than gonotrophic cycle as presented in Fig. 5—with lower rates occurring during the cooler times of the year when dengue transmission is reduced (Costero et al. 1999), (2) with advancing age (Klowden 1990), and (3) at different times in the gonotrophic cycle. Chronological variation in the feeding rate does not necessarily mean that the number of meals per gonotrophic cycle will vary and vice versa (Klowden and Lea 1980). Additional field studies are needed to determine what portion of multiple human meals are taken from different people and, therefore, are epidemiologically relevant (Chow-Schaffer 1997; J. A. DeBenedictis and T.W.S., unpublished data). Results from laboratory studies, like the one by Canyon et al. (1999), are probably best considered as representing the maximum possible blood feeding rates (2.7–5.3 human blood meals per day). Their experimental de-

sign circumvented host-seeking behavior and their estimates did not account for meals taken from nonhuman hosts or from the same person(s).

Experiments in which survival of starved field-collected mosquitoes was studied provide independent support for our conclusion that blood feeding rates vary geographically. Costero et al. (1999) estimated that to avoid death caused by starvation, females in Thailand needed to imbibe a blood meal approximately every other day. In Puerto Rico that interval extended to 3 and 4 d during the high and low dengue transmissions seasons, respectively.

The biological and environmental factors that influence multiple feeding behavior and may have contributed to feeding differences in Thailand and Puerto Rico, are often inter-related. For example, the number of times a female *Ae.aegypti* feeds may be a function of her reproductive and energetic requirements (Van Handel et al. 1994, Scott et al. 1997, Costero et al. 1998a, Naksathit and Scott 1998), especially when it does not feed on sugar (Foster 1995). Thus, smaller mosquitoes would be expected to feed more frequently than larger mosquitoes because they have lower teneral energy reserves (Nasci 1986, 1990; Briegel 1990; Chambers and Klowden 1990) and ingest a smaller volume of blood (Focks et al. 1995). Available energy reserves are important because they affect the amount of blood needed to initiate vitellogenesis, to complete egg maturation, and to carry out maintenance activities (Briegel 1985, 1990; Foster 1995). Although the habitats in Thailand and Puerto Rico where we studied *Ae. aegypti* were distinctly different (Scott et al. 2000) and this may have influenced larval development, the most important environmental factor that we measured was temperature. Maximum and minimum temperatures averaged 3°C warmer in Thailand than Puerto Rico; temperature was inversely related to female size and may have affected blood-feeding rates.

We observed a higher feeding frequency in small than in large females from Thailand but not from Puerto Rico. The nonsignificant regression for the Puerto Rico data indicate that size is only important below a critical value. The wing length of mosquitoes from Thailand ranged from ≈2.3 to 2.7 mm, compared with ≈2.3 to 3.0 mm for those from Puerto Rico. Specimens with wing lengths >2.7 mm decreased the slope of the regression line for feeding frequency and size. Because fecundity is a function of the quantity of blood ingested (Briegel 1985, 1990), multiple feeding may be an evolutionarily advantageous feeding strategy (Scott et al. 1997) that is influenced indirectly by adult size and is more common among small than large females (Naksathit and Scott 1998). In the laboratory, Naksathit et al. (1998b) described patterns of glycogen and lipid accumulation in ovaries and body remnants of large versus small *Ae. aegypti* that are consistent with the notion that small females will need to feed, for nonreproductive energetic reasons, more often on human blood than their larger counterparts. Similarly, Briegel (1990) reported that large *Ae. aegypti*, after imbibing equal amounts of blood, completed their gonotrophic cycle faster than small ones.

We detected a significant relationship between temperature and mosquito wing length at both sites. In contrast, regression analyses of temperature and blood feeding rates were weak. Mosquito size is a function of larval rearing temperature (Rueda et al. 1990; Focks et al. 1993a, 1995) and larval access to adequate food (Gilpin and McClelland 1979, Focks et al. 1993b). The influence of temperature on blood feeding (Yasuno and Pant 1970) may be indirect through factors associated with size, development rate, or energy reserves, as well as, increasing the rate of blood digestion.

Factors other than temperature such as sugar feeding and availability of hosts also may influence blood-feeding frequency. Even though reports of sugar feeding frequencies by *Ae. aegypti* collected close to human hosts are low—2–21% when collected from inhabited houses (Edman et al. 1992, Van Handel et al. 1994, Costero et al. 1998b, Martinez-Ibarra et al. 1997) versus 74% when humans were absent (Van Handel et al. 1994)—fluctuations in the frequency of blood feeding may be associated with variation in sugar feeding. The subtleties of facultative sugar feeding (Van Handel et al. 1994) and its influence on variation in blood feeding rates need to be examined more closely under field conditions. Sugar feeding can inhibit host-seeking behavior (Klowden 1986), blood-feeding frequency (Canyon et al. 1999; L. C. Harrington unpublished data), preoviposition behavior (Klowden and Dutro 1990), oviposition (Canyon et al. 1999), and the amount of blood imbibed (Foster 1995; L. C. Harrington, unpublished data). Similarly, the frequency of blood feeding may be influenced by characteristics of human hosts, such as, number of inhabitants per house, their age, sex, body size, occupation, or activity patterns.

It is important to note within the context of a discussion on the effects of sugar feeding on the frequency of blood feeding that laboratory (Scott et al. 1997, Costero et al. 1998a, Naksathit and Scott 1998; L. C. Harrington, unpublished data) and field studies (Morrison et al. 1999) provide strong support for the conclusion that natural selection favors female *Ae. aegypti* that feed on human blood without feeding on sugar. Results from life table studies cited above, mark-recapture studies that indicated that blood feeding is not an important mortality factor (Day et al. 1994) as well as their own data, are inconsistent with the conclusion by Canyon et al. (1999) that a sugar and blood diet is most likely to occur in nature. Support for their conclusion was limited to the impression that sugar feeding increases female survival even though their data on survival of mosquitoes that fed on blood with water versus blood with sugar do not appear different and females they fed blood with water laid the highest number of total eggs and had the shortest gonotrophic cycle. Selection acts on variation in fitness (the product of survival and reproduction) (Futuyma 1998), which is not necessarily associated with variation in survival alone.

An intriguing implication of the geographic differences among feeding frequencies of mosquitoes in Thailand versus Puerto Rico is that they are consistent with epidemiological patterns of dengue virus transmission. Although the appropriate rigorous epidemiological studies have not been carried out, available information indicates that dengue virus attack rates in Southeast Asia may be higher than in the Americas (Halstead et al. 1969a, b; Waterman et al. 1985; Halstead 1997). Our histologic results, which indicate that *Ae. aegypti* in Thailand feed more often and at shorter time intervals than mosquitoes in Puerto Rico, are consistent with the notion that the frequency of vector-host contact is a determinant of dynamics in dengue virus transmission. Studies over broader geographic areas that simultaneously examine *Ae. aegypti* feeding behavior and dengue incidence rates will determine if the trends we detected are representative of their respective region of the world and, therefore, can be incorporated into region-specific summaries of dengue virus transmission (Focks et al. 1995).

Multiple feeding during a single gonotrophic cycle is a regular part of *Ae. aegypti* biology, but rates of human blood feeding vary geographically and under different climate conditions. At the level of the household, variation in entomological risk of dengue infection (Morrison et al. 1998, Rodriguez-Figueroa et al. 1995) may be the result of a combination of interhouse variation in female *Ae. aegypti* density (Scott et al. 2000), frequency of blood feeding, and survival. Frequent host contact appears to be an important reason why this mosquito species is such an efficient vector of virus and indicates that entomological thresholds for dengue virus transmission will be low; that is, because of their propensity to take frequent blood meals, relatively few *Ae. aegypti* can sustain virus transmission (Kuno 1995, 1997).

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