

Analysis of Reproductive Isolation Between Sibling Species *Anopheles albitarsis* sensu stricto and *Anopheles deaneorum*, Two Malaria Vectors Belonging to the Albitarsis Complex (Diptera: Culicidae)

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ABSTRACT Complexes of sibling species are common among mosquitoes, and their existence within vector species can have important epidemiological consequences. *Anopheles albitarsis* sensu stricto and *Anopheles deaneorum* Rosa-Freitas are two putative vectors of malaria parasites belonging to the *Albitarsis* species complex (Diptera: Culicidae). Using an induced mating technique, we studied the reproductive isolation between these two closely related species and their reciprocal hybrids. Evidence for hybrid male sterility consistent with Haldane's rule was found. The results indicate that male hybrids show very low insemination rates, probably due to abnormalities in their reproductive organs. In addition, the data show that hybrid males carrying an X chromosome derived from *An. deaneorum* perform significantly worse than hybrid males carrying an *An. albitarsis* s.s. X chromosome.

KEY WORDS malaria, speciation, hybrid sterility, Haldane's rule, *Anopheles albitarsis*

SIBLING SPECIES COMPLEXES ARE common among mosquito vectors of *Plasmodium* parasites, the etiological agent of malaria (Krzywinski and Besansky 2003), a disease affecting ≈273 million people worldwide (WHO 2001, 2003). *Anopheles albitarsis* Lynch-Arríbalzaga is considered a complex of sibling species (Kreutzer et al. 1976, Rosa-Freitas et al. 1990, Klein et al. 1991a, Narang et al. 1993). Members of this complex have been incriminated as *Plasmodium* vectors in some areas of South America (Arruda et al. 1986; Deane 1988; Oliveira-Ferreira et al. 1990; Klein et al. 1991b, c, d; Branquinho et al. 1993; Póvoa et al. 2001; Conn et al. 2002). The *Albitarsis* complex is distributed over a large area extending from northern Guatemala to northern Argentina (Rosa-Freitas et al. 1990), and its members may be endophilic or exophilic, depending on their region of occurrence (Deane et al. 1988, Rosa-Freitas et al. 1990). There is also geographical variation in morphological characteristics that are important in the taxonomy of the group (Rosa-Freitas et al. 1990). These behavioral and morphological variations cause difficulties in studies of the vectorial ca-

capacity of the different species of the complex (Deane et al. 1988, Rosa-Freitas et al. 1990; Klein et al. 1991a).

Studies using random amplified polymorphic DNA markers performed by Wilkerson et al. (1995a, b) suggest the existence of four species in this complex: species A, *Anopheles albitarsis* s.s.; species B, not yet described; species C, *Anopheles marajoara* Galvão & Damasceno; and species D, corresponding to *Anopheles deaneorum* Rosa-Freitas. *An. albitarsis* s.s. has been found in Paraguay, southern Brazil, and northern Argentina. The other three species are found in sympatry with *An. albitarsis* s.s. in some regions, but their detailed geographic distributions are unknown (Wilkerson et al. 1995a, b).

Hybridization experiments between species B and *An. deaneorum* from Costa Marques, Rondônia, Brazil (Klein et al. 1991a), indicated that male hybrids between these two species are sterile. In this article, we studied the reproductive isolation between *An. deaneorum* and another member of the complex, *An. albitarsis* s.s.

Materials and Methods

Hybridization experiments were carried out using an induced mating technique (Ow-Yang et al. 1963). The F1 and F2 progeny of *An. deaneorum* females collected in the city of Costa Marques (12° 26' S, 64° 14' W), Rondônia State, Brazil, and the colony of *An. albitarsis* s.s. established from females collected in

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Table 1. Larva-to-adult viability in hybrids of *An. albitarsis* s.s. and *An. deaneorum*

| Genotype | No. initial first instars | Pupae | Adults | Males | Females | Males/females |
|----------------------------|---------------------------|--------|--------|-------|---------|---------------|
| <i>An. albitarsis</i> s.s. | 480 | 227 | 213 | 105 | 108 | 0.972 |
| <i>An. deaneorum</i> | 480 | 203 | 201** | 114 | 87 | 1.310 |
| Hybrid A ^a | 480 | 201 | 114*** | 25 | 89 | 0.281*** |
| Hybrid B ^b | 480 | 286*** | 274 | 151 | 123 | 1.228 |

** $P < 0.01$, *** $P < 0.001$.

^a Hybrid A, F1 of ♀ *An. deaneorum* × ♂ *An. albitarsis* s.s.

^b Hybrid B, F1 of ♀ *An. albitarsis* s.s. × ♂ *An. deaneorum*.

the municipality of Massaranduba (26° 35' S, 48° 58' W), Santa Catarina State (Horosko et al. 1997), were used in the crosses, along with their reciprocal hybrids.

The mosquitoes were reared as described in Horosko et al. (1997) with slight modifications. Wild-caught blood-fed *Anopheles* females from Costa Marques were placed in screen-topped 500-ml cartons and transferred to the laboratory in Rio de Janeiro. On day 3 after the blood meal, gravid females were anesthetized with ethyl acetate and the species identified using a taxonomic key (Faran and Linthicum 1981) and diagnostic characters that allow the correct identification of adult *An. deaneorum* (Rosa-Freitas 1989, Klein et al. 1990). To obtain eggs, the *An. deaneorum* females were transferred to 500-ml cylindrical screened plastic containers (9 cm in width), which were subsequently placed into a white plastic basin (15 by 8 by 4 cm) containing 100 ml of dechlorinated water. Two days after oviposition the female and the cylindrical screened containers were removed. Larvae were fed twice a day with powdered fish food (Tetramin, Tetra Werke, Melle, Germany). On day 4 after oviposition, larvae were separated into groups of 100 and placed in a 18-cm-diameter by 8-cm-deep white plastic basin, with 150 ml of dechlorinated water, where they were reared until pupation. The same procedure was used to rear larvae of the *An. albitarsis* s.s. laboratory colony to be used in the initial crosses.

Pupae were transferred daily to 50-ml plastic cups and placed in screen-topped carton cages (18 cm in diameter by 30 cm in depth). The newly eclosed unmated females were transferred twice a day to a screen-topped 500-ml carton cage and blood fed. Intra- and interspecific crosses were carried out between *An. albitarsis* s.s. and *An. deaneorum* in a room with a temperature range between 22 and 25°C by using an induced-mating technique (Ow-Yang et al. 1963). Mated females were placed into a screened 500-ml carton and provided a cotton plug wetted with a 10% sucrose solution. To induce oviposition, on the third day after mating, one wing was removed from each female, and they were placed individually in 50-ml plastic cups (Lanzaro et al. 1988). After oviposition, the eggs were counted, each female was dissected, and the spermathecae examined for sperm by light microscopy. Because uniseminated females sometimes also lay eggs, hatching was followed for each individual female.

Eggs and larvae from individual females were counted and transferred to rearing pans as described above until they pupated. Pupae from each cross were removed and counted daily and placed in screen-topped carton cages (18 cm in diameter by 30 cm in depth). Newly emerged F1 hybrids were then used in backcrosses with the parental species.

To verify whether there were any differences in viability, the development of 480 first instars from ≈35 females of each of the two species and their hybrids was followed until the adult stage under constant temperature (≈28°C) and with the same amount of food. The number of living larvae and pupae was counted every 2 d. Pupae that died before adult emergence were examined to determine their sex. Newly emerged adult males also were dissected to examine the morphology of their reproductive organs by light microscopy. After dissection, samples were stained with a 1:1 mixture of lactophenol and Diff-Quick solution I (DADE, Düringen, Switzerland) for 1–2 min and mounted in euparal (BioQuip, Santa Monica, CA).

Results

Table 1 shows the results of experiments examining the larva-to-adult viability of *An. albitarsis* s.s., *An. deaneorum*, and their reciprocal hybrids. The observed numbers of surviving pupae and adults of *An. deaneorum* and *An. albitarsis* s.s. × *An. deaneorum* hybrids were compared with *An. albitarsis* s.s., treated here as a control because it is an established colony and is assumed to be adapted to laboratory conditions.

A highly significant ($\chi^2 = 14.573$, $df = 1$, $P < 0.001$) excess of hybrid B (F1 of ♀ *An. albitarsis* s.s. × ♂ *An. deaneorum*) pupae was observed. However, no significant difference was observed in the case of hybrid A (F1 of ♀ *An. deaneorum* × ♂ *An. albitarsis* s.s.) at this developmental stage. Moreover, hybrid A also showed a highly significant reduction, compared with *An. albitarsis* s.s., in the pupae-adult viability. Analysis of the dead pupae indicates that this mortality is mainly due to males failing to emerge from pupae. As a result, hybrid A is the only genotype showing a significant difference in the relative numbers of males and females (Table 1). A significant pupae-adult viability difference also was observed for *An. deaneorum* compared with *An. albitarsis* s.s.

Table 2 shows both the number of forced copulations performed and the number of inseminated fe-

Table 2. Insemination rates in crosses between *An. albitalarsis* s.s., *An. deaneorum*, and their F1 hybrids (percentages in parentheses)

| Cross | No. copulas | No. females inseminated ^a |
|---|-------------|--------------------------------------|
| ♀ <i>An. albitalarsis</i> s.s. × ♂ <i>An. albitalarsis</i> s.s. | 105 | 76 (72) |
| ♀ <i>An. deaneorum</i> × ♂ <i>An. deaneorum</i> | 101 | 71 (70) |
| ♀ <i>An. deaneorum</i> × ♂ <i>An. albitalarsis</i> s.s. | 74 | 53 (72) |
| ♀ <i>An. albitalarsis</i> s.s. × ♂ <i>An. deaneorum</i> | 55 | 27 (49) |
| ♀ <i>An. albitalarsis</i> s.s. × ♂ hybrid A ^b | 31 | 2 (6) |
| ♀ <i>An. deaneorum</i> × ♂ hybrid A | 34 | 0 (0) |
| ♀ <i>An. albitalarsis</i> s.s. × ♂ hybrid B ^c | 53 | 13 (25) |
| ♀ <i>An. deaneorum</i> × ♂ hybrid B | 56 | 25 (45) |
| ♀ hybrid A × ♂ <i>An. albitalarsis</i> s.s. | 58 | 31 (53) |
| ♀ hybrid A × ♂ <i>An. deaneorum</i> | 50 | 17 (34) |
| ♀ hybrid B × ♂ <i>An. albitalarsis</i> s.s. | 54 | 40 (74) |
| ♀ hybrid B × ♂ <i>An. deaneorum</i> | 48 | 19 (40) |

^a Number of inseminated females is equal to the number of females with positive spermathecae plus those with negative spermathecae but with larvae (see *Materials and Methods*).

^b Hybrid A, F1 of ♀ *An. deaneorum* × ♂ *An. albitalarsis* s.s.

^c Hybrid B, F1 of ♀ *An. albitalarsis* s.s. × ♂ *An. deaneorum*.

males obtained in crosses between *An. albitalarsis* s.s., *An. deaneorum*, and their F1 hybrids. The highest frequencies of inseminated females are all ≈70% and include the intraspecific crosses (♀ *An. albitalarsis* s.s. × ♂ *An. albitalarsis* s.s. and ♀ *An. deaneorum* × ♂ *An. deaneorum*) and two of the three crosses involving *An. albitalarsis* s.s. males (♀ *An. deaneorum* × ♂ *An. albitalarsis* s.s. and ♀ hybrid B × ♂ *An. albitalarsis* s.s.). In the other crosses, the percentages ranged between 0 and 53%.

Based on the data of Table 2, comparisons were made between the insemination rates of the different

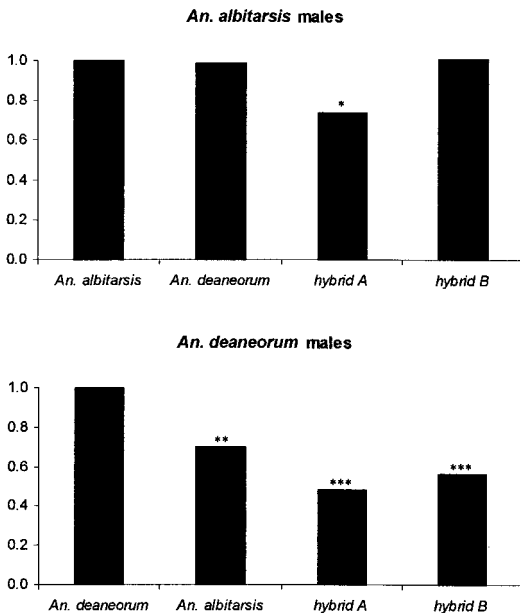


Fig. 1. Normalized insemination rates in crosses between females of different genotypes and *An. albitalarsis* s.s. (top) or *An. deaneorum* (bottom) males. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

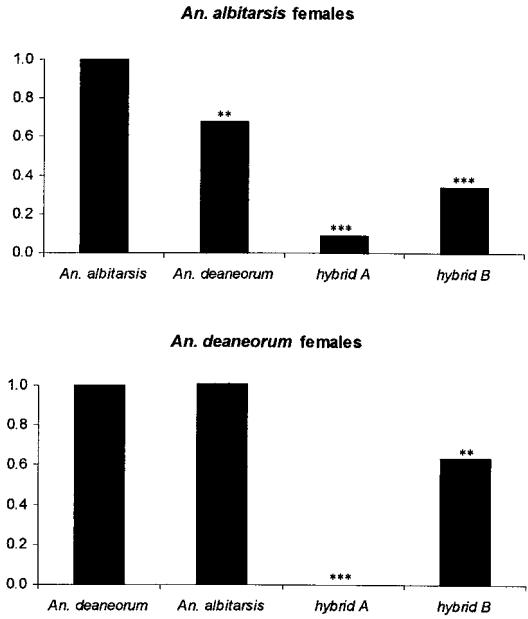


Fig. 2. Normalized insemination rates in crosses between males of different genotypes and *An. albitalarsis* s.s. (top) or *An. deaneorum* (bottom) females. ***P* < 0.01; ****P* < 0.001.

crosses. In each case, the level of insemination of the different crosses was compared (by χ^2 analysis) to the relevant conspecific cross, which was treated as a control (either ♀ *An. albitalarsis* s.s. × ♂ *An. albitalarsis* s.s. or ♀ *An. deaneorum* × ♂ *An. deaneorum*; see below). Figure 1 shows the normalized (by the respective control) insemination rates in crosses of females of different genotypes and *An. albitalarsis* s.s. males (top) or *An. deaneorum* males (bottom). Note that although *An. albitalarsis* s.s. male crosses with *An. deaneorum* females show an insemination rate similar to the control (♀ *An. albitalarsis* s.s. × ♂ *An. albitalarsis* s.s.), in the reciprocal cross (♀ *An. albitalarsis* s.s. × ♂ *An. deaneorum*), the rate is significantly lower than the respective control (♀ *An. deaneorum* × ♂ *An. deaneorum*). Moreover, hybrid females crossed with *An. deaneorum* males also show a lower rate. A significant reduction in the insemination rate was observed for hybrid A females, but not for hybrid B females, when they were crossed to *An. albitalarsis* s.s. males (Fig. 1, top). In fact, the insemination rates in the two types of hybrid females are significantly different ($\chi^2 = 5.126$, *df* = 1, *P* < 0.05) despite the fact that they are expected to be very similar genetically, carrying the same chromosome complement. A similar trend is seen in crosses with *An. deaneorum* males, but in that case the insemination rate difference between the two hybrid females is not significant. Note that in the majority of cases, viable larvae resulted from the inseminated hybrid females (46 of 48 for hybrid A and 48 of 59 for hybrid B), showing that they are mostly fertile.

Figure 2 shows the normalized insemination rates in crosses involving males of different genotypes and

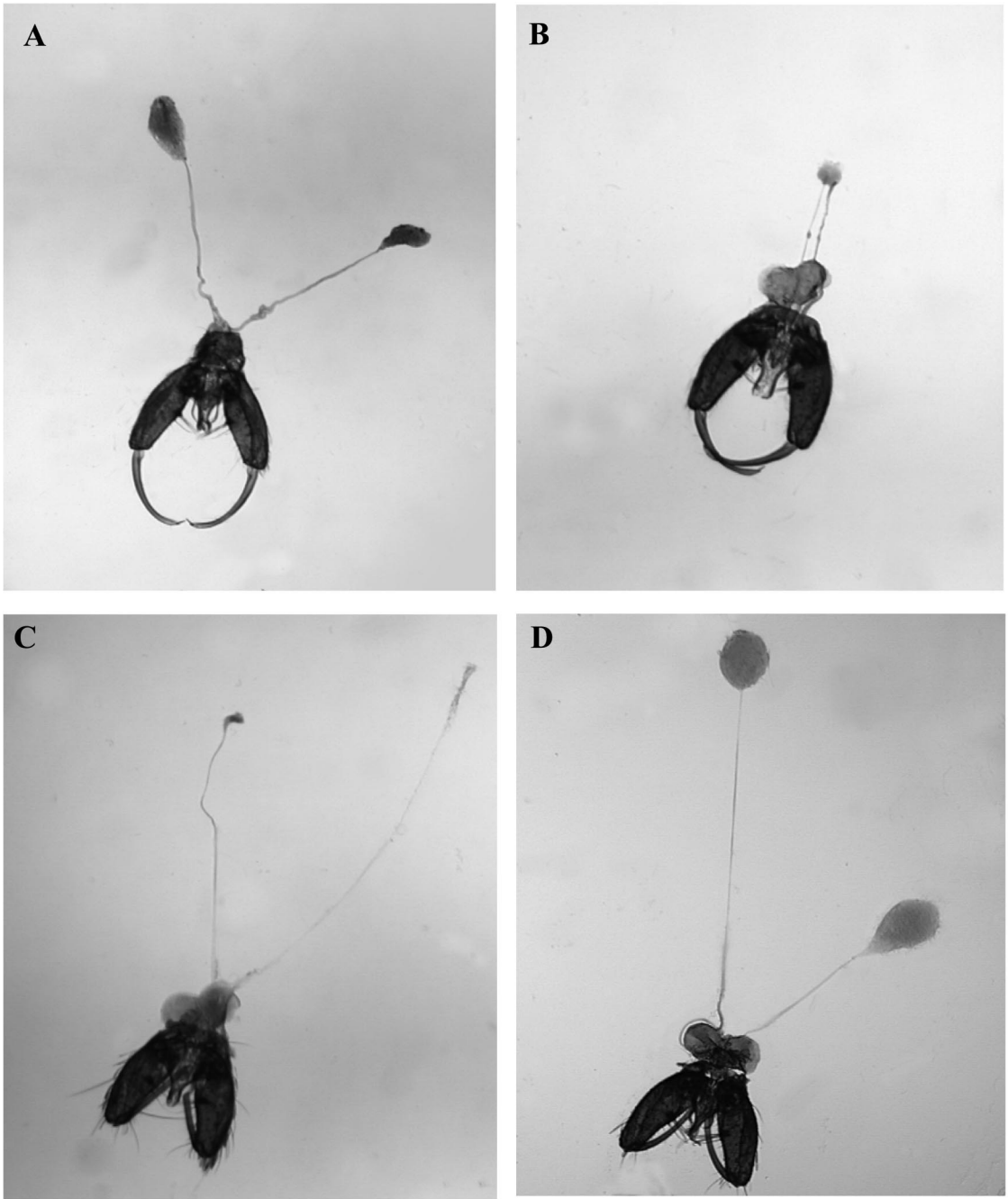


Fig. 3. External genital terminalia and complete male reproductive system of *An. albitarsis* s.s. (A), hybrid A (B and C) and hybrid B (D). In A–D, the male terminalia is at the bottom and the testes are to the top. In some cases, the accessory glands can be observed, adjacent to the terminalia. Note the reduced testis size in hybrids A, compared with *An. albitarsis*. In opposition, testes of hybrid B show an increased volume. Variations in the size of the vas deferens also are observed in the hybrids (A–D are same scale).

An. albitarsis s.s. females (top) or *An. deaneorum* females (bottom). As before, in each case the level of insemination of the different crosses was compared with the conspecific crosses, which were treated as controls ($\text{♀ } An. albitarsis \text{ s.s.} \times \text{♂ } An. albitarsis \text{ s.s.}$ or

$\text{♀ } An. deaneorum \times \text{♂ } An. deaneorum$). Hybrid males show a significantly reduced level of successful insemination of both *An. albitarsis* s.s. and *An. deaneorum* females. In both cases, hybrid A males (carrying an *An. deaneorum* X chromosome) are significantly

less successful than hybrid B males ($\chi^2 = 4.357$, $df = 1$, $P < 0.05$ for ♀ *An. albitarsis* s.s. crosses; $\chi^2 = 21.016$, $df = 1$, $P < 0.001$ for ♀ *An. deaneorum* crosses). These results suggest that *An. albitarsis* s.s. × *An. deaneorum* male hybrids are mostly infertile. To examine this further, the testis and associated organs of *An. albitarsis* s.s., *An. deaneorum* and its reciprocal hybrids were dissected.

Figure 3 shows that the male reproductive organs of hybrids A and B have abnormalities compared with *An. albitarsis* s.s. (and *An. deaneorum*; not shown in figure). Hybrid A males show reduced testis, whereas hybrid B males show very thin vas deferens (see legend for details). These malformations could be responsible for at least part of the reduced insemination rates observed for the hybrid males. However, it should be added that in the majority of cases where insemination did occur (two of two for hybrid A and 36 of 38 for hybrid B) larvae were observed, indicating that sterility is not complete.

Discussion

The existence of species complexes among vectors of malaria parasites has important epidemiological consequences. For example, studies carried out in Costa Marques, Rondônia State, Brazil, showed that *An. deaneorum* is an anthropophilic and endophilic species actively invading houses to bite humans, whereas *An. albitarsis* species B is more zoophilic (Klein et al. 1991b). In addition, laboratory infection studies indicated that although species B (Wilkerson et al. 1995a) is rarely infected by *Plasmodium falciparum*, *An. deaneorum* is very susceptible to infection by both *P. vivax* and *P. falciparum* (Klein et al. 1991c, d), and it might be a very important seasonal vector reaching high densities at the end of the rainy season (Klein and Lima 1990).

Our results confirm that *An. albitarsis* s.s. and *An. deaneorum* are distinct species (Rosa-Freitas 1989) with a high degree of postzygotic reproductive isolation between them. Hybrid males show very low insemination rates due to incomplete sterility probably caused at least in part by the observed abnormalities in their reproductive organs, whereas the insemination rates of hybrid females are higher and in one case completely normal. These results are consistent with Haldane's rule (Orr 1997), which states that in interspecific crosses the heterogametic sex will show sterility or viability problems before the homogametic sex. The same pattern also is observed in crosses between a number of other *Anopheles* species (reviewed by Presgraves and Orr 1998).

The two types of hybrid males show different levels of sterility. Hybrid males carrying an X chromosome derived from *An. deaneorum* show much lower insemination rates than hybrid males carrying an *An. albitarsis* s.s. X chromosome. In addition, the former also shows a reduced pupa to adult viability. This asymmetry in the hybrid sterility and viability effects is often observed in interspecific crosses of very closely related species (Coyne and Orr 1997).

The difference in the insemination rates observed between the two types of hybrid females when crossed to *An. albitarsis* s.s. males is hard to explain but might suggest a significant maternal effect. However, the fact the *An. deaneorum* males are less successful inseminating *An. albitarsis* s.s. females than their conspecific females, whereas no significant difference was observed in the reciprocal cross, suggests that in areas where both species occur in sympatry, asymmetrical introgression might occur. This might be especially true for X-linked loci due to the differential sterility observed between the two hybrid males. Studies of natural populations by using molecular markers might determine whether this is the case.

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References Cited

- Arruda, M., M. B. Carvalho, R. S. Nussenweig, M. Maracic, A. W. Ferreira, and A. H. Cochrane. 1986. Potential vector of malaria and their different susceptibility to *Plasmodium falciparum* and *Plasmodium vivax* in northern Brazil identified by immunoassay. *Am. J. Trop. Med. Hyg.* 60: 1010–1018.
- Branquinho, M. S., C.B.T. Lagos, R. M. Rocha, D. Natal, J.M.S. Barata, A. H. Cochrane, E. Nardin, R. S. Nussenzweig, and J. K. Kloetzel. 1993. Anophelines in the state of Acre, Brazil, infected with *Plasmodium falciparum*, *P. vivax*, the variant *P. vivax* VK247 and *P. malariae*. *Trans. R. Soc. Trop. Med. Hyg.* 87: 391–394.
- Conn, J. E., R. C. Wilkerson, M. N. Segura, R. T. De-Souza, C. D. Schlichting, R. A. Wirtz, and M. M. Póvoa. 2002. Emergence of a new Neotropical malaria vector facilitated by human migration and changes in land use. *Am. J. Trop. Med. Hyg.* 66: 18–22.
- Coyne, J. A., and H. A. Orr. 1997. Patterns of speciation revisited. *Evolution* 51: 295–303.
- Deane, L. M. 1988. Malaria studies and control in Brazil. *Am. J. Trop. Med. Hyg.* 38: 223–230.
- Deane, L. M., C. D. Ribeiro, R. Lourenço-de-Oliveira, J. Oliveira-Ferreira, and A. E. Guimarães. 1988. Study on the natural history of malaria in areas of the Rondônia State—Brazil and problems related to its control. *Rev. Inst. Med. Trop. SP* 30: 153–156.
- Faran, M. E., and K. J. Linthicum. 1981. A handbook of the Amazon species of *Anopheles* (*Nyssorhynchus*) (Diptera: Culicidae). *Mosq. System* 13: 1–81.
- Horosko, S., J.B.P. Lima, and M. B. Brandolini. 1997. Establishment of a free-mating colony of *Anopheles albitarsis* from Brazil. *J. Am. Mosq. Control Assoc.* 13: 95–96.
- Klein, T. A., and J.B.P. Lima. 1990. Seasonal distribution and biting patterns of *Anopheles* mosquitoes in Costa Marques, Rondônia, Brazil. *J. Am. Mosq. Control Assoc.* 6: 700–707.
- Klein, T. A., J. B. Lima, and A. Toda-Tang. 1990. Colonization and maintenance of *Anopheles deaneorum* in Brazil. *J. Am. Mosq. Control Assoc.* 6: 510–513.

- Klein, T. A., J.B.P. Lima, and A. Toda-Tang. 1991a. Hybridization evidence supporting separate species status for *Anopheles albitarsis* and *Anopheles deaneorum* (Diptera: Culicidae) in Brazil. *J. Am. Mosq. Control Assoc.* 7: 301–303.
- Klein, T. A., J.B.P. Lima, and A. Toda-Tang. 1991b. Biting behavior of *Anopheles* mosquitoes in Costa Marques, Rondônia, Brazil. *Rev. Soc. Bras. Med. Trop.* 24: 13–20.
- Klein, T. A., J.B.P. Lima, M. S. Tada, and R. Miller. 1991c. Comparative susceptibility of *Anopheles* mosquitoes in Rondônia, Brazil, to infection by *Plasmodium vivax*. *Am. J. Trop. Med. Hyg.* 45: 463–470.
- Klein, T. A., J.B.P. Lima, M. S. Tada, and R. Miller. 1991d. Comparative susceptibility of anopheline mosquitoes to *Plasmodium falciparum* in Rondônia, Brazil. *Am. J. Trop. Med. Hyg.* 44: 598–603.
- Kreutzer, R. D., J. B. Kitzmiller, and M. G. Rabbani. 1976. Cytogenetically distinguishable sympatric and allopatric populations of mosquito *Anopheles albitarsis*. *Acta Amazonica* 6: 473–481.
- Krzywinski, J., and N. J. Besansky. 2003. Molecular systematics of *Anopheles*: from subgenera to subpopulations. *Annu. Rev. Entomol.* 48: 111–139.
- Lanzaro, G. C., S. K. Narang, S. E. Mitchell, P. E. Kaiser, and J. A. Seawright. 1988. Hybrid male sterility in crosses between field and laboratory strains of *Anopheles quadrimaculatus* Say (Diptera: Culicidae). *J. Med. Entomol.* 25: 248–255.
- Narang, S. K., T. A. Klein, O. P. Pereira, J.B.P. Lima, and A. Toda-Tang. 1993. Genetic evidence for the existence of cryptic species in the *Anopheles albitarsis* complex in Brazil: allozymes and mitochondrial DNA restriction fragment length polymorphism. *Biochem. Genet.* 31: 97–112.
- Oliveira-Ferreira, J., R. Lourenço-de-Oliveira, A. Teva, L. M. Deane, and C. T. Daniel-Ribeiro. 1990. Natural malaria infections in *Anopheles* in Rondônia State, Brazilian Amazon. *Am. J. Trop. Med. Hyg.* 43: 6–10.
- Orr, H. A. 1997. Haldane's rule. *Annu. Rev. Ecol. Syst.* 28: 195–218.
- Ow-Yang, C. F., F. L. Sta Maria, and R. H. Wharton. 1963. Maintenance of a laboratory colony of *Anopheles maculatus* Theobald by artificial mating. *Mosq. News.* 23: 34–35.
- Póvoa, M. M., R. A. Wirtz, R.N.L. Lacerda, M. A. Miles, and D. Warhurst. 2001. Malaria vectors in the municipality of Serra do Navio, State of Amapá, Amazon Region, Brazil. *Mem. Inst. Oswaldo Cruz* 96: 179–184.
- Presgraves, D. C., and H. A. Orr. 1998. Haldane's rule in taxa lacking a hemizygous X. *Science (Wash. DC)* 282: 952–954.
- Rosa-Freitas, M. G. 1989. *Anopheles (Nyssorhynchus) deaneorum*: a new species in the *Albitarsis* complex (Diptera: Culicidae). *Mem. Inst. Oswaldo Cruz* 84: 535–543.
- Rosa-Freitas, M. G., L. M. Deane, and H. Momen. 1990. A morphological, isoenzymatic and behavioural study of ten populations of *Anopheles (Nyssorhynchus) albitarsis* Lynch-Arribalzaga, 1878 (Diptera: Culicidae) including from the Type-locality –Baradero, Argentina. *Mem. Inst. Oswaldo Cruz* 85: 275–289.
- [WHO] World Health Organization. 2001. Malaria. World Health Organization. (<http://www.who.int/tdr/diseases/malaria/files/malaria-poster.pdf>).
- [WHO] World Health Organization. 2003. The transformation of a mosquito, Tropical Diseases Research, World Health Organization. (<http://www.who.int/tdr/diseases/malaria/default.html>).
- Wilkerson, R. C., T. V. Garffigan, and J.B.P. Lima. 1995a. Identification of species related to *Anopheles (Nyssorhynchus) albitarsis* by random amplified polymorphic DNA-polymerase chain reaction (Diptera: Culicidae). *Mem. Inst. Oswaldo Cruz* 90: 721–732.
- Wilkerson, R. C., T. J. Parsons, T. A. Klein, T. V. Gaffigan, E. Bergo, and J. Consolim. 1995b. Diagnosis by random amplified polymorphic DNA polymerase chain reaction of four cryptic species related to *Anopheles (Nyssorhynchus) albitarsis* (Diptera: Culicidae) from Paraguay, Argentina, and Brazil. *J. Med. Entomol.* 32: 697–704.

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