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Biological aspects of immature stage of *Nyssomyia whitmani* (Antunes and Coutinho) (Diptera, Psychodidae, Phlebotominae) in laboratory conditions

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ABSTRACT

Nyssomyia whitmani (Antunes and Coutinho, 1939) has been considered as a complex of cryptic species, and some of the populations of this complex plays an important role in the transmission of *Leishmania* spp. in Brazil. The present study reports the biological aspects concerning the productivity out of eggs and the development time of the descendants of females obtained in Dourados municipality, Mato Grosso do Sul state. The females were captured with modified electric aspirators, fed in hamsters and further individualized in containers for breeding. At the insectary, temperature and relative humidity were maintained on average of 24.5 °C and 67.3%, respectively. From 944 females 3737 eggs were obtained, 748 (20.0%) evolved to the stage of larvae, and 93 (12.4%) of these reached adult stage. The life cycle lasted 80.6 days and the last larval instar was the longest. The use of a higher protein diet revealed a significant improvement in larval development.

Introduction

Nyssomyia whitmani is recorded in 720 Brazilian municipalities and it is distributed over 25 states, including the Federal District (Queiroz et al., 1994; Membrive et al., 2004; Teodoro et al., 2006; Galati, 2014). In Mato Grosso do Sul this species has been recorded in many municipalities (Galati et al., 1996, 2006; Oliveira et al., 2003, 2006; Braga-Miranda et al., 2006; Nascimento et al., 2007; Nunes et al., 2008; Almeida et al., 2010).

The species was found by visualization of flagellate forms or detection of *Leishmania* (*V.*) *braziliensis* DNA in some states as Ceará, Maranhão, São Paulo, Paraná and Mato Grosso do Sul (Pessoa and Coutinho, 1941; Azevedo et al., 1990; Queiroz et al., 1994; Luz et al., 2000; Oliveira-Pereira et al., 2006; Paiva et al., 2010) and therefore, it was implicated as a probable vector of the parasite at the studied sites. The first information about the species life cycle *Nyssomyia whitmani* was obtained by Barretto (1942) from specimens collected from the southeast region of Brazil. However, more research about population dynamics of this sandfly species and its immature stages of biology are still needed to understand the insect's pattern of abundance and its relationship with the environment.

In spite of the few published studies about their immature forms (larvae and pupae) and the duration of these stages, this study aimed to obtain information on oviposition and the life cycle of *Ny. whitmani*, which comprises the stages of egg, larvae and pupae, in order to assist the understanding of this species ecology.

Materials and methods

The captures were held between 2012 and 2013, from 18:00 h to 21:00 h, using an electric aspirator attached to a 6 V battery in a chicken coop located at the Fazenda Coqueiro farm, in the municipality of Dourados, Mato Grosso do Sul (MS), Highway MS162, Km 10 Dourados-Ithaum (22°12′ S and 54°54′ W). The specimens were transported to the Laboratório de Parasitologia Humana da Universidade Federal do Mato Grosso do Sul (UFMS), located at Campo Grande/MS, according to the methodology of Rangel et al. (1985, 1986).

Apple slices were placed inside the cage to feed the males and females until their arrival at the laboratory. In the evening, a hamster (*Mesocricetus auratus*) previously anesthetized with an intramuscular injection (ketamin 75 mg/kg and xylazine 10 mg/kg

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Table 1
Analysis of collected females, total of females that laid eggs, total eggs laid, total hatched larvae, total emerged adults and average temperature (°C) and relative humidity (RH%) during the eight collection periods between 2012 and 2013.

Collection month/year (2012–2013)	Females	Females that laid eggs (%)	Number of eggs (eggs/females)	Total of larvae (%)	Total of pupae	Adults (%)	Temperature (°C) and humidity (UR%)
Collection 1	215	13(6.0)	390(30.0)	52(13.3)	–	–	24.7°/63.9%
Collection 2	151	8(5.3)	324(40.5)	104(32.1)	–	–	25.6°/65.7%
Collection 3	187	18(9.6)	448(25.0)	102(22.7)	–	–	25.4°/64.0%
Collection 4	10	4(40.0)	84(21.0)	6(7.1)	–	–	24.2°/63.0%
Collection 5	90	6(6.7)	86(14.3)	–	–	–	23.9°/68.3%
Collection 6[a]	5	5(100.0)	88(17.6)	41(46.6)	13(31.7)	11(84.6)	23.7°/71.7%
Collection 7	148	78(52.7)	1640(21.0)	431(26.3)	149(34.5)	136(91.2)	24.1°/69.3%
Collection 8	138	33(23.9)	677(20.5)	18(2.7)	3(16.6)	2(66.6)	24.6°/72.7%
Total	944	165(17.48)	3737(22.6)	748(20.02)	165(22.0)	149(90.3)	24.5°/67.3%

[a] Beginning of a new larval diet.

IM in 2:1 rate) was placed inside the cage, in order that the females could blood feed on it during an average time of 40 min. After 48 h, the females were individualized in a 0.5 cm plaster-lined plastic container with 2.5 cm diameter and 3.5 cm height, which were capped with voile fabric. A piece of cotton soaked in undiluted honey was placed on top of the voile fabric, as a source of carbohydrate for the females (Brazil and Brazil, 2003).

The containers were kept in covered plastic boxes (34.0 cm × 24.5 cm × 9.0 cm) with filter paper in the base, or in foam boxes (31.0 cm × 20.0 cm × 22.0 cm) with plaster on the walls and the base, in order to conserve moisture and temperature of the microenvironment. These parameters were controlled with a digital thermo-hygrometer, which maintained an average temperature of 24.5 °C and air relative humidity of 67.3%. After death, females were dissected for species identification, through the observation of spermathecae morphology and identified according Galati (2003). Once the females of *Ny. whitmani* were identified, their eggs were grouped, counted and transferred with fine-tipped brushes to a 0.5 cm plaster-lined Petri dishes (8.5 diameter and 1.5 cm height) according Rangel et al. (1985). After transferring the eggs, soil collected from the hen house where the insects were once collected was added. After the larvae hatched, lyophilized liver (OXOID – dehydrated liver) was introduced in their diet. Daily observations were made until the emergence of the winged adults.

The larvae at all instars (L1, L2, L3 and L4) and the pupae were counted. Larvae hatching and ecdysis were recorded to verify the species biological cycle. To estimate the duration of *Ny. whitmani* life cycle, including incubation period, duration of larval instars and pupal period until adult emergence, the minimum duration (minimum time, in consecutive days, between the observation of the first individual of larval period until the observation of the first individual of the second instar and so on); and the maximum duration (maximum time, in consecutive days, between the observation of the first individual of the instar until the observation of the last individual of the same instar), and median were calculated.

Results

From the 944 individualized females, 3737 eggs were obtained from the oviposition of 165 females. The minimum and maximum times of development of the immature forms were 54.3 and 106.9 days, respectively.

From the total of females fed, 17.48% laid eggs, with the lowest percentage in the second collection (5.3%) and the highest in the 60 (100%), followed by seventh collection (52.7%). The average number of eggs per female was 6.1 (Table 1). The eggs were elliptical, presented dark tonality and were laid singly or in small clusters. The mean incubation period of the eggs was 9.5 days and its median was 12 days. At hatching, first instar larvae (L1) presented a single pair of caudal setae and whitish bodies and heads, with a cephalic portion that became darkened after a few hours. This phase lasted a minimum of 7.0 and the maximum of 11.2 days, with a median of 5 days (Table 2).

The L2 larvae presented two pairs of caudal setae and fed on the hen house soil set in the containers with more avidity when compared to L1 larvae. The maximum and minimum times were 4.9 and 9.4 days, respectively (median of 7 days) (Table 2).

It was possible to check with the naked eye that the third instar larvae was a few times larger than the second and maintained two pairs of caudal setae. This instar lasted a minimum of 6.1 days and maximum of 18.0 days, with a median of 10 days (Table 2).

The fourth and last larval instar (L4) was characterized by the presence of a spot in the last tergite. It was the longest period among the immature forms with a median of 21 days (range 16.1–29.8), and after this time, the larvae had their last ecdysis. It was observed that in this period the larvae reduced its feeding and locomotion. At the beginning of the pupal stage, the pupae presented yellowish and light brown eyes, which changed to a blackened shade as the end of the phase approached and remained until they reach adult form. This period showed a range between a minimum of 10.7 days and a maximum of 22.7 days, with a median of 16.7 days. The biological cycle presented a median of 80.6 days (Table 2). There was no difference between emerged males and females.

Discussion

Barretto (1941, 1942) reported lower values for the life cycle when studying populations of *Ny. whitmani* from Southeast of Brazil, with averages of 50.5 days. When studying the biology of *Nyssomyia intermedia* and *Ny. whitmani*, Barretto (1941, 1942) verified the rapid and gradual development of eggs when temperature was set between 25° and 27 °C. Rangel et al. (1985), reported a range of 26–56 days (average 41 days) when monitoring the life cycle of *Ny. intermedia* at 25 °C and 86% humidity. Andrade-Filho et al. (2004) noted a life cycle of 34.9 days at temperatures ranging between 25 °C and 26 °C and 80% humidity, when studying this last species.

Table 2
Duration (days) of egg, larval and pupal development of *Ny. whitmani* under laboratory conditions at temperature of 24.5 °C (±1 °C) and RH ~67% over the eight collection periods between 2012 and 2013.

Instar	Minimum (days)	Maximum (days)	Median (days)
Egg	9.5	15.9	12.0
Larva 1	7.0	11.2	5.0
Larva 2	4.9	9.4	7.0
Larva 3	6.1	18.0	10.0
Larva 4	16.1	29.8	21.0
Pupa	10.7	22.7	16.7
Total	54.3	106.9	80.6

Brazil et al. (1997) studied the biology of *Evandromyia lenti* and found that its life cycle lasted for 40.2 days at temperatures ranging between 26 °C and 28 °C and relative humidity of 80%. When comparing the cycle of this species with *Lu. longipalpis* and *Ny. intermedia*, the previous authors verified that *Ev. lenti* has the longest cycle, followed by *Ny. intermedia* (32.8 days) and *Lu. longipalpis* (29.5 days). *Evandromyia carmelinoi*, species colonized by Alves et al. (2000), showed an average time of development, from egg to adult form, of 42 days, at average temperature of 25–26 °C and humidity of 80%. Guzmán and Tesh (2000) when observing development time in relation to temperature for the species *Phlebotomus papatasi*, *Ph. perniciosus* and *Lutzomyia longipalpis*, noted that most of the larvae of the last species died at temperatures under 18 °C; which is the opposite of what happened with *Ph. papatasi* and *Ph. perniciosus*. These two species had their cycles extended (150 and 412 days, respectively) at lower temperatures, but had it substantially shortened when the temperature was elevated to 28 °C.

In the present study, temperature remained between 23 °C and 25 °C and the average humidity did not exceed 65% in the first five collections. During the last months, humidity was above 70% and temperature was between 23 °C and 24 °C, which was a better period for larval development and adult emergence. Therefore, it is possible that temperature and humidity may have influenced in the development time of immature stages, and may even have started the process of diapause.

Barretto (1942) studied the biological cycle of *Ny. whitmani* with specimens collected in the southeast region. He observed the occurrence of diapause among the larvae, because the life cycle extended until 176 days. However, Brazil (personal communication) worked with the same species, with individuals originated from Ceará, and reported 37 days life cycle, with no observation of the diapause phenomenon.

Ready and Croset (1980) studied *Ph. perniciosus* and *Phlebotomus ariasi* and noted that environmental stimuli can induce diapause, especially for third and fourth larval instars at temperatures below 21 °C. According to Chaniotis (1967), the diapause is an adaptation period to environmental conditions. Carvalho et al. (2011) suggested that diapause could be an evolutionary mechanism of the sandfly to survive low temperatures and adverse conditions, resuming metabolic activities when conditions become favorable.

Other major factor for the larvae to reach the adult stage is the feeding. In the present study, a mix of rabbit feces and fish food (1:1) was initially used, but it resulted in proliferation of a large amount of fungi and considerable mortality, especially among the first instar larvae. Therefore, an alternative diet consisting of lyophilized liver and soil was tested and it enhanced larval development and the emergence of the winged forms. The addition of soil from the capture site was tried because it was believed that besides stimulating the existing memory (Kelly and Dye, 1997). This material could contain organic matter of high nutritional value, due to the presence of chicken feces, and it could work as a food source for the immature forms (Forattini et al., 1976).

When Rangel et al. (1985) studied the establishment of a colony of *Ny. intermedia*, they used commercial fish food and achieved a ratio of 46.6% adults regarding the number of hatched larvae. Rangel et al. (1986) accompanied the development of *Ny. intermedia* and *Lu. longipalpis* larvae fed with food of animal and vegetal origin. The commercial fish food was well accepted by both species and did not favor fungi development. Gemetchu (1971) studied the larval diet of *Phlebotomus longipes* and observed their preference for lyophilized liver rather than rabbit feces, meat or blood. Guzmán and Tesh (2000) analyzed the effect of two different diets on *Lu. longipalpis* (feces mixed with liver powder and decomposing leaves) and observed that the larvae fed with feces and liver powder had a faster and synchronous development than those fed with leaves. This results supports the findings of Chaniotis (1975), who fed *Lutzomyia trapidoi* larvae with lyophilized liver and noted the same behavior in larval development, instead of when the larvae were fed with cooked lettuce.

Besides food, temperature and humidity, another favorable factor for success in posture and adult emergence was the grouping of females after blood feeding and prior to oviposition. Ten groups of 35 females on average were placed together in plaster-lined glass containers of 10 cm diameter. Through this experiment was observed that the grouped females laid more eggs on average (6.1) than the individualized ones (3.9). However, these data were not used because from 30 emerged females (F1), seven belonged to the species *Psathyromyia bigeniculata* (Sábio et al., 2014).

This fact may be associated with chemical signals released by sandflies to locate hosts and oviposition sites, as proven in the study conducted by Hamilton (2008) with the species *Lu. longipalpis*. It is noteworthy that at the posture moment, females deposit dodecanoic acid in their eggs through the accessory glands, thus attracting other pregnant females to perform posture (Dougherty et al., 1994; Dougherty and Hamilton, 1997). Alves et al. (2003) studied *Lutzomyia renei* and found that females also deposit fatty acids in their eggs, which could probably act as oviposition pheromones.

According to Ward (1977), sandflies life cycle could also be influenced by different populations and methods of breeding, because, as already known, sandflies species require an adaptation to the laboratory environment. And yet, adults of certain species have different feeding preferences of others (Mutinga et al., 1989). Therefore, further studies on sandflies biology are needed to better understand the behavior, feeding habits and life cycle of each species.

Conflicts of interest

The authors declare no conflicts of interest.

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