Revista Brasileira de Parasitologia Veterinária
Brazilian Journal of Veterinary Parasitology

Revista Brasileira de Parasitologia Veterinária
ISSN: 0103-846X
zacariascbpv@fcav.unesp.br
Colégio Brasileiro de Parasitologia Veterinária
Brasil

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Revista Brasileira de Parasitologia Veterinária, vol. 23, núm. 3, julio-septiembre, 2014, pp. 387-392
Colégio Brasileiro de Parasitologia Veterinária
Jaboticabal, Brasil

Disponible en: http://www.redalyc.org/articulo.oa?id=397841493015

Research Note

Braz. J. Vet. Parasitol., Jaboticabal, v. 23, n. 2, p. 387-392, abr.-jun. 2014
ISSN 0103-846X (Print) / ISSN 1984-2961 (Electronic)
Doi: http://dx.doi.org/10.1590/S1984-29612014043

Amblyomma auricularium (Acari: Ixodidae): underwater survival of the non-parasitic phase of feeding females

Amblyomma auricularium (Acari: Ixodidae): sobrevivência subaquática da fase não parasitária de fêmeas alimentadas

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Received February 17, 2014
Accepted April 30, 2014

Abstract

To determine the effects of immersion in water on the biological parameters of engorged females of the tick species *Amblyomma auricularium*, 60 females were distributed in six groups, each comprising 10 individuals. The control group – G1 (not immersed) was fixed dorsally in a Petri dish and incubated at 27 ± 1 °C and 80% RH. The other groups were subjected to immersion periods of 24, 48, 72 and 96 hours, and the sixth group to continuous immersion. After the immersion period, the females were placed in Petri dishes to begin laying. Eggs were collected every 72 hours and kept in biological chambers. All the groups showed significant differences ($p < 0.05$) during the pre-oviposition period. The laying period and the average weight of overall posture did not change. The egg incubation period also did not differ significantly, but the hatching rate in the group immersed for 96h showed a significant difference. Thus, immersion for up to 96 hours does not impair the survival of *A. auricularium* females, although it may delay egg laying and reduce the number of offspring.

Keywords: Immersion, oviposition, Dasypodidae, *Amblyomma auricularium.*

Resumo

A fim de conhecer os efeitos da imersão em água sobre os parâmetros biológicos de fêmeas ingurgitadas de *Amblyomma auricularium*, 60 fêmeas foram distribuídas em seis grupos, cada um contendo 10 indivíduos. O grupo controle G1 (sem imersão) foi fixado dorsalmente numa placa de Petri e incubado a 27 ± 1 °C e 80 % de HR. Os demais grupos foram submetidos a períodos de imersão de 24, 48, 72 e 96 horas e, o último grupo, em imersão continua. Após o período de imersão, as fêmeas foram colocadas em placas de Petri para iniciar a postura. Os ovos foram coletados a cada 72 horas e mantidos em câmaras biológicas. Houve diferença significativa ($p < 0,05$) em relação ao período de pré-oviposição de todos os grupos. O período de postura e o peso médio da postura total não se alterou. O período de incubação dos ovos também não diferiram significativamente, mas houve uma diferença significativa na taxa de eclosão das larvas no grupo imerso por 96 horas. Assim, a imersão por até 96 horas não compromete a sobrevivência de fêmeas de *A. auricularium*, mas pode retardar a postura de ovos e reduzir o número de descendentes.

Palavras-chave: Imersão, oviposição, Dasypodidae, *Amblyomma auricularium.*

Introduction

Amblyomma auricularium (Conil, 1878) is a tick species widely dispersed in Nearctic and Neotropical regions (LORD; DAY, 2000; GUGLIELMONE et al., 2003; GUGLIELMONE; NAVA, 2006; NAVA et al., 2007; BERMÚDEZ et al., 2010; GUZMÁN-CORNEJO et al., 2011). The hosts listed for this species include pigs, rodents, marsupials, and carnivores (ALLAN et al., 2001; GUGLIELMONE et al., 2003; DANTAS-TORRES et al., 2010). There are few records of *A. auricularium* adults in domestic animals such as cattle, horses, and dogs, and according to Guglielmone et al. (2003), these hosts can be considered accidental.

Saraiva et al. (2013) recently isolated *Rickettsia amblyommii* in a female of *A. auricularium*, and found transstadial and transovarial transmission in the laboratory. They used rabbits as hosts for blood

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feeding, although no seropositive symptoms characterizing the transmission of the pathogen were found for this antigen after 21 days of infestation.

Species of Dasypodidae, known as armadillos, seem to be the tick's preferred hosts (EVANS et al., 2000; VENZAL et al., 2002; GUGLIELMONE et al., 2003; GUGLIELMONE; NAVA, 2006; OLEGÁRIO et al., 2006; SZABÓ et al., 2007; BERMÚDEZ et al., 2010). Among them, the nine-banded armadillo, *Dasypus novemcinctus*, (Linnaeus, 1758), is the one most commonly recorded (GUGLIELMONE et al., 2003).

The life cycle of *A. auricularium*, fed on rabbit blood was completed in controlled laboratory conditions (27 ± 1 °C and 80% RH). As is the case of most *Amblyomma* species, it has a three-host life cycle (FACCINI et al., 2010). Besides the records of its hosts, little is known about the life of this tick in the environment.

Several tick species spend most of their life cycle (up to 90%) in the environment. When they are off-host, they experience the rigors of environmental stresses associated with the climate of the immediate habitat (NEEDHAM; TEEL, 1991). The maintenance of ticks collected from wild animals under laboratory conditions and research into injury caused by extreme temperatures, desiccation or flooding are important to predict their behavior in the natural environment and their distribution pattern.

With regard to Neotropical species of importance to livestock production, several studies have revealed important features about temperature and moisture stress (CARDOSO et al., 2006; FREITAS et al., 2004; CHACÓN et al., 2003; CHACÓN et al., 2002; SUZUKI et al., 2000; SILVA et al., 2000). In addition, submergence at some stage of the life cycle has been studied and considered to be a limiting factor for some species (CAMPBELL; GLINES, 1979; SMITH, 1973; SUTHERST, 1971; BENNETT, 1974; KOCH, 1986; GAZÊTA et al., 1995; PAULA et al., 2000; LOUZADA; DAEMON, 2003; CANÇADO et al., 2006; FIELDEN et al., 2011; GIANNELLI et al., 2012; SANTOS et al., 2012). However, little is known about the ability of ticks on wild animals to survive this condition.

To add to research on the bioecology of ticks of domestic and wild animals, specifically the effects of abiotic components as limiting factors for its development, this study focused on the non-parasitic phase of engorged *A. auricularium* females under water. The specific questions addressed here were: How long can engorged females of this species survive under water? Are engorged females still able to lay eggs after different immersion times? Does the immersion time in water affect the larval hatching rate? And are females able to lay eggs underwater?

The knowledge obtained in this study will shed light on the ecology, tick-host relationships and distribution of *A. auricularium*.

Materials and Methods

Ticks

Engorged females of *A. auricularium* were obtained from a colony (10th generation/5 years). This colony originated from two engorged females collected manually from wild *D. novemcinctus* in the municipality of Mossoró (5°11" south latitude and 37°20" west longitude) in the state of Rio Grande do Norte, Brazil. The non-parasitic stages are kept in an incubator with controlled temperature and humidity (B.O.D, ELETROlab®) at 27 ± 1 °C, 80% RH and scotophase. All the parasitic stages are placed on healthy crossbred rabbits (New Zealand × Californian), about 2 months old, of both sexes, without prior contact with ticks and acaricides.

Ten rabbits were infested with 15 *A. auricularium* couples each. The parasite stages were placed in chambers attached to the shaved backs of rabbits of both sexes (NEITZ et al., 1971). All the rabbits were kept under proper sanitary conditions (Protocol no. 23011443/2011 of the University's Animal Ethics Committee). The engorged females naturally that detached on the same day were washed with tap water, dried with paper towels and weighed individually on an analytical balance with 0.001g precision.

Bioassay

To determine the ability of engorged females of *A. auricularium* to withstand immersion in water, 60 females (100-560 mg) were distributed in six groups (G1, G2, G3, G4, G5 and G6), each comprising 10 individuals.

By using adhesive tape, all the engorged females of the control group (G1) were fixed in the dorsal position in Petri dishes (100×15 mm) to lay eggs individually. All ticks were placed in a B.O.D. incubator at 27 ± 1 °C, 80 ± 5% RH and scotophase throughout the experimental period. Furthermore, the incubator was equipped with a thermometer and hygrometer, which were checked daily to ensure that the temperature and humidity shown in the incubator display window remained constant.

The ticks (n=10) of groups G2, G3, G4, G5 and G6 were placed in individual test tubes (40 ml) filled with distilled water and closed with cloth and an elastic band. The tubes of each experimental group were placed in 600-ml beakers containing distilled water and kept in the B.O.D. incubator (PAULA et al., 2000).

The females of groups G2, G3, G4, and G5 remained immersed for 24, 48, 72, and 96 hours, respectively. They were then dried, weighed again on an analytical balance, and placed in Petri dishes for oviposition under the same conditions as those described above for the control group.

The females were inspected every 24 hours until the first eggs were visible. After that, the egg masses were collected at 72-hour intervals, weighed on a precision balance and stored in syringes (5 mL), and sealed with cotton wool.

Group 6 remained under continuous immersion until egg laying or death was observed.

Survival was measured by the ability of the females to lay eggs, and the ticks were not handled to check for death.

The biological parameters of oviposition were assessed, namely: estimated time of the pre-oviposition period, in days; the time elapsed from when the engorged females dropped off the host until the first egg was laid; the estimated time of the oviposition period, in days; the time of onset of oviposition until the last egg was laid (BELLATO; DAEMON, 1997); egg mass weight, i.e., weight (in mg) of all the eggs from the onset of oviposition of a female;

egg production index (EPI = Weight of the egg mass x 100/Female weight before oviposition); nutrient index (NI= Weight of the egg mass × 100/Weight loss of the female during oviposition) (BENNETT, 1974); incubation period, which comprises the period, in days, from the onset of oviposition to the first larval hatching; larval hatching period, i.e., interval, in days, of hatching of the first larva in the pool of eggs until hatching of the last larva; and the larval hatching rate, i.e., estimated percentage of hatched larvae relative to the total mass of eggs laid (BELLATO; DAEMON 1997).

Statistical analysis

The biological parameters of the non-parasitic phase of females were evaluated to detect intra- and inter-group variations. The normality of data was assessed using the Kolmogorov-Smirnov test. The data were subjected to an analysis of variance (ANOVA) and the Tukey-Kramer test, with significance of $P< 0.05$. The tests were performed using Instat 3.0 GraphPad software.

Results

The engorged females of *A. auricularium* gained weight after immersion in distilled water. The females immersed for 24 hours (G2) showed an average weight gain of 0.69%, and G3 (48 hours), G4 (72 hours), and G5 (96 hours) showed average weight gains of 1.74%, 1.66%, and 1.32%, respectively.

The oviposition of the females subjected to immersion for 24 hours was not affected (n= 10). In G3, eight females (80%) laid eggs, while in G4 and G5 only six females (60%) laid eggs.

The females of G6 did not lay any eggs. After 15 days of immersion, they presented an abnormal blackish color and no mobility and were considered dead. In addition, the water in 70% of the tubes showed a reddish-brown color.

The average weight of egg mass and the overall oviposition time did not differ in any of the groups. The only parameter that showed differences among the groups was the average pre-oviposition period of *A. auricularium* females.

The pre-oviposition period was directly proportional to the immersion time and all the groups showed differences ($p< 0.05$) from the control group (G1) and from each other, except for G3 (48 hours) and G4 (72 hours), whose pre-oviposition periods did not differ from each other. The average oviposition periods of *A. auricularium* females in G2, G3, G4, and G5 did not differ significantly from that of the control group. In this group (G1), peak egg production occurred in the first three days of oviposition.

The egg production index (BENNETT, 1974) did not differ ($p> 0.05$) among the experimental groups of *A. auricularium* females. Differences in the nutrient index (BENNETT, 1974) were observed ($p <0.05$) between G5 (96 hours) and both G1 (control) and G2 (24 hours).

There was no difference ($p> 0.05$) among treatments in the incubation period. The larval hatching period of G5 (96 hours) differed from that of the other groups, except for G4 (48 hours).

The average egg incubation period of Group 5 (96 hours) differed from that of all the other groups (Table 1).

Discussion

The variability in the females' pre-oviposition weight before immersion can be considered random and common to this species, since, under the same laboratory conditions, Faccini et al. (2010) completed the cycle of this tick and found significant variations in the weight of engorged females. However, a comparison of the mean weight of the females showed no statistically significant differences, thus eliminating any potential interference in the results.

The weight gain observed in *A. auricularium* females after different periods underwater, as has already been observed in other tick species (PAULA et al., 2000), can be explained by the flow of water into the body through the spiracles (SUTHERST, 1971). However, there may be a limit to this water flow, since the groups of *A. auricularium* females immersed for 48, 72 and 96 hours showed no difference in the average percentage of weight gain. It is likely that after about 48 hours underwater, *A. auricularium* females are already water saturated (turgid).

The reddish-brown color of the water of G6 may be due to the extravasation of hemoglobin contained in the females' midgut cells. Since these ticks were subjected to an extended period of submersion, it is possible that the imbalance in osmotic pressure caused the cells of their internal organs to swell and burst, releasing their contents through natural orifices. Animal cells perform exchanges with the external environment, since they do not have cell walls, and when placed in strongly hypotonic media such as distilled water, these cells can burst (STRANGE, 2004). Paula et al. (2000) observed the same water coloration in tubes containing *Amblyomma cajennense* and *Anocentor nitens* ticks on the 10th day after continuous immersion, and concluded that the blood ingested was eliminated through the insects' natural orifices.

Louzada and Daemon (2003) found that a 24-hour immersion period was deleterious to *R. (Boophilus) microplus*, with 40% mortality of females before oviposition. After 48 hours underwater, 77.8% of *R. (B.) microplus* died, and 72 hours of immersion was lethal to all females.

Females of *Rhipicephalus sanguineus* immersed in distilled water survived for 24 hours under this condition. After 48 hours of submersion, 40% of the females died and after 72 hours the mortality rate was 100% (GIANNELLI et al., 2012).

Paula et al. (2000) reported that after 48 hours, immersion in water interfered negatively with the oviposition of *A. nitens* females, and 72 hours completely prevented oviposition. The mortality rate of *A. cajennense* was 90% after 48 hours underwater, reaching 100% after 72 and 96 hours.

We compared the *A. auricularium* immersion data obtained in this study with those found in the literature on *A. cajennense*. Although both tick species belong to the same genus and have a heteroxenous life cycle, *A. auricularium* seems to be more resistant to this type of stress than *A. cajennense*.

Amblyomma auricularium is a parasitic tick mostly found on armadillos, whose habits include diving (BITTNER, 2010) and digging in wetland areas (McDONOUGH et al., 2000). Hence, this tick species may have undergone different forms of selective pressure to survive over long periods of flooding.

Table 1. Average values of the parameters related to oviposition of engorged *Amblyomma auricularium* subjected to different periods of immersion in distilled water.

Immersion period	Pre-oviposition female weight (mg)		Weight gain (%)	Survival (n)	Period (days)	
	Before	After immersion			Pre-oviposition	Oviposition
Control	275.91[a] ± 57.41 (n=10)	_	_	10	5.5[d] ± 0.85 (n=10)	19.9[a] ± 6.23 (n=10)
24hours	273.5[a] ± 56.00 (n=10)	286. 75 ± 132.8 (n=10)	0.69[b]	10	7.4[c] ± 1.51 (n=10)	21.9[a] ± 5.67 (n=10)
48 hours	331.49[a] ± 88.92 (n=10)	395.64 ± 116.31 (n=10)	1.74[ac]	08	9.88[b] ± 0.83 (n=8)	20.25[a] ± 6.43 (n=8)
72 hours	337,04[a] ± 50,88 (n=10)	410, 6 ± 111,84 (n=10)	1,66[ac]	06	11,33[b] ± 1,86 (n=6)	22,00[a] ± 2,45 (n=6)
96 hours	271,16[a] ± 45,17 (n=10)	280, 62 ± 64,48 (n=10)	1,32[bc]	06	13,6[a] ± 1,03 (n=6)	15,83[a] ± 5,34 (n=6)

Immersion period	Period (days)		Egg mass weight (mg)	Larva hatching success (%)	Egg Production Index (EPI%)	Nutrient Index (NI%)
	Incubation	Hatching				
Control	34.17[a] ± 1.56 (n=10)	5.68[b] ± 1.15 (n=10)	135.45[a] ± 63.27 (n=10)	96.0[a] ± 0.06 (n=10)	44.48[a] ± 8.17 (n=10)	77.97[a] ± 12.98 (n=10)
24hours	34.6[a] ± 1.99 (n=10)	7.35[a] ± 1.13 (n=10)	120.75[a] ± 41.29 (n=10)	83.0[a] ± 0.23 (n=10)	43.06[a] ± 12.32 (n=10)	68.22[a] ± 15.86 (n=10)
48 hours	32.6[a] ± 1.33 (n=8)	6.18[ab] ± 0.52 (n=8)	184.87[a] ± 53.82 (n=8)	94.0[a] ± 0.03 (n=8)	50.85[a] ± 14.73 (n=8)	66.96[a] ± 15.88 (n=8)
72 hours	33,25 [a] ± 1,40 (n=6)	5,21[bc] ± 0,65 (n=6)	185,85[a] ± 66,24 (n=6)	87,0[a] ± 0,11 (n=6)	47,76[a] ± 10,99 (n=6)	65,73[ab] ± 16,14 (n=6)
96 hours	34,6[a] ± 1,20 (n=6)	3,67[c] ± 0,57 (n=3)	102,6[a] ± 39,93 (n=6)	25,0[b] ± 0,23 (n=3)	37,64[a] ± 8,53 (n=6)	42,68[b] ± 15,87 (n=6)

Means followed by the same letters in the same column do not differ significantly at 5% level.

Cançado et al. (2006) also reported high resistance of larvae and engorged nymphs of *Amblyomma dubitatum* when immersed in distilled water. The authors associated the ability of these ticks to support immersion with the primary host, the capybara, which is also a mammal with aquatic habits. Therefore, their ectoparasites are exposed to water during parasitism or when the ticks drop off in wetlands. The relationship between ticks and primary hosts is a still speculative issue and needs further research.

Balashov (1972) reported that after ixodid females became engorged, they were unable to move over long distances due to the weight of their bodies. However, this should make it very difficult for engorged females when they drop off animals in wetland ecosystems and seek a sheltered place to lay their eggs. On the other hand, if engorged *A. auricularium* females dropped off in an environment that is only temporarily flooded, or if they were able to move a sufficiently long distance to leave the immersion condition, this would enable them to continue their life cycle.

The delay in oviposition of *A. auricularium* females after immersion can be ascribed to morphogenetic diapause, which is characterized by arrested development when environmental conditions are not ideal. This seems to be common in other insects (BELOZEROV, 2009) as well as in ticks (RANDOLPH, 2004).

Fielden et al. (2011) recently found that *Dermacentor variabilis* is able to utilize dissolved oxygen in the water through a plastron formed by its complex spiracular plates. However, these authors reported that even when their plastron disabled, or in water with very low oxygen content, some ticks can still survive for several days. They also suggested that metabolic depression or some anaerobic pathways could possibly be involved.

Thus, the immersion of engorged females in distilled water may have led to a stagnation of oogenesis, which, according to Sonenshine (1991), is completed between the moment the female drops off the host animal into the environment and the beginning of oviposition.

Also, the pre-oviposition period, especially that of G5 (11-17 days), was much longer than that of the upper limit of the control group (4-7 days). Under similar laboratory conditions, Faccini et al. (2010) reported a maximum pre-oviposition period of *A. auricularium* of nine days.

The pre-oviposition period of other ixodid tick species has also been found to increase after immersion. The average increase after 48 hours of immersion was 3.5 days for *R. (B.) microplus* (LOUZADA; DAEMON, 2003), and 3.0 and 4.93 days for *A. cajennense* and *A. nitens*, respectively.

The average pre-oviposition period of *A. variegatum* is 10 to 21 days, and more than 21 days is considered diapause (PEGRAM et al., 1988). Although this is purely speculative, the results of this study suggest that the delay in oogenesis of *A. auricularium* may be due to diapause.

The groups showed no statistically significant difference in egg mass weight. However, from a biological standpoint, G5 (96 hours) had the lowest egg mass (mean=102.60 mg), which would also reduce the number of individuals of the next generation. The average egg mass weights of G2, G3, and G4 (120.75 mg, 184.87 mg, and 185.85 mg, respectively) were higher than those

of the females in the control group. However, this does not mean that the immersion of females or the initial weight contributed to a better oviposition performance. Based on the nutrient index (NI) (BENNETT, 1974), the females of the control group exhibited better performance than those of the other groups.

The ability of engorged *A. auricularium* females to ensure the continuity of a new generation was assessed based on the egg incubation period, larval hatching period and larval hatching percentage. With regard to the egg incubation period, there was no difference ($p > 0.05$) between treatments. The larval hatching period of G5 (96 hours) differed from that of the other groups, except for G4 (48 hours). The average incubation period of G5 (96 hours) differed from that of all the other groups.

The larval hatching period of *A. auricularium* was significantly shorter in G5 (mean= 3.67 days), but this does not mean that long periods of immersion hasten the larval hatching process. In this case, the shorter period was due to the low percentage of larval emergence of this group (mean= 25%).

When *A. nitens* females were subjected to 48 hours of immersion, there was a prolonged period of egg incubation (PAULA et al., 2000). However, 24 hours of immersion did not change the egg incubation period of *A. cajennense* (PAULA et al., 2000) and *R. (B.) microplus* (LOUZADA; DAEMON, 2003).

As for the egg mass laid by females in G5 (96 hours), there were large numbers of shriveled eggs. This explains the low average larval hatching rate, and allows us to state that immersion in water for a period of 96 hours impairs the reproduction of *A. auricularium*.

However, the results of this study do not us to state that this immersion period (96 h) is lethal to this species, since larval hatching occurred, albeit at low rates, which probably enabled the continuation of the cycle.

An important note about studies involving immersion is that so far the engorged female ticks of domestic animals (PAULA et al., 2000; LOUZADA; DAEMON, 2003; GIANNELLI et al., 2012) appear to be more sensitive to flooded conditions than those of wildlife species.

This reinforces the hypothesis that the host-parasite relationship and the host's lifestyle are important factors for the physiological adaptation of these ectoparasites, enabling the individuals to enter new habitats, even under adverse conditions.

Acknowledgments

This research was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and PPGCV- UFRRJ. Sá-Hungaro IJB, Pinheiro MC and Ribeiro CCDU were recipients of CAPES scholarships. Authors thank Crespi MPAL and Timóteo P for supplying naive rabbits.

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