

BIOLOGY OF AMAZONIAN ANOPHELINE MOSQUITOES. XVI. EVIDENCE OF MULTIPLE INSEMINATION (POLIANDRY) IN *Anopheles nuneztovari* GABALDON, 1940 (DIPTERA: CULICIDAE)

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ABSTRACT

Genetic segregation among the descendants of *Anopheles nuneztovari* for the isoalleles *EST1*F*, *EST1*S*; *EST2*F*, *EST2*S*; *EST6*F*, *EST6*M*, *EST6*S* were analyzed in our laboratories. These analyses indicated that effective multiple insemination (poliandry) occurs in natural populations of *A. nuneztovari*. 40 females were collected in the field, and eight descendants per female were submitted to electrophoresis. The results indicated that at least 15% of the females had semen from two males and the remaining 85% had brood fathered by a single male.

INTRODUCTION

Studies of the reproductive behavior of different species of mosquitoes have shown that the females are either exclusively or predominantly monandric (monogamic)* (French and Kitzmiller, 1963; Jones and Wheeler, 1965; Craig, 1967; Bryan, 1968, 1972; Asman, 1975; Santos *et al.*, 1981 and Baimai and Green, 1987). This apparently is related to a pheromone (matrone) produced by the males which is transferred to the females during copulations and prevents subsequent inseminations (Craig, 1967; Spielman *et al.*, 1967; Fuchs *et al.*, 1969; Bullini *et al.*, 1976). Leahy and Craig (1965) suggested the

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* Monogamic, in older literature.

possibility that this pheromone could be involved with an increase of oviposition in *Aedes aegypti*. However, the literature also reports the occurrence of multiple inseminations (polyandry) in species of *Anopheles*, *Culex* and *Aedes* (Vande Hey and Craig, 1958; Goma, 1963; French and Kitzmiller, 1963; Gwadz *et al.*, 1971; Bullini *et al.*, 1976; Baimai and Green, 1987).

Females of *Anopheles nuneztovari* and their descendants were studied to verify the occurrence of monandry or multiple insemination using as genetic markers the segregation of alleles of esterase isozymes.

MATERIALS AND METHODS

The samples analyzed were collected in the rain forest of Base 4 which is located on the right bank of the Tucuruí reservoir (Pará State, Brazil). After capture, the females were fed chicken blood (*Gallus gallus*) and then separated individually into cups for oviposition. Following the hatching of the eggs, the larvae of each brood were kept in separate trays until the emergence of the adults, according to Scarpassa (1988) and Scarpassa and Tadei (1990). Forty females and eight descendants of each, collected at random, resulted in a total of 320 descendants analyzed. The females collected in the field and their respective descendants (fourth instar larvae, pupae and young adults) were submitted to electrophoresis in 12.5% starch gel. The buffer solutions were prepared according to Lima and Mestriner (1985) and Falcão and Contel (1990), and the reaction mixtures according to Harris and Hopkinson (1976). For the designation of the loci and alleles, the nomenclature proposed by Shows *et al.* (1979) was used. After staining of the esterase activity zones, the readings of the phenotypes were done for loci *EST1*, *EST2* and *EST6*. Based on the segregation of the alleles *EST1*F*, *EST1*S*; *EST2*F*, *EST2*S*; *EST6*F*, *EST6*M*, *EST6*S*, the genotypes of the mother and descendants were easily recognizable and the paternal genotypes could be inferred. For the paternity tests, we took into account the segregation of the alleles in each progeny, and the possibility of fertilization of eggs by spermatozooids from one (H_0) or two (H_1) males. The frequencies obtained were compared by Chi-square.

RESULTS AND ANALYSES

The results of the analyses of the 320 descendants and the 40 females for esterases 1, 2 and 6, respectively are shown in Tables I, II and III. For esterase 1 the data permitted a deduction of the following four types of crosses: ♀ *EST1*F/EST1*S* x ♂ *EST1*F/EST1*S*; ♀ *EST1*F/EST1*S* x ♂ *EST1*S/EST1*S*; ♀ *EST1*S/EST1*S* x ♂ *EST1*F/EST1*S*; ♀ *EST1*S/EST1*S* x ♂ *EST1*S/EST1*S*. For the second and third

crosses the frequency of the phenotypes observed showed that only one male is involved in the fertilization of the eggs ($X_1^2 = 1.0 - P > 0.05$; $X_1^2 = 0.00 - P > 0.05$, respectively for the second and the third). The hypotheses of a second male was tested only for the 1st crossing ($\text{♀ } EST1^*F/EST1^*S \times \text{♂ } EST1^*F/EST1^*S$), but the chi-squared values indicated spermatozoids of only one male ($H_0 - X_2^2 = 3.7$; $P > 0.05/H_1 - X_2^2 = 7.8$; $P < 0.01$). For the fourth crossing, where all the descendants showed a $EST1\ S/EST1\ S$ phenotype, the chi-square was not calculated ($\text{♀ } EST1^*S/EST1^*S \times \text{♂ } EST1^*S/EST1^*S$).

Table I -Phenotypes observed for esterase 1 in the offspring of the crossings and chi-square values of the paternity tests.

1st Crossing: $EST1^*F/EST1^*S^{\#} \times EST1^*F/EST1^*S^{\#\#}$										
Phenotype observed	Descendants				Total	H_0		H_1		
	Expected probability	Frequency in progeny				Esp1 FS	X_2^2	Esp2 FS	X_2^2	
		1	2	3						4
EST1 F/EST1 F	0.25	3	3	3	3	12	8	2.0	12	0.0
EST1 F/EST1 S	0.50	3	3	2	3	11	16	1.6	16	1.6
EST1 S/EST1 S	0.25	2	2	3	2	9	8	0.1	4	6.2
Total						32	32	3.7	32	7.8**

2nd Crossing: $EST1^*F/EST1^*S^{\#} \times EST1^*S/EST1^*S^{\#\#}$								
Phenotype observed	Descendants				Total	H_0		
	Expected probability	Frequency in progeny				Esp 1 SS	X_1^2	
		1	2					
EST1 F/EST1 S	0.50	4	2		6	8	0.5	
EST1 S/EST1 S	0.50	4	6		10	8	0.5	
Total					16	16	1.0	

Continued

Table I - Continued

3rd Crossing: $EST1^*S/EST1^*S^{\#} \times EST1^*F/EST1^*S^{\#\#}$

Phenotype observed	Descendants			H ₀	
	Expected probability	Frequency in progeny		Esp 1 FS	X ₁ ²
		1	2		
EST1 F/EST1 S	0.50	5	3	8	0.0
EST1 S/EST1 S	0.50	3	5	8	0.0
Total				16	0.0

4th Crossing: $EST1^*S/EST1^*S^{\#} \times EST1^*S/EST1^*S^{\#\#}$

Phenotype observed	Descendants		Total
	Frequency in progeny		
	1		
EST1 S/EST1 S	8		8

- Observed maternal genotype.

- Inferred paternal genotype.

H₀ - Hypothesis of insemination by one male (Expected 1)H₁ - Hypothesis of insemination by two males (Expected 2).

** - P < 0,01.

The results of esterase 2 showed six types of crosses (Table II). The hypothesis of a second male was considered for the first cross. Although the values of chi-square are not significant in these cases, the data give more support to the hypothesis of insemination by two males ($\text{♀ } EST2^*F/EST2^*S \times \text{♂ } EST2^*F/EST2^*S - X_2^2 = 2.75 [H_0] \text{ and } 0.55 [H_1]$; $\text{♀ } EST2^*S/EST2^*S \times \text{♂ } EST2^*F/EST2^*S - X_1^2 = 2.20 [H_0] \text{ and } 0.28 [H_1]$, $P > 0.05$). For the crossing - $\text{♀ } EST2^*F/EST2^*S \times \text{♂ } EST2^*S/EST2^*S$ (third types) the data indicate fertilization by a single male ($X_1^2 = 3.33$; $P > 0.05$) which can be extended to the homozygote crossings - $\text{♀ } EST2^*F/EST2^*F \times \text{♂ } EST2^*F/EST2^*F$ and $\text{♀ } EST2^*S/EST2^*S \times \text{♂ } EST2^*S/EST2^*S$ (fifth and sixth types, respectively).

Table II - Phenotypes observed for esterase 2 in the offspring of the crossings occurred in nature and chi-square values of paternity tests.

1st Crossing: $EST2^*F/EST2^*S^{\#} \times EST2^*F/EST2^*S^{\#}$

Descendants				H ₀		H ₁	
Phenotype observed	Expected probability	Frequency in progeny		Esp1 FS	X ₂ ²	Esp 2 FS	X ₂ ²
		1	Total				
EST2 F/EST2 F	0.25	4	4	2	2.00	3	0.30
EST2 F/EST2 S	0.50	3	3	4	0.25	4	0.25
EST2 S/EST2 S	0.25	1	1	2	0.50	1	0.00
Total			8	8	2.75	8	0.55

2nd Crossing: $EST2^*S/EST2^*S^{\#} \times EST2^*F/EST2^*S^{\#}$

Descendants				H ₀		H ₁	
Phenotype observed	Expected probability	Frequency in progeny		Esp1 FS	X ₁ ²	Esp 2 FS	X ₁ ²
		1	Total				
EST2 F/EST2 S	0.50	6	5	8	1.1	12	0.08
EST2 S/EST2 S	0.50	2	3	8	1.1	4	0.20
Total			16	16	2.20	16	0.28

3rd Crossing: $EST2^*F/EST2^*S^{\#} \times EST2^*S/EST2^*S^{\#}$

Descendants				H ₀		
Phenotype observed	Expected probability	Frequency in progeny		Total	Esp 1 SS	X ₁ ²
		1	2			
EST2 F/EST2 S	0.50	2	2	4	8	2.0
EST2 S/EST2 S	0.50	6	6	12	8	1.33
Total				16	16	3.33

Table II - Continued

4th Crossing: $EST2^*F/EST2^*S^{\#} \times EST2^*F/EST2^*F^{\#\#}$

Phenotype observed	Descendants			H ₀	
	Expected probability	Frequency in progeny	Total	Esp 1 FF	X ₁ ²
		1			
EST2 F/EST2 F	0.50	7	7	4	2.25
EST2 F/EST2 S	0.50	1	1	4	2.25
Total			8	8	4.50*

5th Crossing: $EST2^*F/EST2^*F^{\#} \times EST2^*F/EST2^*F^{\#\#}$

Phenotype observed	Descendants				Total
	Frequency in progeny				
	1	2	3	4	
EST2 F/EST2 F	8	8	8	8	32

6th Crossing: $EST2^*S/EST2^*S^{\#} \times EST2^*S/EST2^*S^{\#\#}$

Phenotype observed	Descendants			Total
	Frequency in progeny			
	1			
EST2 S/EST2 S	8			8

- Observed maternal genotype.

- Inferred paternal genotype.

H₀ - Hypothesis of insemination by one male (Expected 1).H₁ - Hypothesis of insemination by two males (Expected 2).* - $P < 0.05$.

The results of the 4th crossing - ♀ *EST2*F/EST2*S* x ♂ *EST2*F/EST2*F* which showed significant chi-squared values ($X_1^2 = 4.50$; $P < 0.05$) can be explained by the low viability of the heterozygotes (*EST2 F/EST2 S*). For esterase 6 (Table III) 10 types of crossings were detected. As in the former cases, considering the phenotypes of the progeny, the test for a second male was run for the first, third and fourth crossing. However, for the first crossing where chi-squared values were significant for H_0 and H_1 ($X_2^2 = 16.0$; $P < 0.001$ - $X_2^2 = 21.9$; $P < 0.001$, respectively), the results indicate that the hypothesis of insemination by a single male is a better fit. On the other hand, the low viability of the heterozygotes (*EST 6 F/EST6 M*) should be considered. The hypothesis of a single male also extends to the fourth crossing because the chi-square was not significant for H_0 ($H_0 - X_1^2 = 0.50$; $P > 0.05$ / $H_1 - X_1^2 = 6.0$; $P < 0.01$). Based on the chi-square values, in the third crossing, the hypothesis of insemination by two different males is a better fit ($H_0 - X_1^2 = 2.20$; $P > 0.05$ / $H_1 - X_1^2 = 0.33$; $P > 0.05$). For the crossing - ♀ *EST6*M/EST6*M* x ♂ *EST6*F/EST6*M*; ♀ *EST6*F/EST6*M* x ♂ *EST6*F/EST6*F* and ♀ *EST6*F/EST6*M* x ♂ *EST6*M/EST6*S* (second, fifth and sixth types), based on the phenotypes detected, the data are consistent with the fertilization of eggs by spermatozooids of one male. The same was observed for the crossings - ♀ *EST6*F/EST6*F* x ♂ *EST6*M/EST6*M*; ♀ *EST6*F/EST6*F* x ♂ *EST6*F/EST6*F*; ♀ *EST6*M/EST6*M* x ♂ *EST6*M/EST6*M* (seventh, eight and ninth types), only one phenotype was observed.

Table III - Phenotypes observed for esterase 6 in the offspring of the crossings and chi-square values of the paternity tests.

1st Crossing: *EST6*F/EST6*M[#]* x *EST6*F/EST6*M[#]*

Phenotype observed	Expected probability	Descendants						Total	H_0		H_1	
		Frequency in progeny							Esp1	X_2^2	Esp2	X_2^2
		1	2	3	4	5	6		FM		FM	FF
EST6 F/EST6 F	0.25	4	1	4	5	5	3	22	12	8.3	18	0.9
EST6 F/EST6 M	0.50	0	2	1	2	3	3	11	24	7.0	24	7.0
EST6 M/EST6 M	0.25	4	5	3	1	0	2	15	12	0.7	6	14.0
Total								48	48	16.0***	48	21.9***

Continued

Table III - Continued

2nd Crossing: $EST6^*M/EST6^*M^{\#} \times EST6^*F/EST6^*M^{\#\#}$

Phenotype observed	Expected probability	Descendants				Total	H ₀	
		Frequency in progeny					Esp 1 FM	X ₁ ²
		1	2	3	4			
EST6 F/EST6 M	0.50	3	5	4	4	16	0.0	
EST6 M/EST6 M	0.50	5	3	4	4	16	0.0	
Total						32	0.0	

3rd Crossing: $EST6^*F/EST6^*F^{\#} \times EST6^*F/EST6^*M^{\#\#}$

Phenotype observed	Expected probability	Descendants			H ₀		H ₁	
		Frequency in progeny			Esp1 FM	X ₁ ²	Esp 2 FM	X ₁ ²
		1	2	Total				
EST6 F/EST6 F	0.50	4	7	11	8	1.1	12	0.08
EST6 F/EST6 M	0.50	4	1	5	8	1.1	4	0.25
Total				16	16	2.20	16	0.33

4th Crossing: $EST6^*F/EST6^*F^{\#} \times EST6^*F/EST6^*S^{\#\#}$

Phenotype observed	Expected probability	Descendants			H ₀		H ₁	
		Frequency in progeny			Esp1 FS	X ₁ ²	Esp 2 FS	X ₁ ²
		1	Total	FF				
EST6 F/EST6 F	0.50	3	3	4	0.25	6	1.5	
EST6 F/EST6 S	0.50	5	5	4	0.25	2	4.5	
Total			8	8	0.50	8	6.00**	

Continued

Table III - Continued

5th Crossing: $EST6^*F/EST6^*M^{\#} \times EST6^*F/EST6^*F^{\#}$

Descendants				H ₀	
Phenotype observed	Expected probability	Frequency in progeny	Total	Esp 1 FF	X ₁ ²
		1			
EST6 F/EST6 F	0.50	3	3	4	0.25
EST6 F/EST6 M	0.50	5	5	4	0.25
Total			8	8	0.50

6th Crossing: $EST6^*F/EST6^*M^{\#} \times EST6^*M/EST6^*S^{\#}$

Descendants				H ₀	
Phenotype observed	Expected probability	Frequency in progeny	Total	Esp 1 MS	X ₃ ²
		1			
EST6 F/EST6 M	0.25	4	4	2	2
EST6 F/EST6 S	0.25	3	3	2	0.5
EST6 M/EST6 M	0.25	0	0	2	2
EST6 M/EST6 S	0.25	1	1	2	0.5
Total			8	8	5.0

7th Crossing: $EST6^*F/EST6^*F^{\#} \times EST6^*M/EST6^*M^{\#}$

Descendants		
Phenotype observed	Frequency in progeny	Total
	1	
EST6 F/EST6 M	8	8

Continued

8th Crossing: $EST6^*F/EST6^*F^{\#}$ x $EST6^*F/EST6^*F^{\#\#}$

Descendants		
Phenotype observed	Frequency in progeny 1	Total
EST6 F/EST6 F	8	8

9th Crossing: $EST6^*M/EST6^*M^{\#}$ x $EST6^*M/EST6^*M^{\#\#}$

Descendants		
Phenotype observed	Frequency in progeny 1 2	Total
EST6 M/EST6 M	8 8	16

10th Crossing: $EST6^*F/EST6^*M^{\#}$ x $EST6^*F/EST6^*S^{\#\#}$
 $EST6^*M/EST6^*S$

Descendants		
Phenotype observed	Expected probability	Frequency in progeny 1
EST6 F/EST6 F	0.125	1
EST6 F/EST6 S	0.250	0
EST6 F/EST6 M	0.250	0
EST6 M/EST6 S	0.250	2
EST6 M/EST6 M	0.125	5
Total		8

$\#$ - Observed maternal genotype.

$\#\#$ - Inferred paternal genotype.

H_0 - Hypothesis of insemination by one male (Expected 1).

H_1 - Hypothesis of insemination by two males (Expected 2).

** - $P < 0,01$; *** - $P < 0.001$.

The segregation of the alleles observed in the progeny of the last crossing (10th) showed that the eggs were fertilized by spermatozoids from two males -
 $\text{♀ EST6}^*F/\text{EST6}^*M \times \text{♂ EST6}^*F/\text{EST6}^*S$
 $\text{♂ EST6}^*M/\text{EST6}^*S$

Considering the results of the esterase 1, 2 and 6, H_0 was rejected in six cases, which means a percentage of at least 15% of double insemination, for the 40 females studied.

DISCUSSION

Knowledge of the occurrence of monandry and multiple insemination is relevant to the interpretation of biological data of mosquitoes populations, particularly for the genetic control of vector species. We registered low frequencies of multiple insemination in *A. nuneztovari*. These data are in agreement with other records of multiple insemination in *Anopheles* at low frequencies for example for *Anopheles gambiae* (Gillies, 1956; Goma, 1963), *Anopheles quadrimaculatus* (French and Kitzmiller, 1963) and *Anopheles dirus* (Baimai and Green, 1987).

In other genera of mosquitoes multiple insemination has also been observed, for example, *Aedes aegypti* (Vande Hey and Craig, 1958; Gwadz *et al.*, 1971) and *Culex pipiens* L. (Bullini *et al.*, 1976). In the case of *A. aegypti*, Gwadz *et al.* (1971) observed multiple insemination only when several males copulated with the same female in a short span of time. The same was found for *C. pipiens* L. (Bullini *et al.*, 1976).

Although multiple insemination has been registered in different species of mosquitoes, monandry is the predominant form. Predominance of monandry (85%), which is what we report for *A. nuneztovari*, has also been shown for *A. gambiae*, *A. quadrimaculatus*, *A. dirus*, *A. aegypti* and *C. pipiens* L. Exclusive monandry has been registered in *Anopheles darlingi* (Santos *et al.*, 1981), *Anopheles maculatus* (Baimai and Green, 1987) and in three subspecies of the *Culex pipiens* complex, *C. p. pipiens*, *C. p. fatigans*, *C. p. molestus* (Kitzmiller and Laven, 1958) and in *Culex tarsalis* (Asman, 1975).

Various authors have discussed two mechanisms which act during copulation to guarantee the predominance of monandry. The first was emphasized by Gilles (1956) and discussed by Bullini *et al.* (1976). This mechanism is represented by a mucoid mating plug, formed during the first copulation. However, Bullini *et al.* (1976) mention that the efficiency of this mechanism is not complete, fertilization of the eggs by spermatozoids of a second male can occur though at low percentages (10%). The other mechanism was proposed by Craig (1967) and Fuchs *et al.* (1969) and apparently is the more efficient (Bullini *et al.*, 1976). Monandry is guaranteed by the action of a proteic substance produced by the accessory glands of the males, a pheromone (matrone) which is

transferred to the females during copulation. This substance inhibits further fertilization of the eggs (Craig, 1967; Fuchs et al., 1969; Bullini et al., 1976; Santos et al., 1981).

The way in which these mechanisms operate was discussed by Bullini et al. (1976). These authors demonstrated that the multiple insemination observed for *C. pipiens* (4.4%) was the result of cases in which the female copulated twice within 48 hours. They also showed that it takes a little while for the pheromone to act. After which, it apparently prevents subsequent inseminations. Similar conclusions had already been proposed by Gwadz et al. (1971) for *A. aegypti* in which multiple insemination occurs only when various males copulate with a female within a short span of time.

Considering the data we have on *A. nuneztovari*, in which multiple insemination was observed at low frequencies, we suppose that this was a result of subsequent copulations occurring within a short time, as reported for *A. aegypti* (Gwadz et al., op. cit.) and *C. pipiens* L. (Bullini et al., op. cit.). With regards to *C. pipiens* L. the authors estimated the time span to be 48 hours, based on laboratory experiments. As in our study the females were collected in the field the time has yet to be determined.

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RESUMO

Com base nos resultados da segregação dos alelos de isoenzimas de esterase *EST1*F EST1*S; EST2*F, EST2*S; EST6*F, EST6*M, EST6*S*, foi registrada a ocorrência de múltipla inseminação efetiva (poliandria) em populações naturais de *Anopheles nuneztovari*. Foram estudadas 40 fêmeas capturadas na natureza, e 8 descendentes por progênie, resultando em um total de 320 indivíduos analisados. Os resultados evidenciaram que a múltipla inseminação ocorre em pelo menos 15% das fêmeas, porém, como nas demais espécies de mosquitos, a monandria (monogamia) é mais freqüente (85%). Os seis casos de múltipla inseminação constatados para *A. nuneztovari*, provavelmente, resultam de sucessivas cópulas, ocorridas no espaço de 48 horas, igualmente ao descrito para *Culex pipiens* L..

REFERENCES

- Asman, M. (1975). Observations of mating behavior in *Culex tarsalis*. *Ann. Entomol. Soc. Am.* 68: 777-778.
- Baimai, V. and Green, C.A. (1987). Monandry (Monogamy) in natural populations of Anopheline Mosquitoes. *Journal Am. Mosq. Control. Assoc.* 3: 481-484.

- Bryan, J.H. (1968). Results of consecutive matings of female *Anopheles gambiae* B with fertile and sterile males. *Nature* 218: 489.
- Bryan, J.H. (1972). Further studies on consecutive matings in the *Anopheles gambiae* complex. *Nature* 239: 519-520.
- Bullini, L., Coluzzi, M. and Bianchi-Bullini, A.P. (1976). Biochemical variants in the study of multiple insemination in *Culex pipiens* L. (Diptera, Culicidae). *Bull. Ent. Res.* 65: 683-685.
- Craig Jr., G.B. (1967). Mosquitoes: Female monogamy induced by male accessory gland substance. *Science* 156: 1499-1501.
- Falcão, T.M.M.A. and Contel, E.P.B. (1990). Genetic variability in natural populations of Brazilian social bees. I. Isozyme patterns and polymorphism for esterases and total protein. *Rev. Bras. Genet.* 13: 731-754.
- French, W.L. and Kitzmiller, J.B. (1963). Evidence for multiple fertilization in *Anopheles quadrimaculatus* using genetic markers. *Am. Soc. Zool.* 2: 524.
- Fuchs, M.S., Craig Jr., G.B. and Despommier, D.D. (1969). The protein nature of the substance inducing female monogamy in *Aedes aegypti*. *J. Insect Physiol.* 15: 701-709.
- Gillies, M.T. (1956). A new character for the recognition of nulliparous females of *Anopheles gambiae*. *Bull. World Health Org.* 15: 451-459.
- Goma, L.K.H. (1963). Tests for multiple insemination in *Anopheles gambiae* Giles. *Nature* 197: 99-100.
- Gwadz, R.W., Craig Jr., G.B. and Hickey, W.A. (1971). Female sexual behavior as the mechanism rendering *Aedes aegypti* refractory to insemination. *Biol. Bull.* 140: 201-214.
- Harris, H. and Hopkinson, D.A. (1976). Handbook of enzyme electrophoresis in human genetics. North Holland.
- Jones, J.C. and Wheeler, R.E. (1965). Studies on spermathecal filling in *Aedes aegypti* (Linnaeus). I. Description. *Biol. Bull.* 129: 134-150.
- Kitzmiller, J.B. and Laven, H. (1958). Tests for multiple fertilization in *Culex* mosquitoes by the use of genetic markers. *Am. Journal Hyg.* 67: 207-213.
- Leahy, M.G. and Craig Jr., G.B. (1965). Male accessory gland substance as a stimulant for ovoposition in *Aedes aegypti* and *A. albopictus*. *Mosquito News* 25: 448-452.
- Lima, L.M.K.S. and Mestriner, M.A. (1985). Starch gel electrophoretic patterns of esterases and non-specific proteins in 11 different species of Meliponine bees. *Rev. Brasil. Genet.* VIII: 639-647.
- Santos, J.M.M., Contel, E.P.B. and Kerr, W.E. (1981). Biologia de anofelinos amazônicos. 2. Fêmeas de *Anopheles darlingi* produzem filhos de um só macho. *Acta Amaz.* 11: 413-414.
- Scarpassa, V.M. (1988). Estudo do ciclo biológico e de isoenzimas na ontogênese de *Anopheles* (*Nyssorhynchus*). *nuñez-tovari* Gabaldon, 1940. Masters Thesis, INPA/FUA.
- Scarpassa, V.M. and Tadei, W.P. (1990). Biologia de Anofelinos Amazônicos. XIII. Estudo do ciclo biológico de *Anopheles nuñez-tovari* (Diptera, Culicidae). *Acta Amaz.* 20: 95-118.
- Shows, T.B., Alper, C.A., Bootsma, D., Dorf, M., Douglas, T., Huisman, T., Kit, S., Klingler, H.P., Kozak, C., Lalley, P.A., Lindsley, D., McAlpine, P.J., McDougall, J.K., Meerakhan, P., Meisler, M., Morton, N.E., Opitz, J.M., Partridge, C.W., Payne, R., Roderick, T.H., Rubinstein, P., Ruddle, F.H., Shaw, M., Spranger, J.W. and Weiss, K. (1979). Human gene Mapping. Fifth International Workshop on Human Gene Mapping. *Cytogenet. Cell. Genet.* 25: 96-116.

- Spielman, A., Leahy, Sr. M.G. and Skaff, V. (1967). Seminal loss in repeatedly mated female *Aedes aegypti*. *Biol. Bull.* 132: 404-412.
- Vande Hey, R.C. and Craig Jr., G.B. (1958). Multiple fertilization demonstrated in *Aedes aegypti*. *Bull. Ent. Soc. Am.* 4: 102.

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