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Bisexual population of the parthenogenetic tick *Amblyomma rotundatum*: bioecological data from the field and laboratory

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Research Article

Keywords: Biological vector, Ixodidae, Bisexual behavior, Amazon region

Posted Date: June 25th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-10020665/v1

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Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Parasites & Vectors on September 24th, 2026. See the published version at https://doi.org/10.1186/s13071-026-07682-w.

Abstract

Background

The tick *Amblyomma rotundatum* parasitizes primarily cold-blooded animals and is widely distributed in the Neotropics. It is a tick considered parthenogenetic, with rare reports of males in nature. In the present study, we identified for the first time a bisexual population of *A. rotundatum* which generates males and females in the same proportion, both in nature and in the laboratory.

Methods

Ticks were collected from reptiles and amphibians in Amazon biome Maranhão state, Brazil. The ticks were identified as *A. rotundatum* using a combination of morphological and molecular characterization. Specimens of *A. rotundatum* collected in the field were kept in BOD incubators for laboratory observations of their biology.

Results

Overall, 517 ticks were collected in the larval (n = 82), nymphal (n = 95), and adult (n = 340 [130 males and 210 females]). The sex ratio of males/females in the field was of ~1M/0.6F ($P > 0.05$). A total of 25 nymphs, engorged and semi-engorged, were collected from iguanas in nature, and 17 molted into 10 males and 7 females (ratio ~1M/0.7F). The cycle in the laboratory was carried out in the presence of female and male. In the laboratory, toads (*Rhinella marina*) were artificial infested with *A. rotundatum* and monitored for two consecutive generations. Approximately 27% of the engorged larvae molted to nymphs, and 61% of the nymphs molted to adults, with 23 males and 18 females (ratio 1M/0.8F). The mean egg incubation period was 33.7 ± 1.5 days, and mean egg hatching percentage was 90.0% ± 7.0.

Conclusions

The tick *A. rotundatum* exhibited bisexual population both in the field and in the laboratory. This indicates the existence of a bisexual population of this species in Amazon biome, with the presence of males and females in equal proportions.

Background

The tick *Amblyomma rotundatum* Koch, 1844 (Acari: Ixodida: Ixodidae) parasitizes mainly cold-blooded animals (e.g., Amphibians and Reptiles), but there are reports of this species parasitizing birds and mammals, including humans [1–5]. It is a Neotropical species distributed from northern Argentina to southern Mexico [2, 6], but established populations have been reported in the Nearctic region along with

the introduced toad *Rhinella marina* [2]. This species has also been reported in Canada parasitizing migratory birds, but to date there is no evidence of established populations in that country [7].

Amblyomma rotundatum is a three-host tick, with a single report showing a two-host life cycle when experimentally fed on snakes [8]. Furthermore, it is one of the few species that reproduce by parthenogenesis [6]. The first record of *A. rotundatum* males was published by Keirans and Oliver [9] from a laboratory colony. Subsequently, additional records of males were reported in natural environments one specimen parasitizing lizard of the genus *Tropidurus* [1], one specimen on the snake *Boa constrictor* [10], one specimen on the lizard *I. iguana* [11], two specimens on the tortoise *Chelonoidis denticulatus* [12], one specimen on the snake *Bothrops atrox* [13], and twelve on the lizard *Iguana iguana* [14]. Curiously, all records in nature occurred in the Brazilian Amazon, suggesting the possible existence of bisexual populations in this biome. Despite this, the few reports of males cited above are sporadic, isolated and in geographically distant populations. Thus, the existence of a bisexual population of *A. rotundatum* has never been reported, despite the wide geographical distribution.

Similarly to *A. rotundatum*, a few other tick species have been reported to reproduce by obligatory parthenogenesis, such as *Amblyomma cordiferum* [15], *Ixodes jellisoni* [16] and *Ixodes schulzei* [17]. The male stage has remained unreported for *A. cordiferum* and *I. schulzei* [17, 18], whereas a single male specimen has been recorded for *I. jellisoni* [16]. Interestingly, the tick *Haemaphysalis longicornis* is represented by populations that are either parthenogenetic or bisexual throughout its range [19, 20], with the parthenogenetic populations being more widely distributed than the bisexual populations.

Reports of *A. rotundatum* males are rare in nature [14], and studies on the biology of *A. rotundatum* in the presence of males are nonexistent. In this context, all studies that evaluated the life cycle of *A. rotundatum* in the laboratory have used parthenogenetic populations exposed to amphibians and reptiles [8; 21]. Therefore, in the present study we identified at the first time a bisexual population of *A. rotundatum* that produces males and females in similar proportions, both in nature and in the laboratory, and described the bioecology of this population.

Methods

Capture of animals and collection of ticks

The ticks in the present study were collected in their natural habitat, in the municipalities of São José de Ribamar (2°32'43.3"S, 44°07'06.5"W), Centro Novo do Maranhão, Gurupi Biological (ReBioGurupi) (3°48'22"S, 46°46'8"W), and Sao Luís (2°31'48"S, 44°18'10"O) Maranhão state, Brazil (Fig. 1 and Table 1). However, the bisexual population reported in this study was identified only in the municipality of São José de Ribamar, parasitizing exclusively *I. iguana* (Fig. 1, red triangle and Table 1).

Table 1
Bisexual population of *Amblyomma rotundatum* collected in the Amazon biome in the present study, Maranhão state, Brazil.

Host (PA)	N° of ticks			Sexual ratio	Municipality
	L	N	A		
Iguana iguana (42)	**82**	**95**	**337 (128M; 209F)**	**~ 1M/0.6F**	**São José de Ribamar**
Rhinella marina (1)			1 M		Gurupi Biological (ReBioGurupi)
Chelonoidis sp. (1)			1M;1F		Sao Luís
Total	82	95	130M;210F		

Where: PA = number of parasitized individuals; L = larva, N = nymph, A = adult (M = male; F = female). **Highlighted in bold: bisexual population identified in this study.**

In the municipalities of Centro Novo do Maranhão (ReBioGurupi) and Sao Luís, only two males were collected: one specimen of *Chelonoidi*s and one specimen of *Rhinell*a sp., therefore these data were not included in the study of the biology and sex ratio at field and laboratory. Host captures were carried out in four field campaigns (each of 1 to 5 days), distributed between rainy (April and May) and dry (August and September) seasons in the years 2024 and 2025. Hosts were captured using a scoop and gloves, without the use of chemical restraint. All hosts were inspected for ticks, which were manually collected when found. The collected ticks were taken alive to the laboratory and morphologically identified under a stereoscope microscope. Unengorged specimens were stored in plastic microtubes containing 70% alcohol for further molecular analysis. Engorged ticks (larvae, nymphs and females) were kept in an incubator at 27°C and 90% RH for ecdysis (larvae and nymphs) or oviposition (females). The larval offspring from engorged females were used in studies of the biology of *A. rotundatum* under experimental conditions at the Parasite Control Laboratory of the Federal University of Maranhão (UFMA). After collecting the ticks, the iguanas and tortoises were released into their natural habitats, whereas toads retained for colony maintenance under laboratory conditions.

Morphological identification of ticks

Larvae were identified morphologically to the genus level according to Barros-Battesti et al. [22]. Nymphs and adults were identified to species level based on Martins et al. [23] and Dantas-Torres et al. [24], respectively. Morphological identification of *A. rotundatum* males followed the characteristics reported by Labruna et al. [1] and Luz et al. [14].

Biology of Amblyomma rotudatum in the laboratory

The larvae derived from females collected in the municipality of S.J. de Ribamar (bisexual population) were used to initiate a laboratory colony and, in parallel, to observe the biological parameters of *A. rotundatum*. Nymphs and adults (males and females) were generated from these larvae. Infestations of larvae, nymphs, and adults (males and females) from two consecutive generations consisted of exposing toads (*R. marina*) to ~ 500 larvae/host, ~ 25 nymphs/host, or 4 adults (2 males, 2 females)/host. In total, three *R. marina* hosts were used; the same three infested simultaneously by each tick stage. In each infestation, ticks were unfed and approximately 30 days old. In an effort to determine if females without males maintained parthenogenetic behavior, a total of four females were fed without any contact with males before and during feeding.

During infestations, toads were kept individually in glass desiccators (300 x 385 millimeters), maintained in an incubator at 27ºC and ~ 90% RH. Toads were fed daily with insects (commercial mealworms); water was offered *ad libitum* in a plastic chamber, in which toads were frequently observed with half their bodies submerged. Engorged and detached ticks were collected in the morning (between 9 and 10 am) and in the afternoon (between 4 and 5 pm) and immediately transferred to an incubator at 27°C and 90% RH.

The following biological parameters were obtained for the ticks: feeding period (time from infestation to detachment of the engorged tick), premolt period (number of days from larval or nymphal detachment to ecdysis), pre-oviposition period (number of days from female detachment to the onset of oviposition), oviposition period (number of days from the onset of oviposition to the last egg) and incubation period (number of days from the onset of oviposition to the hatching of the first larva). Each engorged female was weighed on the day of detachment. The hatching percentage for each female's egg mass was estimated visually according to Labruna et al. [25].

Morphological redescription of males

For the redescription of *A. rotundatum* males, 10 specimens generated in the laboratory during this study were used. Ticks were initially cleaned in an ultrasonic bath (USC1600A) for approximately 30 minutes in an Eppendorf tube containing distilled water. Thereafter, they were individually transferred to a new Eppendorf tube containing absolute alcohol to remove excess water. Next, the ticks were removed from the alcohol solution and placed on paper towels for approximately 5 minutes to dry. Ticks were measured and photographed using the Image-Pro-Plus 5.1 program for analysis of images and morphometry, fitted to an Olympus SZX stereoscope microscope (Carl-Zeiss, Germany, 16x zoom). Each male was individually placed dorsally and ventrally on carbon-coated tape and examined by SEM/EDS (Hitachi TM3000, Eindhoven, Netherlands).

In the description that follows, all measurements are given in millimeters; first the range, followed by the mean ± the standard deviation in parentheses. Light microscopy color photographs were prepared to show the natural scutal ornamentation pattern. Males used for redescription were deposited in the tick collection “Coleção Nacional de Carrapatos Danilo Gonçalves Saraiva” of the Faculty of Veterinary

Medicine of the University of São Paulo, São Paulo, Brazil (CNC). Illustrations were prepared showing the dorsal and ventral views of the specimens.

Molecular analyses

Genomic DNA was extracted from specimens identified morphologically as *A. rotundatum* (n = 66) using a guanidine isothiocyanate protocol [26]. Tick DNA samples were tested by two PCR assays, one targeting a ~ 460 base pair (bp) fragment of the tick mitochondrial 16S rDNA gene [27], and another targeting a ~ 1200 bp fragment that includes the entire ITS2 spacer region [28], at the University of São Paulo (USP).

Aliquots of DNA of 59 ticks were subsequently transported to the Multi-User Laboratory of Molecular Biology (LBioMol/UFRRJ) without refrigeration, using an isopropanol precipitation method. Briefly, 30 µL of extracted DNA were combined with an equal volume of isopropanol in screw-cap microcentrifuge tubes (Sarstedt), mixed by inversion and were shipped at ambient temperature. Upon arrival, tubes were centrifuged briefly to collect the DNA–isopropanol mixture, and the isopropanol was evaporated by incubation at 70°C for 20 minutes. To ensure complete drying of the DNA, the tubes were maintained at 70°C for an additional two hours. The dried DNA´s were re-suspended in 30 µL of AE buffer (10 mM Tris-Cl, 0.5 mM EDTA, pH 9.0) for use in PCR assays.

A subset (n = 20 ticks), of the resuspended samples were employed as template using the PCRs targeting mitochondrial 16S rDNA using Mangold primer pairs and published reaction cited above. In addition, a modified 16S rDNA primer pair was designed by adapting the Mangold primers cited above. Specifically, each primer was extended at the 3' end with additional nucleotides determined from alignment of *A. rotundum* mitochondrial 16S rDNA sequences available in GenBank, with the objective of improving amplification efficiency for this species. The modified primers were designated ROT16S-F (5'-CCGTC TGA ACT CAG ATC AAG TAG GAA-3') and ROT16S-R (5'-GCT CAA TGA TTTT TTA AAT TGC TGT GGT AT-3'), with the bold/underlined bases representing the nucleotides introduced at the 3' ends relative to the original forward and reverse primers. The PCR assays with the modified primers were subsequently applied to all samples, being performed in a final volume of 12.5 µL, consisting of 11.0 µL of master mix and 1.5 µL of DNA template. The master mix contained 2.0 mM $MgCl_2$, 200 µM of each dNTP, 5 pmol of each primer, and 0.5 U of Platinum Taq DNA polymerase (Thermofisher Scientific) in the manufacturer's supplied buffer. Amplifications were performed via a touch-up protocol consisting of an initial denaturation at 94°C for 5 min, followed by three cycles of 94°C for 15 s, 45°C for 15 s, and 72°C for 15 s, then 36 cycles of 94°C for 15 s, 49°C for 15 s, and 72°C for 15 s, with a final extension at 72°C for 5 min.

PCR products were analysed on 1.5% (w/v) agarose gels prepared in 1× Tris–acetate–EDTA (TAE) buffer. Amplicons were purified with ExoSAP-IT Express (Thermofisher Scientific) according to the manufacturer's instructions and sequenced in both directions using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) with the same primers as those used for amplification. Sequencing

was performed on a 3500 genetic Analyzer (Applied Biosystems) and sequences were aligned, trimmed to remove primer sequences, and entered into the BLAST search algorithm and the NCBI nucleotide database to determine gene identity.

Statistical analyses

Prevalence, mean intensity, and mean abundance of tick infestations were calculated following Bush et al. [29]. The chi-square test was used to compare the proportion of female and male ticks, considering the null hypothesis of the proportion of 1 male for 1 female.

Results

Field collected ticks

In total, 517 ticks were collected in the larval (n = 82), nymphal (n = 95), and adult (n = 340; 130 males, 210 females) stages from 44 hosts specimens (Table 1). In the municipality of S.J. de Ribamar, 82 larvae, 95 nymphs, and 337 adults (128 males, 209 females) were collected exclusively from *I. iguana* (42 host specimens). In this scenario, ticks were collected from Iguanas mainly in the head region (65 larvae, 37 nymphs, and 73 adults) (Fig. 2). The least parasitized site was the tail, with 2 larvae, 5 nymphs, and 15 adults. Additionally, one male and one female were collected on *Chelonoidis* in the municipality of Centro Novo do Maranhão (ReBioGurupi), and one male from *Rhinella* sp. in the municipality of São Luís.

The mean intensity of tick parasitism was 11.7 ± 7.3 ticks/host. Tick burden ranged from 1 to 54 ticks, with ~ 10% of hosts infested with at least one or two tick specimens. No lesions was observed at the tick bite site, but a hemorrhagic process was observed after removal of adult ticks.

Molecular analyses of ticks

Ticks (nymphs and adults) were identified using a combination of morphological characterization and nucleotide sequence analysis of mitochondrial 16S rDNA and nuclear ITS2 DNA. Morphologically, all ticks (95 nymphs and 340 adults [n = 130 males and 210 females]) were identified as *A. rotundatum*, which was corroborated by molecular analyses, as stated below.

A total of 66 DNA samples were extracted from ticks morphologically identified as *A. rotundatum*. Five samples showed no detectable DNA and were excluded, resulting in 61 samples for subsequent analyses. Amplification of the mitochondrial 16S rDNA fragment using the protocol of Mangold et al. (1998) was not obtained for any samples, while ITS2 amplification yielded products for 8/61 samples. Following 1:10 dilution of the DNA extracts, ITS2 amplification was obtained in an additional 44 samples, resulting in 52/61 (85.2%) positive samples. In contrast, no amplification of the mitochondrial 16S rDNA fragment was obtained using the original protocol. Using the modified PCR assay targeting the mitochondrial 16S rDNA, amplification was obtained in 55/59 samples without dilution and in the remaining 4 samples following 1:10 dilution, resulting in successful amplification of all samples tested

with this assay. Sequences of ITS2 amplicons were obtained from three males and one female. After trimming, the resulting sequences (644 bp) were identical, and BLAST analysis showed 100% nucleotide identity with previously deposited ITS2 sequences of *A. rotundatum* from Peru (KU720373) and French Guiana (MT000680). A representative sequence was deposited in GenBank under accession number PX574309. Six mitochondrial 16S rDNA amplicons were sequenced: four from males, one from a female, and one from a nymph. After trimming of primer sequences, all sequences (397 bp) were identical and showed 100% nucleotide identity with previously deposited *A. rotundatum* sequences, including KX278439 (Amazon region), MG023145 (Minas Gerais, Brazil), and MK511995 (Maranhão, Brazil). A representative sequence was deposited in GenBank under accession number PX426845. The sequences obtained were 11 nucleotides shorter than *A. rotundatum* 16S sequences available in the GenBank, corresponding to the 3'-end extensions incorporated into the modified primers.

Sex ratio of field-collected Amblyomma rotundatum ticks

Due to the high number of male and female *A. rotundatum*, the sex ratio was calculated only for the population found in the municipality of S.J. de Ribamar. Based on adult specimens collected from naturally infested hosts, a total of 128 *A. rotundatum* males were identified morphologically and molecularly identified, with an overall male:female sex ratio of ~1M/0.6F ($P > 0.05$), equivalent to 62% (males) to 38% (females). In light of the observation that only *A. rotundatum* was identified in the present study, we speculate that all field-collected larvae also belonged to this species.

Among a sample of 30 engorged larvae and 25 nymphs (17 engorged and 8 semi-engorged), all collected from iguanas in nature, none of the larvae molted into nymphs whereas the 17 (68%) engorged nymphs molted into 10 males and 7 females, a sexratio of ~1M/0.7F. The prevalence of infestation was high (~ 90%) in the parasitized iguanas, with 269 ticks collected in the dry season (July - September) and 245 in the rainy season (March - May) ($P > 0.05$).

Life cycle under laboratory conditions

The biological data for two consecutive generations of *A. rotundatum* were grouped and are presented in Table 2. Feeding success was 23.5%, 55.7%, and 66.7% for larvae, nymphs, and females, respectively. Mean feeding periods were 11.8 ± 1.4 days for larvae, 13.0 ± 1.3 days for nymphs, and 19.5 ± 0.5 days for females. Mean premolt periods were 13.5 ± 0.9 and 15.9 ± 0.7 days for engorged larvae and nymphs, respectively. Approximately 27% of the engorged larvae molted into nymphs, and 61% engorged nymphs molted to adults (23 males and 18 females [sex ratio 1M/0.8F]). The mean egg incubation period was 33.7 ± 1.5 days, and mean egg hatching percentage was 90.0% ± 7.0. The four females that fed without the presence of males showed an incomplete life cycle, not reaching the adult stage.

Table 2
Biological data of a bisexual population of *Amblyomma rotundatum* under laboratory conditions and for field collected-engorged larvae and nymphs. Feeding periods were assessed by exposing ticks to toads (*Rhinella marina*), which were held in an incubator at 27°C and ~ 90% RH. Tick off-host stages were kept in the same incubator.

Biological Parameters	**Laboratory**			**Field-collected ticks**
	Larva	**Nymph**	**Female**	**Nymph**
No. exposed ticks	1780	75	6	
No. recovered ticks (%)	418 (23.5)	41 (54.7)	5 (66.7)	
Feeding period (days)*	11.8 ± 1.4 (10–15)	13 ± 1.3 (11–16)	19.5 ± 0.5 (19–20)	
			-	
Premolt period (days) *	13.5 ± 0.9 (12–15)	15.9 ± 0.7 (14–17)	-	14 ± 1.0 (13–15)
Molting success (%)	27.3	61 (23M/18)	-	68 (10M/7F)
Weight of engorged females (mg)*	-	-	573.4 ± 67.4 (490–641)	
Preoviposition (days)*	-	-	6 ± 1.0 (6–8)	
Oviposition (days)	-	-	20 ± 1.0 (19–21)	
Egg incubation period (days)*	-	-	33.7 ± 1.5 (31–36)	
Egg hatching (%)*	-	-	90% ± 7.0 (80–90)	
*Values presented as: mean ± standard deviation (range in parentheses)				

Redescription of the male of Amblyomma rotundatum

The measurements and description of males of *A. rotundatum* are presented in mm and illustrated in Figs. 3 and 4.

Idiosoma: Length from apices of scapulae (rounded) to posterior body margin 2.87–3.90 (3.63 ± 0.32), maximum breadth 2.33–3.29 (3.02 ± 0.29). Outline oval, broadest at the level of spiracular plates. Genital aperture located at level of coxa II, U-shaped. Spiracular plate 0.67–0.85 (0.77 ± 0.05) long, 0.38–0.54 (0.44 ± 0.05) broad, comma-shaped with elongate macula, numerous minute globlets. Scutum ornate with pale iridescent patches of orange ornamentation on lateral fields, postero-accessory and postero-

median spots narrow, lateral spots small but distinct; in some cases, presence of a soft central ornamentation resembling a pseudo-scutum. Scutum with numerous punctations, more densely distributed on antero-lateral field. Cervical grooves short, deep, comma-shaped. Eyes flat. Marginal groove absent. With 11 festoons without tubercles, central festoon 0.31–0.43 (0.35 ± 0.04) breadth. Gnathosoma. Palpi long; article I short; article II long; article III shorter than article II; article IV ventrally to article III. Length of palpal apices to cornua apices 0.72–0.99 (0.91 ± 0.08), breadth 0.63–0.85 (0.73 ± 0.06). Basis capituli length 0.42–0.53 (0.48 ± 0.04), subrectangular, posterior margin slightly concave, with the presence of short, rounded cornua; few small punctations dorsally. Palp length 0.72–0.90 (0.82 ± 0.06); length of palpal article I 0.06–0.21 (0.16 ± 0.04); length of palpal article II 0.41–0.56 (0.49 ± 0.05); length of palpal article III 0.17–0.29 (0.22 ± 0.04), suture between II and III distinct. Hypostome elongate, broadly rounded apically (spatulate) with corona of fine denticles. Total length of hypostome 0.52–0.79 (0.66 ± 0.08); length of toothed portion 0.24–0.38 (0.33 ± 0.05). Hypostomal dentition 3/3 with 5–6 teeth per row. Legs. Coxa I with two distinct, short, blunt spurs, subequal in size; coxae II-IV each with two short, bluntly rounded spurs. Trochanters without spurs. Tarsus I 0.36–0.88 (0.74 ± 0.15) long, 0.17–0.28 (0.22 ± 0.04) broad. Tarsus IV 0.48–0.66 (0.61 ± 0.05) long; 0.15–0.20 (0.17 ± 0.02) broad.

Discussion

Tick – hosts relationships in the environment

In the present study, we report parasitism by *A. rotundatum* on reptiles and amphibians in the Amazon biome, and highlight the elevated number of males collected on iguanas (*I. iguana*) (sex ratio: 1 M/0.7 F), which indicates the existence of a bisexual population. After morphological and molecular analyses, all ticks collected on iguanas were identified as *A. rotundatum*. In addition, we also report for the first time the parasitism by one male tick on a toad (*R. marina*) and the second record of a male tick on a tortoise (*Chelonoidis* sp.) in the Amazon Biome. Furthermore, we report for the first time the life cycle of the bisexual population of *A. rotundatum* under laboratory conditions.

Amblyomma rotundatum is a tick primarily associated with cold-blooded animals (reptiles and amphibians) in the Neotropics [3, 18]. Importantly, all adults of *A. rotundatum* that have been reported in the Neotropical region, outwith the Amazon Biome, have been females, consistent with parthenogenetic populations. In Brazil, reptiles and amphibians also sustain populations of another tick species, *Amblyomma dissimile*, which occurs sympatrically with *A. rotundatum* in the Pantanal and Amazon biomes, often in coinfestations [30, 31, 32]. In contrast to *A. rotundatum*, all known populations of *A. dissimile* are bisexual [22, 32].

Parthenogenesis in ticks is a phenomenon in which the female produces viable eggs without mating or fertilization by a male [33]. The first record of asexual reproduction in ticks was in *A. rotundatum* (formerly *Amblyomma gamum*) [34]. Later other species with obligatory or facultative parthenogenetic biological behavior were discovered in both the families Ixodidae (hard ticks) and Argasidae (soft ticks)

[16, 15, 18, 35–38]. In Brazil, *A. rotundatum* and *I. schulzei* are the only known tick species with obligatory parthenogenetic [17, 34]. Parthenogenesis in *A. rotundatum* has been reported both under experimental and natural conditions [8, 34, 39, 40], with occasional reports of males [1, 10, 12, 14]. For *I. schulzei*, the parthenogenetic reproduction has been reported in the laboratory, consistent with the absence of records of male specimens at field [17]. Parthenogenesis for *A. dissimile* was reported by Bodkin [36] under experimental conditions in Jamaica but the possibility of a species misidentification with *A. rotundaum* cannot be ruled out. It is pertinent to emphasize that sympatry between *A. rotundatum* and *A. dissimile*, two morphologically similar species, is common in the Neotropics [32, 41].

Although *A. rotundatum* is widely distributed in the Americas, from the southern United States to northern Argentina [2, 6, 14, 18, 40], there is a report of one male obtained under experimental conditions [9]. To date, males of *A. rotundatum* collected on hosts in nature have been reported from four different locations, totaling 18 specimens collected exclusively on reptiles in different locations of the Amazon biome [1, 10, 12–14]. Mendoza-Roldan et al. [42] reported only one record of a male *A. rotundatum* parasitizing reptiles in the Neotropics. In the study of Luz et al. [14], 1 male and 3 females were collected on *Boa constrictor*, 9 males and 6 females on *I. iguana*, with a male:female sex ratio of ~ 1/0.9 in natural environment of the host. Based on these reports, Luz et al. [14] speculated the existence of a possible bisexual population of *A. rotundatum* in Amazon biome associated with reptiles, and that exclusively parthenogenetic populations would be present in the rest of the country. In the present study, we recorded nearly six times more male specimens of *A. rotundatum* (n = 103) than the total number reported in the literature to date (n = 18). In addition, 63 females specimens were collected, with a sex ratio male:female of 1/0.6, corroborating the ratio reported by Luz et al. [14].

Life cycle under laboratory conditions

The life cycle of a bisexual population of *A. rotundatum* was investigated for the first time under laboratory conditions. In the laboratory, most adults (male and female) and immature ticks attached and fed to repletion on toads, indicating successful feeding. The fact that some ticks did not attach or were not recovered may be related to host grooming, possibly involving ingestion, or to failure to attach or feed, potentially reflecting host specificity, as the population used for infestation was collected from iguanas rather than amphibians.

Larvae and nymphs showed mean feeding period of 12 and 13 days, respectively. These records are below those reported for parthenogenetic populations of *A. rotundatum* by Oba and Shumaker [39], Luz et al. [21] and Rodrigues et al. [8]. On the other hand, the nymphal feeding period was high in relation to the study by Rodrigues et al. [8]. Similarly, the female mean feeding period (19.5 days) in the present study was shorter than those reported by Oba and Shumaker [39] and Luz et al. [21], with 36 days and 31 days, respectively. However, it was longer than the period reported by Rodrigues et al. [8] (13 days, maximum 21 days of engorgement). According to Apanaskevich and Oliver [43], numerous factors can interfere with the feeding time of hard ticks, with the host and ambient temperature being the most important. In our study, the feeding period of larvae, nymphs, and adults was obtained from toads kept at a controlled temperature of up to 27ºC. On the other hand, in the studies by Oba and Shumaker [39],

Rodrigues et al. [8] and Luz et al. [21], it is unclear whether the feeding period was monitored under controlled abiotic conditions as in the present study. Although Rodrigues et al. [8] used snakes in their study, Oba and Shumaker [39] and Luz et al. [21] used the same taxonomic host group (toads, *Rhinella* spp.) as the present study. Although this cannot be demonstrated here, the influence of intrinsic factors on the parasitic biology of this bisexual population of *A. rotundatum* cannot be ruled out. Taken together, these results indicate that the bisexual population of *A. rotundatum* tends to have a shorter life cycle, especially the feeding period. A similar pattern was observed by Chen et al. [44], who reported that the life cycle periods of the parthenogenic population of *H. longicornis* are longer than those of the bisexual population. The egg hatching rate averaged ~ 90%, a value higher than those reported for parthenogenetic populations of *A. rotundatum* [8, 21, 39]. Furthermore, the high hatching rate represents greater success in the life cycle of the bisexual population of *A. rotundatum*.

Nymphs reared in the laboratory molted to males and females in a sex ratio of 1M/0.8F. This result is similar the field findings of the present study, in which a sex ratio ~1M/0.6F was observed. In summary, these results reinforce the hypothesis proposed by Luz et al. [14] on the existence of a bisexual population of *A. rotundatum* with males and females that parasitizes primarily iguanas in part of the Amazon biome. The reasons for the presence of a bisexual population of *A. rotundatum* in the Amazon biome (present study), plus several previous reports of *A. rotundatum* males in this biome [1, 10, 12–14] are unknown, but factors associated with the environment and hosts cannot be ruled out. However, due to the spatial limitations of field data, it is not possible to determine whether this bisexual population is present throughout the Amazon biome.

According to Kiszewski et al. [45] parthenogenetic and bisexual populations of ticks occur allopatrically. For example, the tick *H. longicornis* comprises both parthenogenetic and bisexual populations, with the parthenogenetic population being widely distributed and less demanding in terms of habitat, therefore more generalist in relation to its bisexual population [19]. Thus, on the Asian continent, *H. longicornis* has its parthenogenetic population widely distributed, with some bisexual populations occurring in certain locations. The fact that the bisexual population of *A. rotundatum* was found exclusively in the Amazon biome may be related to the biome's abiotic conditions, where the average annual temperature is 27°C and relative humidity is high (~ 80 to 90%), in addition to an annual rainfall regime of ~ 2.300 mm. Although these abiotic conditions could be related to the absence of bisexual populations of *A. rotundatum* in other Brazilian biomes, this hypothesis is to be evaluated in further studies. In addition, males were found almost exclusively on reptiles, corroborating with the previous hypothesis that *A. rotundatum* males may be primarily associated with reptiles in the Amazon biome [14]. This hypothesis requires further evaluations, particularly because we found a male parasitizing a toad in nature during the present survey. Additionally, the experimental life cycle of the bisexual population was also conducted on toads, indicating the suitability of this type of host for a bisexual population of *A. rotundatum*, at least under experimental condition.

The rarity with which males of the bisexual population of *A. rotundatum* had been found until the beginning of this research may have been due to misidentification with *A. dissimile*. According to Luz et

al. [41], the separation of the female stage of these two species by the external morphology is a difficult task, as they present only slight differences in the coxal spurs, and the scutal punctuations and shape, while they parasitize the same hosts (reptiles and amphibians) and may occur in sympatry. In many cases, the finding of a male specimen was considered a valid indication of *A. dissimile*, since no bisexual population of *A. rotundatum* was known. Indeed, extreme caution is needed to perform morphological identification of *A. rotundatum* and *A. dissimile*, regardless of the presence of male, especially in the Amazon biome.

Partial sequences of ITS2 and 16S rDNA indicated that the ticks examined in this study belonged to *A. rotundatum*, consistent with the morphological identifications. The modified 16S PCR assay enabled successful amplification, overcoming the failure observed with the protocol of Mangold et al. [27]. In addition, the DNA transport method based on isopropanol precipitation, ambient shipment, and subsequent drying at 70°C performed reliably, providing a practical and low-cost option for DNA transfer between laboratories without reliance on refrigerated transport.

The sequences obtained were identical or highly similar to previously deposited *A. rotundatum* sequences, with minimum nucleotide identity values of 99.8% for 16S rDNA and 99.5% for ITS2. Notably, the 16S rDNA sequences showed 100% identity with the vast majority of full-length *A. rotundatum* sequences available in GenBank, excluding the terminal regions affected by primer design. This elevated level of similarity supports the taxonomic assignment of the specimens and is consistent with the low intraspecific divergence reported for these markers in this species.

These findings suggest that commonly used markers such as ITS2 and 16S rDNA may have limited resolution for detecting potential genetic variation associated with distinct biological traits, such as the occurrence of males. Further studies using additional molecular markers may be required to explore this possibility. The potential influence of endosymbionts and other components of the microbiome also warrants investigation, as such factors may contribute to the occurrence of bisexual populations.

Redescription of the male

Due to the rarity of records, male *A. rotundatum* specimens have been morphologically characterized using only 10 specimens. The first description was reported by Keirans and Oliver [9] of a specimen generated in the laboratory. A decade later, the male *A. rotundatum* was morphologically described by Labruna et al. [1] from a single specimen collected naturally. Both authors reported the following morphological characteristics for male *A. rotundatum*: body outline oval, marginal grooves absent, scutum densely punctate (especially at periphery), pale iridescent patches of orange ornamentation present along scutal margins; basis capituli dorsally subrectangular, with bluntly rounded cornua; hypostome with 3/3 dental formula; coxae I-IV each with two short, bluntly rounded spurs; genital aperture possessing a flap, typical of male *Amblyomma* ticks. In our study, we redescribed the male of *A. rotundatum* based on 10 specimens from the laboratory and natural environment. The morphological characteristics found here for males support the original descriptions of Keirans and Oliver [9] and Labruna et al. [1]. In some specimens, it was possible to observe an apparent ornamentation in the

central region of the shield, resembling an orange-colored pseudo-scutum, in addition to those reported on the sides by Labruna et al. [1]. The pseudo-scutum can also be observed in males of *A. dissimile* [24], which may contribute to misidentification between these two species when sympatry occurs. Therefore, the morphological identification of male *A. rotundatum* is based on host type and characteristics (restricted to cold-blooded animals, especially reptiles) and some morphological characteristics typical of the species: coxa IV with a short, rounded external spur. Scutum with pale-orange patches inateral fields. Punctuations numerous, more densely distributed in antero-lateral fields.

Conclusions

We report for the first time a bisexual population of *A. rontundatum*, currently restricted to the Amazon biome. It is noteworthy that we collected most males of *A. rotundatum* on reptiles (iguanas), indicating that the bisexual population of this species may be primarily associated with these hosts in nature, although association with toads and tortoises cannot be ruled out because we also collected one male from each of these hosts.

Declarations

Acknowledgements

The authors thanks Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)–"Amazônia-Legal" (number 0762/2020 / 88887.510068/2020- 00 / Code 001), Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) – "Biodiversidade, potencialidades, preservação e saúde ambiental da Amazônia Oriental" PPBio (Biodiversity Research Program) (number 441189/2023-7) and Instituto Nacional de Ciência e Tecnologia em Parasitologia Animal – INCT-IPA (number 408812/2024-9) for their financial support. The sequencing acivities at LBioMOL/UFRRJ recived finacial support by Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro - FAPERJ. The Fundação de Amparo à Pesquisa do estado do Maranhão (FAPEMA).

Author contributions

R.M. de M.S, J.L.H.F., M.B. L., L.M. C-J, H.R. L.: Writing – original draft, Investigation. R.M. de M.S, T.F. M., I. S. S., M.P.M., L.M.C-J, H.R. L.: Methodology, Data curation. R.M. de M.S, E.B.A.J., L.T. D.,R.M. S. N., J.M. S.S., A.C. M. F., L.F.O. C., J.R.F. P.,D.M.S., C.N.F.B, H.R. L.: Field data collection and investigation.

Data availability

All relevant data generated during this study are included in the article.

Ethics approval and consent to participate

The study was evaluated and approved by the Animal Experimentation Ethics Committee of the Federal University of Maranhão and was conducted with the permission of ICMBIO; process number 23115.012423/2023-85 e 3484-4, respectively.

Consent for publication

Not applicable.

Declaration of competing interest

The authors declare no conflict of interest.

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Figures

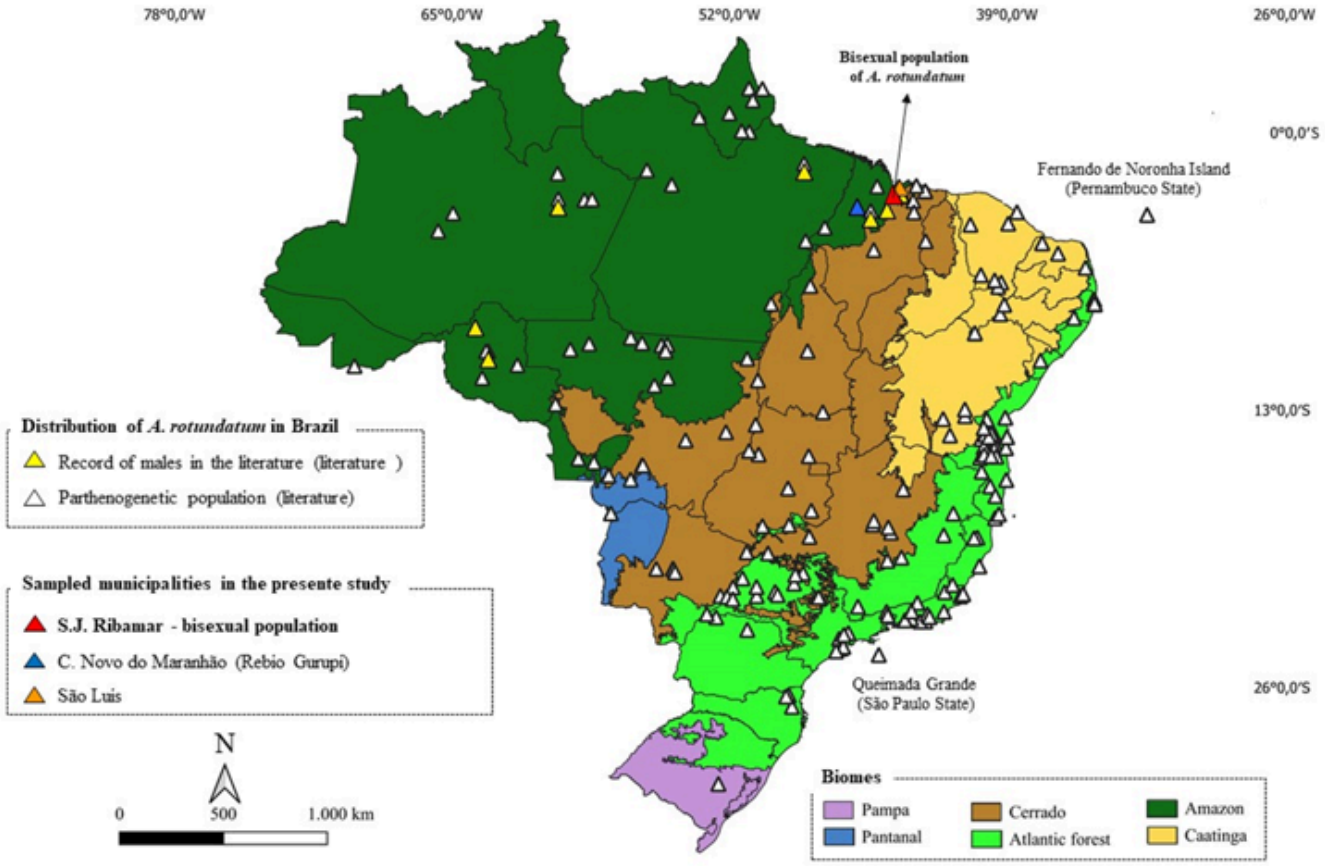


Figure 1

Distribution of *Amblyomma rotundatum* and *Amblyomma dissimile*in Brazil

(Polo et al. 2021) in Brazil, highlighting the bisexual population found in the present

study.

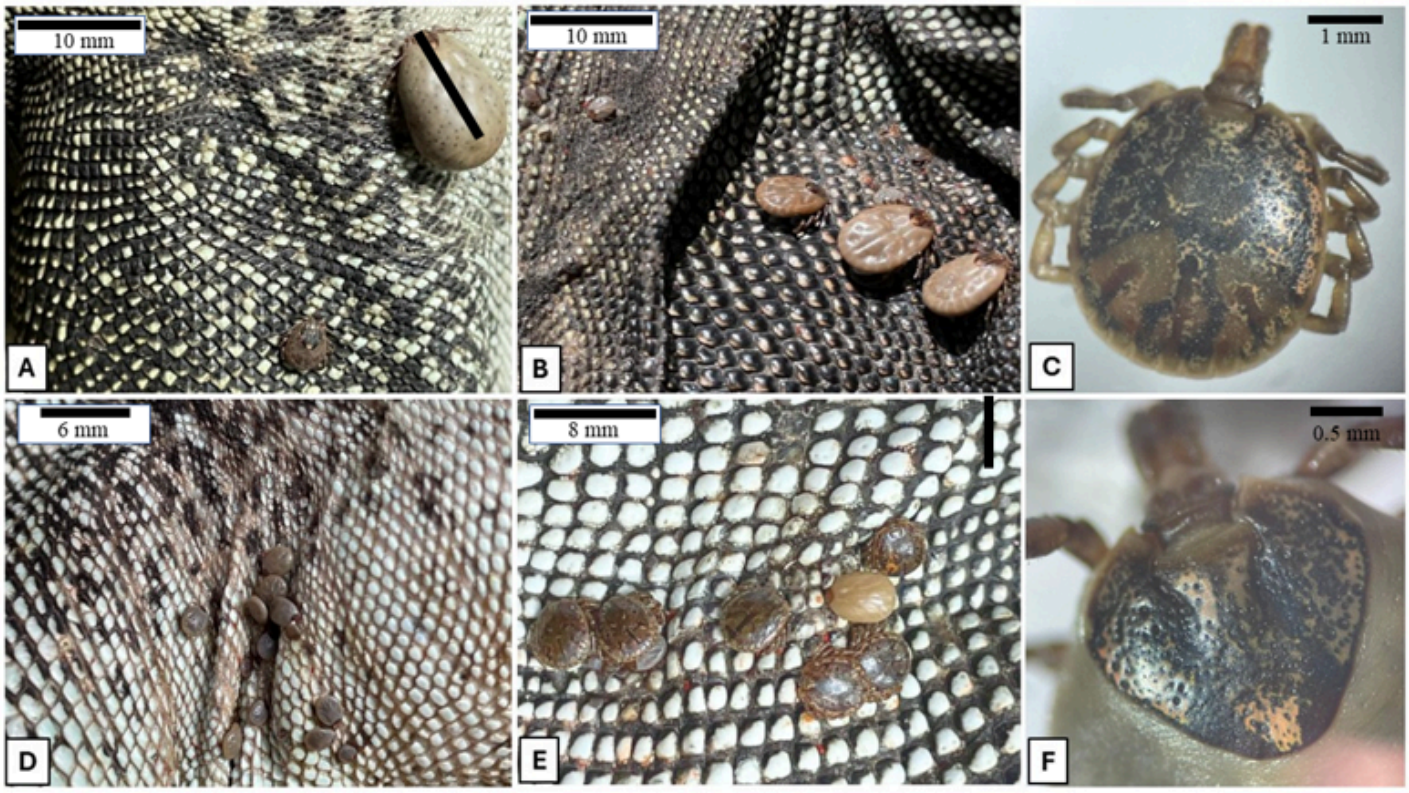


Figure 2

Males, females and nymhps of *Amblyomma rotundatum* (bisexual population) parasitizing *Iguana iguana* in the Amazon biome, Maranhão state, Brazil. Where A - (females), B - (females and nymphs), C - (Male collected from *I. iguana*), showing the lateral colorations in the scutum, center, and a slight pseudo-scutum, D - (nymphs), E-(males and nymphs) and F - (female).

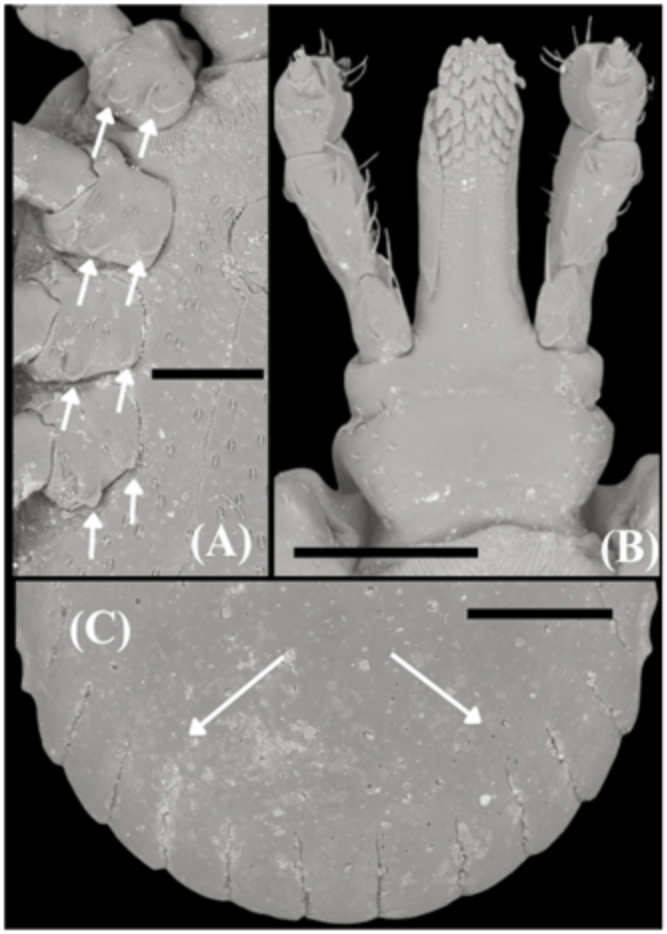


Figure 3

Amblyomma rotundatum, male, scanning electron micrography. (A) Ventral:Coxae I-IV with short spines, subequal length and rounded. (B) (Ventral) hypostomewith 3/3 dentition. (C) Dorsal: absence of marginal groove.

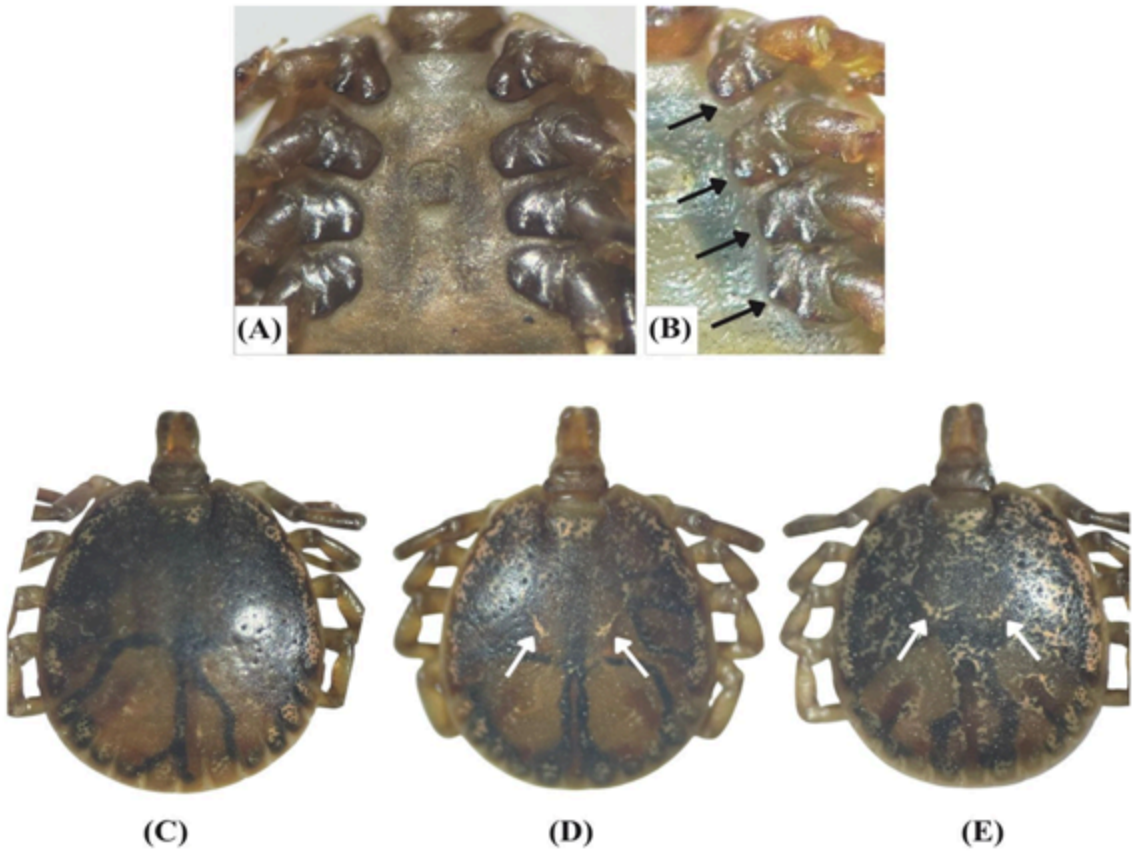


Figure 4

Amblyomma rotundatum, male, stereoscopic microscope. (A-B) Ventral:

Coxae I-IV with short spines, subequal length and rounded. (C-E) variation in dorsal ornamentation: (C) ornamentation on the side of the body, (D) ornamentation on the side and sign of a pseudo-escutum, (E) ornamentation distributed on the sides and center of the scutm, presence of a pseudo-scutum.