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Physiological Age and Longevity of *Anopheles* (*Kerteszia*) *cruzii* Dyar & Knab (Diptera: Culicidae) in the Atlantic Forest of Southern Brazil

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ABSTRACT - We analyzed the reproductive status, ovarian development, daily survival rate, and length of the gonotrophic cycle in females of *Anopheles* (*Kerteszia*) *cruzii* Dyar & Knab, to determine how these factors influence the risk of malaria transmission in the coastal region of the state of Paraná, southern Brazil. In the Palmito State Forest, Paranaguá, females were captured at dawn and dusk by aspiration, bimonthly from December 2006 through March 2007. A total of 2,268 females were captured, of which 454 were dissected. Of these, 48% were parous, 50% not reproductive, 73% in Christopher and Mer stages I and II, 23% in stages III to V, 55% nulliparous, 14% uniparous, and 11% had blood in their midgut. Daily survival was 0.24 ± 0.03 overall, 0.51 ± 0.04 for females captured at dusk, and 0.25 ± 0.03 for those captured at dawn. The Davidson equation for calculation of the gonotrophic cycle was inadequate for *An. cruzii* populations. Females captured at dusk had a higher survival rate than those from dawn, which means that more females of the dusk population enter the parasite extrinsic cycle. The continuous activity and abundance of *A. cruzii* in the Palmito State Forest suggests that the conditions are very favorable for its development, with a potential for participation in the protozoan's transmission cycle.

KEY WORDS: Parity, daily survival rate, ovarian development, malaria

Anopheles (*Kerteszia*) *cruzii* Dyar & Knab is a Neotropical anopheline, exophilic mosquito (Forattini *et al* 1996) found in forests along the Brazilian coast, and is a vector for malaria (*Plasmodium vivax* Grassi & Feletti) and simian plasmodia (Deane *et al* 1984, Carvalho-Pinto & Lourenço de Oliveira 2004). This species is responsible for transmission of "bromeliad malaria" in southern and southeastern Brazil (Aragão 1956), which in 1944 infected 45% of the coastal population in an outbreak in Paraná (Luz *et al* 1979).

Despite the success of the physical and chemical control methods adopted (Correa *et al* 1943, Luz *et al* 1979), *Kerteszia* has persisted in protected natural areas with many bromeliads and due to the impossibility of specific control of mosquitoes in these areas because of environmental conservation issues (Bertoli & Moitinho 2001, Ueno *et al* 2007). Monitoring of *An. cruzii* populations is indispensable for evaluation of the risk of transmission of *P. vivax*.

Vector capacity is the estimate of the ability of an infection to multiply due to a biological vector (Forattini 2002). "In practice, it is a complex interaction of several factors, including population density of vectors and hosts, frequency of feeding on a host, pathogen transfer competence, along with the probability of survival long enough to permit pathogen transmittance" (Beerntsen *et al* 2000).

The daily survival rates of females are of primary importance for understanding vector capacity, since female survival and the probability of infection are intimately related (Fernandez & Forattini 2003). Knowledge of the survival probability, combined with estimates of the reproductive state of females and the duration of the gonotrophic cycle, permit estimation of the vector capacity (Kakitani & Forattini 2000). Here, we measured the reproductive state, ovarian development, daily survival, and gonotrophic cycle of *A. cruzii* females to estimate the current potential of this mosquito species as a vector for malaria.

Material and Methods

Mosquitoes were studied in a Sustainable Use Conservation Unit, with ~530 ha of native vegetation (Palmito State Forest – FEP; 25º 35' S, 48º 32' W), in the municipality of Paranaguá, near the coast in the state of Paraná. Located approximately 90 km from Curitiba, the area borders highway PR-407 at km 4. The climate is subtropical with hot wet summers, but without a clearly defined dry season (Köeppen classification Cfa, IAPAR 2000). Annual rainfall averages 1950 mm, with most rainfall during January and February. Average relative humidity is ~85% (Boeger & Wisniewski 2003). The local vegetation includes pioneer formations with marine influence, mangroves, and some areas with human activity, including buildings (Carrano 2006). The original vegetation is dense lowland rainforest, with abundant and diverse epiphytes and lianas (Boeger & Wisniewski 2002).

The mosquitoes were captured in the human-modified area surrounded by native vegetation.

Captures were made bimonthly from December 2006 to March 2007 (summer), at dawn and dusk. Captures began 1h before sunrise and sunset, and ended 1h after sunrise and sunset. At this latitude, the crepuscular period (dawn and dusk) lasts nearly 30 min (23-26 according to the Nautical Almanac), so we used 30 min as the standard to define dawn and dusk. Thus, captures lasted a total of 2.5 h each, dawn and dusk, divided into three parts: pre-dawn or dusk (1h), dawn or dusk (30 min), and post-dawn or dusk (1h).

We used aspiration to capture mosquitoes, following Forattini (2002). Two people captured mosquitoes at ground level, and wore special clothing, gloves, and head-nets to prevent mosquito bites so that the captured mosquitoes did not have a recent meal from their captors. Collecting jars were changed regularly as mosquitoes were captured. Subsequently, insects were placed in cages and separated according to the six capture periods described above. Mosquitoes were transported alive in cages to the laboratory, where they were maintained in controlled conditions (25ºC, 85% relative humidity) and fed with a 10% honey solution.

Fifteen females from each sampling period were randomly selected for dissection. The specimens were identified by direct observation of morphological characters under a stereo dissecting microscope, using keys for the Anophelinae (Consoli & Lourenço de Oliveira 1994, Forattini 2002). If an individual was difficult to identify, it was not dissected, and all dissections were of clearly identifiable females.

Females were dissected within 12h (those captured at dusk) and 24h (captured at dawn) after capture. If the number of females was less than 15 in any sample, all females in that sample were dissected.

Dissection followed the Polovodova technique, with separation of nulliparous from parous females based on the number of dilatations on the ovariolar pedicel. Evaluation of follicular stages followed Christophers & Mer, in which follicles are classified into five stages. Tracheal terminations, following Detinova, separate reproductive females (anautogenous females that had already fed on blood, or had completed the gonotrophic cycle) from non-reproductive females (females that had never fed on blood nor oviposited) (Charlwood *et al* 1980). From analysis of the midgut, females were separated into three categories: without blood, with red blood, orand with brown blood (Barata *et al* 2001).

The ovaries of each female were examined. One was dried for determining the state of the trachea, and the other was used for examination of the ovariole pedicle. Pedicular dilatations and ovariolar sacs were noted, when present, in all females, following precepts of the "old school" (Forattini 2002). All *Anopheles* specimens that were not dissected were mounted, labeled, identified, and placed in the Padre Jesus Santiago Moure Entomological Collection at the Zoology Department of the Universidade Federal do Paraná (UFPR).

Analysis. We followed Vecruysse (1985) to estimate the daily survival rate (*p*) for *Anopheles cruzii*. Females were separated into three age groups:

(1) NP_1: Nulliparous females at their first meal (Christophers and Mer's stage I or II).

(2) NP_2: Nulliparous females at their second meal (after Christophers and Mer's stage II).

(3) *P*: Parous females

The proportion of each age group in the population:

(4) $n_1 = Np_1/(NP_1+NP_2+P)$: Proportion of females in age class NP_1

(5) $n_2 = Np_2/(NP_1+NP_2+P)$: Proportion in age class NP_2

(6) $n_3 = P/(NP_1+NP_2+P)$: Proportion in age class *P*

(7) $n_1 + n_2 + n_3 = 1$

Estimates of survival rate derived from the proportions of each group in the total population:

(8) $E(n_1) = 1\text{-}p^2/(1 - p^2+p) \Rightarrow (1 - n_1)\, p^2+n_1p - (1 - n_1)$

(9) $E(n_2) = p\,(1 - p^2) / (1\text{-}p^2 + p) \Rightarrow p^3 - n_2p^2 + p\,(n_2 - 1) + n_2$

(10) $E(n_3) = p^3/(1 - p^2 + p) \Rightarrow p^3 + n_3p^2 - n_3p - n_3$

The best estimate of *p* is that which minimizes the following sum of squares:

(11) $\sum_{i=1}^{3} [\, n_i - E_{(ni)} \,]^2 = p$

To solve this equation, we used the program Maple V, release 4. An approximate standard error was given by (from Vercruysse 1985):

(12) $\text{S.E.p.}_{i=1} = {}^{3}(\sum [\, n_i - E_{(ni)} \,]^2 / \text{Total caught})^{1/2}$

With these values, we estimated the duration of the gonotrophic cycle following Kakitani & Forattini (2000) as:

(13) $\log p = (1 / g) \log$ (parous females / total number of females)

where
p: daily survival rate
g: duration (in days) of the gonotrophic cycle.

We compared dawn and dusk captures for mosquito density, parous and non-parous females, stages I-V, nulliparous, and uniparous females, using the Mann-Whitney U test ($P \leq 0.05$). Among the dissected females, we compared the number of parous, nulliparous, and uniparous, in stages I-V using the sign test. Analyses were carried out by means of Statistica 7.0 (StatSoft).

Results

We collected 2,268 *A. cruzii* females, which were relatively equally distributed between dusk (53%, n = 1193) and dawn (47%, n = 1075, Mann-Whitney U test $P > 0.10$). Of these, 454 were dissected, 59% (n = 270) from those captured at dusk and 41% (n = 184) from those at dawn (Table 1).

Approximately 48% of the dissected females were parous, while the remaining were non-parous. The proportion of non-parous females was higher at dawn (Mann-Whitney U test, P = 0.018, U = 174.5000, Table 2). Follicles were in stages I and II in 73% (n = 330) of them, and 23% (n = 104) of females had had at least one blood meal. The proportion of stage I and II females was higher at dusk (Mann-Whitney

Table 1 Aspiration captures of females of *Anopheles cruzii* from December 2006 through March 2007 in the Palmito State Forest, Paranaguá, state of Paraná (mH is mean hourly capture rate).

Periods	*Anopheles cruzii*		
Sunset	N	%	mH
Before sunset	106	9	5,3
Sunset	171	14	8,6
After sunset	916	77	45,8
N total	1193	100	59,7
Effort of capture		20h	
Dawn	N	%	mH
Before dawn	885	82	44,3
Dawn	108	10	5,4
After dawn	82	8	4,1
N total	1075	100	53,8
Effort of capture		20h	

U test, P = 0,007, U = 159.0000), and the number of females in stages I and II was larger than that in stages III-V (Sign test P < 0.01, Table 3).

At dusk, only one female that had oviposited twice was captured. The number of uniparous females was higher at dusk (n = 45) than dawn (n = 17) (Mann-Whitney U test, P = 0.002, U = 146.0000). A larger number of females were nulliparous than uniparous (Sign test P < 0.01, Table 4). Ovariolar sacs were found in 26% (n = 16) of the females, while dilatations were found in 74% (n = 46). It was not possible to determine the ovariolar state of 62 females because their follicles had passed stage III, when accurate visual determination of the ovariolar state becomes difficult or impossible.

Table 2 Parity of females of *Anopheles cruzii* by time of day and totals, based on dissected females (Detinova technique) captured by aspiration from December 2006 through March 2007 in the Palmito State Forest, Paranaguá, state of Paraná.

Periods	Parous		No parous		Not determined	
Sunset	N	%	N	%	N	%
Before sunset	28	23	35	25	3	33
Sunset	46	37	54	39	4	44
After sunset	49	40	49	36	2	22
N	123		138		9	
% Sunset	46		51		3	
Dawn	N	%	N	%	N	%
Before dawn	40	43	55	63	3	75
Dawn	21	23	15	17	0	0
After dawn	32	34	17	20	1	25
N	93		87		4	
% Dawn	51		47		2	
N Total	216		225		13	
% Total	48		50		3	

Table 3 Follicular stages (after Christophers and Mer) of *Anopheles cruzii* by time of day and totals, based on dissected females (Detinova technique) captured by aspiration from December 2006 through March 2007 in the Palmito State Forest, Paranaguá, state of Paraná.

Periods	Follicular stage					
	I, II		III, IV, V		Not determined	
Sunset	N	%	N	%	N	%
Before sunset	47	22	12	24	7	64
Sunset	81	39	20	40	3	27
After sunset	81	39	18	36	1	9
N	209		50		11	
% Sunset	77		19		4	
Dawn	N	%	N	%	N	%
Before dawn	70	58	26	48	2	22
Dawn	26	21	8	15	2	22
After dawn	25	21	20	37	5	56
N	121		54		9	
% Dawn	66		29		5	
N Total	330		104		20	
% Total	73		23		4	

Most females captured at dusk had no signs of blood (89%, n = 402), while 4% (n = 16) contained red blood and 7% (n = 31) had brown blood in their midgut. Seven percent (n = 33) of *A. cruzii* females were nulliparous, with follicles past stage II and attempting to perform the hematophagic activity for the second time in the same gonotrophic cycle. The same occurred with 2% (n= 10) of the nulliparous females that contained red blood in the midgut and ovaries in stages III –V (Table 5). Daily survival rates (Vercruysse 1985) and the duration of the gonotrophic cycle (Davidson 1954) estimates for the total and for each time period (dawn, dusk) can be found in Table 6.

Discussion

In Palmito State Park, as in other locations (Forattini *et al* 1986), the majority of *A. cruzii* activity, and therefore captures, was prior to dawn and following sunset. Here, about half of the population of females was nulliparous and had not consumed a blood meal. These females were actively pursuing a blood source during the evenings; light levels seem to be critical for initiating blood feeding (Forattini *et al* 1981).

Developed follicles (stage V) were found in few females, and these were captured while searching for an oviposition site. Follicles in most females were in stages I and II, and these individuals were in search of a blood meal (mostly at

Table 4 Parity of females of *Anopheles cruzii* by time of day and totals, based on dissected females (Polovodova technique) captured by aspiration from December 2006 through March 2007 in the Palmito State Forest, Paranaguá, state of Paraná.

Periods	Nulliparous		Uniparous		Biparous		Not determined	
Sunset	N	%	N	%	N	%	N	%
Before sunset	35	24	11	24	0	0	20	26
Sunset	54	37	17	38	1	100	32	42
After sunset	58	39	17	38	0	0	25	32
N	147		45		1		77	
% Sunset	54		17		0		29	
Dawn	N	%	N	%	N	%	N	%
Before dawn	60	59	9	53	0	0	29	44
Dawn	23	23	3	18	0	0	10	15
After dawn	18	18	5	29	0	0	27	41
N	101		17		0		66	
% Dawn	55		9		0		36	
N Total	248		62		1		143	
% Total	55		14		0		32	

dusk) to initiate vitellogenesis. Since fewer stage I and II females were captured at dawn, we deduce that these females at this time are engorged.

Throughout this study, young females were much more common, especially at dusk. Only 14% of the captured females had passed the critical point for oviposition, and only one female captured had passed through two gonotrophic cycles. This may indicate a high mortality for parous females, or a continuous emergence of adults, or both. In an earlier study in coastal Paraná (1976-1977), 80% of the captured *A. cruzii* at dusk were young and nulliparous (Luz *et al* 1979). At the same location in 2004-2005, 58% (n = 120) of the daytime population of this mosquito was nulliparous (Dalla Bona & Navarro 2006). Malaria transmission in this region is usually through young females. Thus, to be an important vector for disease transmission, the vector must be abundant (Luz *et al* 1979).

The number of dilatations in each ovariole has been used as an indicator of the number of gonotrophic cycles, in which uniparous females are defined by their dilatations and

Table 5 Stages and phases of ovariole development of *Anopheles cruzii* by time of day and totals, based on dissected females (Polovodova technique) captured by aspiration from December 2006 through March 2007 in the Palmito State Forest, Paranaguá, state of Paraná.

Periods	Stage I or II						Stage III, IV, V					
	Nulliparous		Uniparous		Biparous		Nulliparous		Uniparous		Not determined	
Sunset	N	%	N	%	N	%	N	%	N	%	N	%
Before sunset	27	22	9	23	0	0	7	35	2	33	21	27
Sunset	48	38	13	33	1	100	5	25	4	67	33	42
After sunset	50	40	17	44	0	0	8	40	0	0	25	32
N	125		39		1		20		6		79	
% Sunset	46		14		0		7		2		29	
Dawn	N	%	N	%	N	%	N	%	N	%	N	%
Before dawn	55	63	7	50	0	0	5	38	2	67	29	44
Dawn	18	20	2	14	0	0	5	38	1	33	10	15
After dawn	15	17	5	36	0	0	3	23	0	0	27	41
N	88		14		0		13		3		66	
% Dawn	48		8		0		7		2		36	
N Total	213		53		1		33		9		145	
% Total	47		12		0		7		2		32	

Table 6 Parameters for and estimates of daily survival rates and gonotrophic cycle duration, for females of *Anopheles cruzii* by time of day and totals, based on dissected females captured by aspiration from December 2006 through March 2007 in the Palmito State Forest, Paranaguá, state of Paraná.

Parameters	Population of *Anopheles cruzii*		
	Total	Sunset period	Dawn period
NP1	213	125	88
NP2	33	20	13
P	63	46	17
n1	0,69	0,65	0,75
n2	0,11	0,10	0,11
n3	0,20	0,24	0,14
E(n1)	0,38	0,43	0,3
E(n2)	0,12	0,11	0,12
E(n3)	0,62	0,66	0,55
Daily survival ± standard error	0.2365 ± 0.0277	0.505 ± 0.0404	0.2450 ± 0.0282
Duration of gonotrophic cycle	0.89 days	0.48 days	1.38 days

ovariolar sac (Forattini 2002), although controversies have arisen, as some investigators believe that the dilatations are products of abortive oogenesis and that only the follicular sacs indicate normal oogenesis (Fox & Brust 1994). Only 26% (n = 16) of the uniparous females had ovariolar sacs and normal oogenesis, according to Fox & Brust (1994), but this interpretation may underestimate their longevity (Telles de Deus & Kakitani 2006). Based on the number of dilatations in the ovarioles, we considered 100% (n = 62) females to be uniparous. Any sequence of abortive and normal oogenesis in the same ovariole was considered as a diagnostic ovariole (Fox & Brust 1994).

Anopheles cruzii is anautogenous, and its complete ovariolar maturation occurs following one or more blood meals. Autogeny may be adaptive in areas or periods when blood sources are lacking (Forattini 2002). Apparently there are no such periods in coastal Paraná, since 11% of the females were engorged. Autogeny may make age estimates more difficult and reduce the efficiency of the vector, because it delays the first potentially infective blood meal (Russell 1987a).

The frequency of nulliparous females that passed stage II of Christophers and Mer in our samples demonstrates that a female may take more than one blood meal within one gonotrophic cycle at any time. In dissected females, 2% had ovaries at either stage III, IV, or V, as well as red blood in their midgut. This also indicates gonotrophic discordance in *A. cruzii* females (Forattini *et al* 1993, 1996, Dalla Bona & Navarro-Silva 2006).

It is often suggested that the daily survival rate is constant (age-independent), while in a study using capture, marking and recapture (Harrington *et al* 2001), older females had higher survival than younger females. Here, we also found that parous females had higher survival rates than nulliparous females ($E_{(n3)} > E_{(n1)}$, Table 6). To be considered as a potential vector, survival must be > 60% and usually above 80%. Since the extrinsic period of *Plasmodium vivax* is eight days (Russell 1987b), a population of 100,000 females in coastal Parana would include five females (dawn and dusk summed), 897 dusk females, and six dawn females that survived long enough to become infective.

Thus, the dusk population is more likely to become a vector problem in higher densities. In another study, on the *Anopheles* (*Nyssorhynchus*) *albitarsis* Lynch-Arribálzaga complex, 50% daily survival rates were shown to yield only 1% of the population that survived long enough to become infective (Kakitani & Forattini 2000).

Female daily survival rates, population density, human associations, the period of extrinsic incubation, and vector competence should all be analyzed together with environmental variables. Populations that have low vectorial competence but are abundant may still cause infection transmission (Miller *et al* 1989).

The gonotrophic cycle in females in nature requires on average 0.89 days in *A. cruzii*. During dusk, the cycle is nearly half of this, 0.48 days, whereas at dawn it is 1.38 days (Davidson 1954). In two populations of *A. albitarsis*, the gonotrophic cycle lasted 1.99 and 2.04 days (Kakitani & Forattini 2000). In *A. cruzii*, other studies have calculated gonotrophic cycles of 4.01 days (Chahad-Ehlers *et al* 2007) and 6-7 days (Kakitani 1992), both much longer than we found here.

The duration of the gonotrophic cycle of about a day, obtained by the equation of Davidson, differs from those reported in the literature. *Anopheles cruzii* shows gonotrophic discordance, and one day would be too short a time for the female to locate more than one host. The temperature, non-obligatory resting period between meals, distance to and ease of finding an oviposition site, and finally, finding a host, all influence the length of the gonotrophic cycle (Charlwood *et al* 1980).

The region of the Serra do Mar (Coastal Range) is considered a hypoendemic area of malaria, where the epidemiology of the atypical native cases has been debated. Evidence of the variant circulation of *P. vivax* and simian plasmodium has been found recently (Ueno *et al* 2007). The

duration of the gonotrophic cycle obtained with the aid of the Davidson equation did not show a straight-line relationship with the biology of the species; this equation is possibly inadequate because of the species' gonotrophic discordance. Females of the dusk population had a higher survival rate than the dawn females, which means that more females of the dusk population cross the parasite extrinsic cycle, becoming infective. The continuous activity and abundance of the mosquito *A. cruzii* in the Palmito State Forest suggest that the conditions are very favorable for the development of this species, with potential participation in the protozoan's transmission cycle.

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