ECOLOGY OF THE MALARIA VECTOR, *ANOPHELES* (*NYSSORHYNCHUS*) *MARAJOARA* GALVÃO AND DAMASCENO IN TRINIDAD, WEST INDIES

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ABSTRACT. The taxonomy and ecology of wild-caught *Anopheles marajoara* mosquitoes derived from rice fields in Frederick Settlement, Trinidad, were studied in the laboratory using specimens identified with species-specific random amplified polymorphic DNA (RAPD) profiles and recently developed rDNA ITS2 polymerase chain reaction methods. Adults were collected using Shannon traps and human bait in 2 houses over a 1-year period. All mosquitoes collected were taken to the laboratory, where they were identified, wing-length measured, and parity rates determined using standard methods. In addition, 25 females were blood fed and subsequently offered a blood meal every 2 h for a 60-h period. Based on the morphological keys and molecular tools used, the presence of *An. marajoara* is confirmed in Trinidad for the first time. Analysis of the seasonal distribution of *An. marajoara* revealed that over 58% were collected during the rainy season. The wing length of 660 females measured averaged 2.90 ± 0.130 mm, with no significant differences being observed among the parous and nulliparous females' wing sizes (2.90 and 2.92 mm, respectively). In addition, the monthly parous rate was not significantly correlated with mean wing-length over time ($r = 0.157$, $df = 16$, $P > 0.07$). Results from the blood feeding studies showed 85% of females blood fed immediately (hour 0) after capture in the field. However, blood feeding declined thereafter until 24 h later, when over 40% refed. This study clearly identified the presence of *An. marajoara* in Trinidad and provides information of the seasonal abundance and blood-feeding behaviors. These results suggest that this species can play a significant role in the transmission of malaria within its geographical range in the Neotropics.

KEY WORDS *Anopheles marajoara*, taxonomy, PCR methods, seasonal distribution, blood-feeding, Trinidad

INTRODUCTION

Anopheles (*Nyssorhynchus*) *marajoara* Galvão and Damasceno is a widespread Neotropical species found from Costa Rica to southern Brazil. This species was thought to be, at most, a secondary local vector of malaria parasites (reviewed by Linthicum 1988), but recently, it was shown that it is the primary malaria vector in the northeastern Brazilian state of Amapá (Conn et al. 2002). Because of this, the importance of documenting the presence of *An. marajoara* and studying its basic biology in Trinidad and elsewhere in its distribution, is essential. It is one of the 4 members of the Albitarsis Complex, the others being *An. albitarsis* Lynch-Arribálzaga, *An. deaneorum* Rosa-Freitas, and *An. albitarsis* B (Wilkerson et al. 1995a, 1995b). To date, *An. marajoara* is the only species in the Albitarsis Complex found north of Belem, Brazil, leading to the assumption that *An. marajoara* is the only species in the complex found in Trinidad (Map 1). The species-specific random amplified polymorphic DNA (RAPD) profiles (Wilkerson et al. 1995a, 1995b) used to confirm the existence of the 4 species, and a recently developed rDNA ITS2 polymerase chain reaction (PCR) method for identification of the 4 species (Li and Wilkerson 2005) both corroborate this assumption. Based on these methods, we present here verification of the presence of *An. marajoara* in Trinidad and give the results of basic seasonal abundance and life-cycle measures for this species on the island.

MATERIALS AND METHODS

Study site: *Anopheles marajoara* adults used in laboratory observations were collected in the rice fields of Frederick Settlement (10°86′N, 61°25′W), a village located on the Caroni Savanna Road and the Southern Main Road, 5 km west of Piarco International Airport, Trinidad. The Caroni River flows on the northern side of the village, whereas the eastern, western, and southern sides contain >1,500 ha of rice cultivation. In Trinidad, there are 2 distinct seasons: the rainy season, from May to November, and the dry season, from December to May (Chadee 1992).

Mosquito collections: Between April 1993 and June 1994, *An. marajoara* collections were made weekly using human bait between 1500 and 2300 h, similar to the methodology used for *An. aquasalis* Curry collections (Chadee and Beier 1995a, 1995b). Informed consent was obtained from all mosquito collectors and the Insect Vector Control Division Internal Review Board approved the project. Collections were made in 2 houses with 2 men stationed outdoors in the yard and 2 indoors in the living room in each house. Each collector was provided with a flashlight, aspirator, and net. Mosquitoes caught on the exposed lower legs and ankles of the collectors were transferred into moistened jars lined with Plaster of Paris and kept separate by location, time, and collector. In the laboratory, mos-

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Report Documentation Page

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1. REPORT DATE **2006**	2. REPORT TYPE	3. DATES COVERED **00-00-2006 to 00-00-2006**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Ecology of the Malaria Vector, Anopheles (Nyssorhynchus) Marajoara Galvao and Damasceno in Trinidad, West Indies	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Walter Reed Army Institute of Research,Department of Entomology,Silver Spring,MD,20910**	8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
see report

15. SUBJECT TERMS

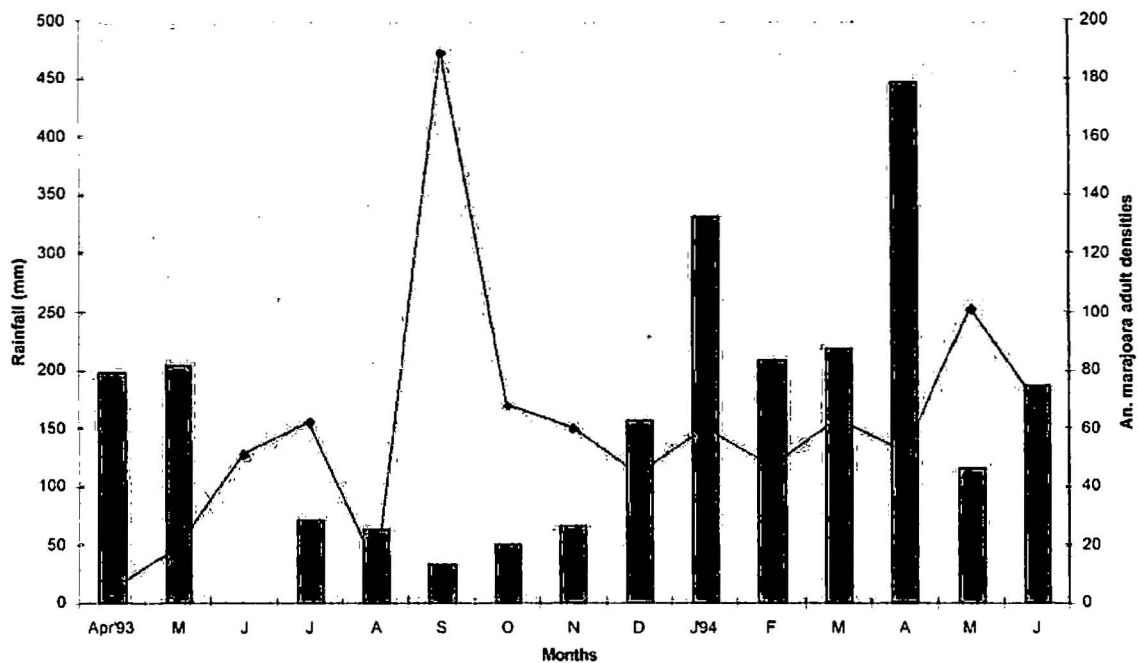| 16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**	**Same as Report (SAR)**	**7**	

Fig. 1. Seasonal variation in rainfall patterns (bars) and *An. marajoara* mosquito (line) densities in Frederick Settlement, Trinidad, West Indies.

quitoes were anesthetized, identified, and counted. The ovaries of 60% of the specimens were dissected in distilled water to determine parity (Detinova 1962).

Wing-length measurements: The wings of all dissected mosquitoes were removed and placed on glass slides in small drops of dilute saline. For each wing, the distance from the axillary incision to the apical margin was measured as described by Nasci (1986), using a binocular microscope with a calibrated eyepiece graticule.

Laboratory experiment: In January 1994, 50 female *An. marajoara* with no visible signs of blood were placed into a 450-ml net-screened, cylindrical ice-cream container (18 cm in height × 16.5 cm in diameter) to acclimatize to laboratory conditions. At 0500 h, 25 females were selected and individually placed into a 5-ml glass tube covered with mosquito netting and, at 0600 h, females were allowed to engorge to repletion on 2 human forearms. For the experiment, 0600 h was designated 0 h, and for the next 60 h, the 25 mosquitoes were individually offered a blood meal every 2 h.

Morphological and molecular identification: *Anopheles* mosquitoes of the subgenus *Nyssorhynchus*, collected for this study, were identified using Faran (1980) and Linthicum (1988). A sample of presumed *An. marajoara* was collected in August 2000 at Frederick Settlement using a Shannon trap. Larvae were also collected from a rice field at the same site and were reared to the adult stage, and exuviae retained and slide mounted. Morphological

and molecular vouchers are deposited in the Smithsonian Institution, National Museum of Natural History. Sixty specimens were identified using the techniques described in Wilkerson et al. (1995a, 1995b) (RAPDs) and Li and Wilkerson (2005) (ITS2-based PCR primers).

RESULTS

A total of 1,157 *Anopheles* females were captured during the experimental study; 95.2% were *An. marajoara* and 4.8% were *An.* (*Nys.*) *aquasalis* Curry. Table 1 shows the seasonal distribution of *An. marajoara*, with 58% being collected in the wet season and 42% in the dry season. The rainfall patterns revealed that 71.4% of all rainfall was measured during the months May–November, with the highest monthly rainfall in June 1993, when 446.8 mm (17%) was collected (Fig. 1). It should be noted that, in October, the wet season is usually interrupted for about 2 weeks by a dry spell, the Indian summer, or *petit careme*.

The wing length of 660 females averaged 2.90 ± 0.130 mm (±SE), the coefficient of variation (CV) of wing length measurements was 4.48, and the range was 2.71–3.10 mm (Table 1). Size variation was greatest in October 1993 (5.54 mm), at almost the end of the rainy season (Fig. 2). The overall mean wing length of parous and nulliparous females was 2.90 and 2.92 mm, respectively. The means were not significantly different in a 2-way analysis of variance (ANOVA) (F = 0.21, df =

Table 1. Monthly mean (SE) wing length (mm) of parous and nulliparous *Anopheles marajoara* in Frederick Settlement, Trinidad (1993-94).

Months (year)	N	Parous		N	Nulliparous		Combined		CV[1]
April (1993)	43	2.89	(0.088)	25	2.90	(0.107)	2.89	(0.095)	3.29
May	63	2.92	(0.091)	39	2.93	(0.071)	2.93	(0.088)	3.00
June	35	2.91	(0.097)	17	2.92	(0.088)	2.91	(0.094)	3.23
July	57	2.81	(0.128)	6	2.86	(0.076)	2.82	(0.124)	4.40
August	44	2.81	(0.123)	3	2.82	(0.216)	2.81	(0.127)	4.52
September	49	2.87	(0.131)	11	2.96	(0.136)	2.89	(0.135)	4.67
October	135	2.96	(0.168)	9	2.96	(0.101)	2.96	(0.164)	5.54
November	157	2.93	(0.118)	3	2.98	(0.032)	2.93	(0.118)	4.03
December	63	2.92	(0.132)	5	2.95	(0.132)	2.92	(0.131)	4.49
January (1990)	179	2.87	(0.140)	10	2.87	(0.119)	2.87	(0.139)	4.84
February	12	2.79	(0.072)	0	0	(0)	2.79	(0.072)	2.58
March	41	2.96	(0.138)	21	2.93	(0.096)	2.95	(0.126)	4.27
April	27	2.91	(0.125)	24	2.94	(0.087)	2.92	(0.109)	3.75
May	18	2.96	(0.106)	1	3.02	(0)	2.96	(0.104)	3.51
June	5	2.97	(0.137)	0	0	(0)	2.97	(0.137)	4.61
Total	927	2.90	(0.135)	174	2.92	(0.135)	2.90[2]	(0.130)	4.48

[1] Coefficient of variation.
[2] n = 1,101.

1,865, $P = 0.93$). The interaction between months (time) and parity indicated that differences between the wing lengths of parous and nulliparous females changed over time, but these changes were not statistically significant (F = 1.23, df = 12,811, $P = 0.06$). Monthly parous and nulliparous wing lengths were compared. Wing lengths of parous and nulliparous females also showed monthly variations, but were not consistent over time. In addition, the monthly parous rate was not significantly correlated

with mean wing lengths over time ($r = 0.157$, df = 16, $P > 0.07$).

The overall parous rate was 57.0% (376/660) (Table 2). During the dry season 55.7% (152/273) of all females were parous, indicating a high proportion of physiologically old mosquitoes being present in the rice fields of Fredrick Settlement. This is very different from the number of females found nulliparous (44.3%). During the wet season, the overall parous rate was 57.9% (224/387), which
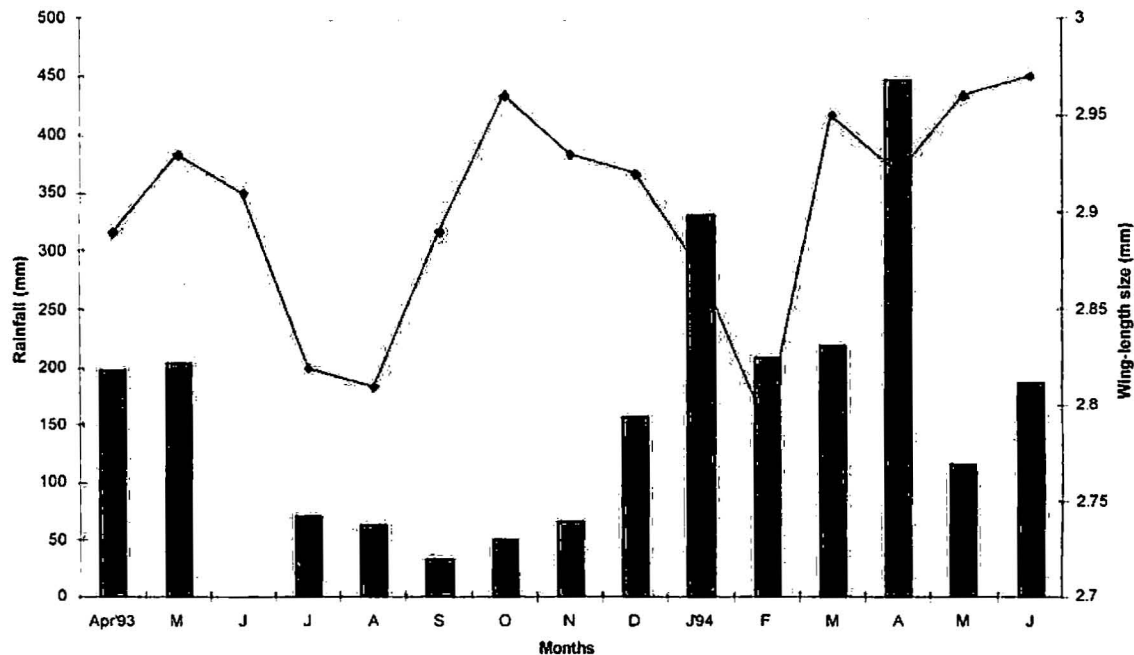

Fig. 2. Seasonal variation in rainfall patterns (bars) and wing lengths (line) of *An. marajoara* mosquitoes from rice fields in Frederick Settlement, Trinidad, West Indies.
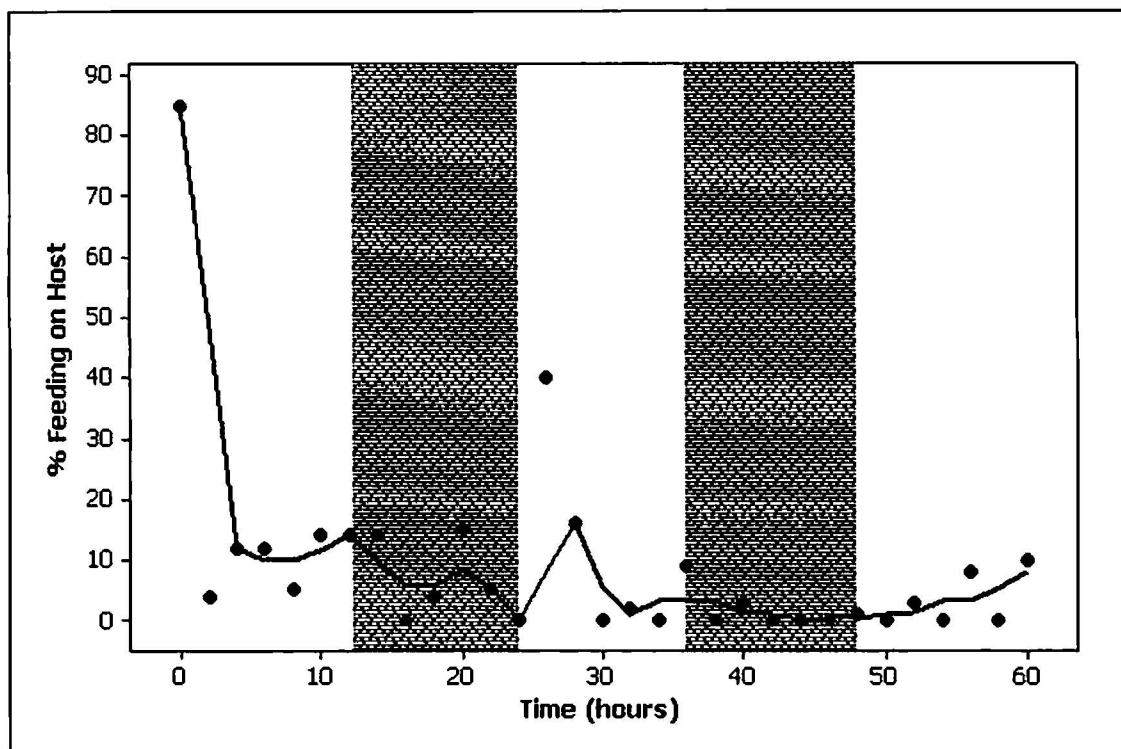
Fig. 3.   Host-seeking responses of *An. marajoara* under laboratory conditions in Trinidad, West Indies. Each point represents the response of a different population of 25 females. Shaded bars represent the scotophase.

is significantly different ($P > 0.02$) from that found among nulliparous females 42.1% (163/387). The parous rates during the wet and dry seasons were very similar (57.9% vs. 55.7%) (Table 2).

Feeding and gonotrophic cycle: From the 25 field-collected *An. marajoara* females offered a blood meal at 0600 h, at hour 0, 85% responded to host stimuli, immediately blood feeding to repletion (Fig. 3). However, few responded immediately afterward but showed variable responses to host stimuli. A small proportion of the *An. marajoara* population responded after 4 h, with the most significant ($P > 0.01$) multiple feeding occurring 24 h postfeeding, that is, when 40% refed. As eggs developed, host seeking declined but feeding continued in a proportion of the population for the remainder of the gonotrophic cycle (Fig. 3).

Molecular identification: All 60 specimens tested using the above-described molecular techniques were *An. marajoara.*

DISCUSSION

We give here a brief review of the systematics of the Albitarsis Complex in order to put *An. marajoara* in context with the other members of the group. This is especially important because it is possible that reports of malaria susceptibility, or lack of it, found in the literature may refer to one or more of the other species in the complex or to population variability (Linthicum 1988). Until recently, morphological identifications were difficult or impossible, resulting in a rather involved taxonomic history (reviewed by Linthicum 1988).

Table 2.   Number of *Anopheles marajoara* females (and percentage parous) collected during the wet and dry seasons at Frederick Settlement, Trinidad.

Category	Season		
	Wet (May–November)	Dry (December–April)	Total
No. of females	639	462	1,101
No. of females age graded	387 (60.6%)	273 (59.1%)	660 (60.0%)
No. of nullipars	163 (42.1%)	121 (44.3%)	284 (43.0%)
No. of parous	224 (57.9%)	152 (55.7%)	376 (57.0%)
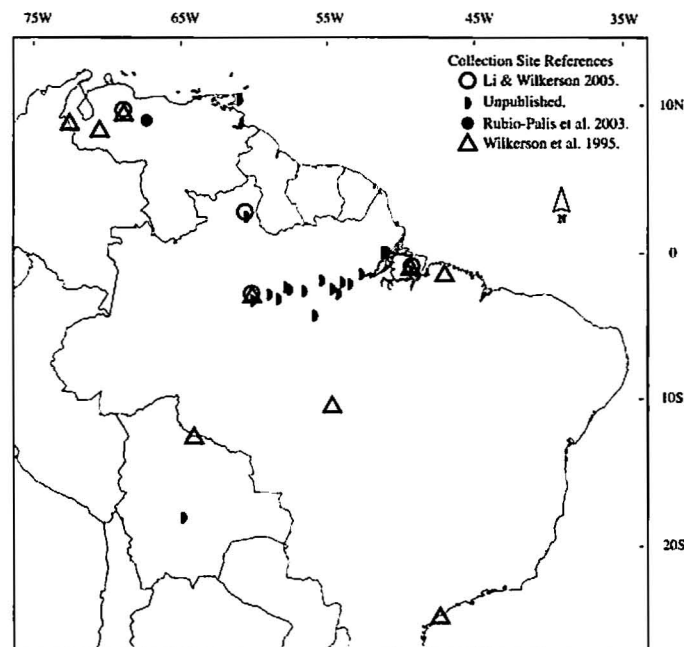
Map 1.   Distribution of the nominal taxon *Anopheles (Nyssorhynchus) marajoara* as determined by one or more of the following methods: random amplified polymorphic DNA (RAPD), ribosomal DNA internal transcribed spacer 2 sequence (rDNA ITS2), a PCR diagnostic based on ITS2 sequence, and DNA microsatellite markers.

When Linthicum (1988) revised the Argyritarsis Section of *Anopheles* subgenus *Nyssorhynchus*, he recognized 2 species recognized in the Albitarsis Subgroup, *An. albitarsis* and *An. marajoara*. However, he observed a great deal of variation and speculated that cryptic species were probably present. Using various techniques, such as polytene chromosomal banding patterns, isozyme analysis, morphology, and behavior, the same conclusion was reached by Kitzmiller (1976), Kreutzer et al. (1976), Steiner et al. (1982), Rosa-Freitas et al. (1990), Narang et al. (1993), and Rubio-Palis et al. (2003). To add to the confusion, other names have been applied to what is now *An. marajoara*, including *An. alopha* (now a nomen dubium) and the rather appealing name, reflecting indoor biting behavior, of *An. albitarsis domesticus*, which was synonymized by Linthicum (1988), and also rather convincingly confirmed earlier by Rios et al. (1984). Subsequent to Linthicum (1988), *An. deaneorum* Rosa-Freitas (1989) and the undescribed species B (Wilkerson et. al 1995a, 1995b) were recognized. Because of RAPD diagnostic markers (Wilkerson et al. 1995a, 1995b), a general idea of the distribution of the species is now known: *An. albitarsis* sensu stricto is found in Argentina, Paraguay, and southern Brazil; *An. marajoara* is found from Costa Rica to Bolivia; *An. deaneorum* has a distribution from Rondônia and Mato Grosso states in Brazil to northern Argentina; and species B has a wide distribution approximately 1–25°N and 38–

54°W in Brazil and Paraguay (Wilkerson et al. 1995a, 1995b).

Because morphological separation of the 4 species in the Albitarsis Complex is difficult or impossible, it was not feasible until RAPD markers were available to make identifications. Subsequently, Li and Wilkerson (unpublished data) carried out a detailed study of the rDNA ITS2 in the complex and found inter- and intragenomic variation in all 4 species. However, it was possible to design species-specific primers based on constant sites to make reliable identification (Li and Wilkerson 2005). Lehr et al. (in press), using the entire mitochondria COI gene, suggest a 5th species nested in an *An. marajoara* clade in northern Brazil and Venezuela. The Li and Wilkerson (in press) ITS2 results do not corroborate this. In addition, population genetic studies of these same populations, using microsatellite markers, in northern Brazil, Venezuela, and Trinidad do show populational differences but not what we consider at this time to be species-level differentiation (unpublished data). Therefore, the question of the presence of another cryptic species, which would probably be the taxon we studied in Trinidad, awaits further study. For purposes of this article, because at this time most evidence does not support a 5th species, we are assuming that the Trinidad population is conspecific with true *An. marajoara* from the type locality of Ilha do Marajó, Brazil (Map 1).

Little information is available on the ecology of

An. marajoara in the Neotropics. The present study confirms the presence of *An. marajoara* in Trinidad and replaces the mosquito species *An. albitarsis*, whose geographical distribution is restricted to the temperate and subtropical areas of South America (Linthicum 1988, Rosa-Freitas et al. 1990). The results show a clearly defined seasonal pattern, with more than 58% of the *An. marajoara* collected during the wet season, but a substantial number were also collected during the dry season (42%). This is to be expected because of the presence of suitable breeding sites throughout the year. In the study area, the breeding sites were mainly rice fields, which were supplied with water either from rainfall or water pumped into the rice fields from the nearby rivers. Consequently, the almost constant supply of water helped in maintaining elevated populations of *An. marajoara* during both the wet and dry seasons. The late wet-season peak recorded during October and November (54%) may be due to a buildup in the number of females from eggs hatching after the resumption of the rains, after *petit careme*. Similar studies conducted on *Anopheles* (*Kertezia*) *homunculus* Komp showed a distinct seasonal pattern, with over 73% of females collected during the rainy season (Chadee 1994), compared with 58% collected during the present study. The differences observed may be due to the different breeding habitats, that is, *An. homunculus* breeding in bromeliads while *An. marajoara* breeds in rice fields. In addition, parous rates of *An. homunculus* were found to be similar during both the wet and dry seasons (58.9% vs. 56.1%; Chadee 1994), and during the present study, *An. marajoara* parous rates were also found to be similar (57.9% vs. 55.7%). These results suggest that the high population densities of physiologically old mosquitoes from both species may play a significant role in the transmission of malaria.

Results from the blood-feeding study suggest an initial inhibition of feeding after engorgement, which is consistent with the working mechanism of the abdominal distention receptors, which are known to reduce blood feeding among mosquitoes (Klowden and Lea 1978). However, Briegel and Rezzonico (1985) reported that many *Anopheles* concentrate their blood meal during feeding to compensate for their relatively small midgut capacity. Chadee and Beier (1995a) showed 89.5% of *An. albitarsis* (*An. marajoara*) prediuresed, thus concentrating their blood meal, so the volume of blood ingested is not as great as that of comparable-sized *Aedes aegypti* Linn. These results suggest that multiple feeding is a common feature, which possibly compensates for the small blood-meal capacity or midgut size. Therefore, the distention-induced inhibition of host seeking does not last long but results, in the case of *An. marajoara*, in a large proportion of the population host-seeking within 12 h of their initial blood meal in the laboratory.

Several studies have suggested that more than 1 blood meal may be necessary for egg development in field populations of *Anopheles* (Gillies 1954, 1955; Klowden and Briegel 1994), while other workers (Briegel and Horler 1993, Chadee and Beier 1997) have demonstrated that multiple feeding occurred when mosquitoes were small and were malnourished during their immature stages. It is noteworthy that we measured the tendency for multiple feeding under laboratory conditions using field populations of *An. marajoara*. The observed tendency to multiple feed is a real feature for field populations of *An. marajoara* in Trinidad and is similar to that reported for *An. aquasalis* in Trinidad (Senior-White 1953).

It should be noted that studies on the blood-feeding kinetics of field-collected *An. marajoara* (*An. albitarsis*) females from Trinidad revealed that blood feeding to repletion took approximately 164.8 ± 88.7 sec under laboratory conditions (Chadee and Beier 1995b), while 2 peaks of activity were observed near the end of the photophase and at the beginning of the scotophase, which are features of 2 different kinds of behavior in *An. marajoara*: landing on humans in the field (Chadee 1992) and egg laying under laboratory conditions (Chadee 1995). That is, with peaks of landing on humans falling close to 1900 h and the time of peak oviposition between 2000 and 2200 h (Chadee 1992, 1995). These studies showed that peak landing and oviposition both occurred after sunset, thus suggesting that disease transmission may also occur at this time during the diel.

In conclusion, this study has clearly identified the presence of *An. marajoara* in Trinidad and the high proportion of parous females supports previous studies that showed that this species is a primary vector of malaria in Brazil (Conn et al. 2002). In addition, the large number of parous mosquitoes collected in both the wet and dry seasons and multiple blood-feeding behaviors identified during the present study suggest that this species may play a significant role in the transmission of malaria not only in Trinidad but throughout its geographical range within the Neotropics.

ACKNOWLEDGMENTS

Research. There is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting true views of the Department of the Army or the Department of Defense.

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