doi:10.12741/ebrasilis.v13.e899
e-ISSN 1983-0572
Publication of the project Entomologistas do Brasil
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Taxonomy and Systematic

Description of nymphs and ontogenetic morphometry of *Triatoma ryckmani* Zeledón & Ponce, 1972 (Hemiptera: Heteroptera: Reduviidae: Triatominae)

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EntomoBrasilis 13: e899 (2020)

Edited by:
William Costa Rodrigues

Article History:
Received: 29.v.2020
Accepted: 23.vii.2020
Published: 00.viii.2020

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Funding agencies:
Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)

Abstract. *Triatoma ryckmani* Zeledón & Ponce, 1972 is a species found in sylvatic habitat at Central America. Considered rare in the past and poorly studied, nowadays can be found inside houses with potential of dispersal and colonization. All five instars of *T. ryckmani* are described based on optical and scanning electron microscopy. The postembryonic development of the head analyzed by ontogenetic morphometry showed the largest changes occur in the first three instars. Information about morphology of the immature stages of disease vectors can be helpful in the identification process increasing speed and efficiency of control strategies.

Keywords: Chagas disease; immature stages; geometric morphometry; scanning electron microscopy; vectors.

The species of the subfamily Triatominae (Hemiptera: Heteroptera: Reduviidae) are true bugs specialized in blood-sucking. The subfamily is composed of 151 extant and three fossil species assigned to five tribes and 18 genera (Oliveira *et al.* 2020, Peixoto *et al.* 2020). All species are potential vectors of *Trypanosoma cruzi* (Chagas, 1909), the causative agent of Chagas disease (Galvão *et al*. 2003; Schofield & Galvão 2009). *Triatoma* Laporte, 1832 is the most speciose genus with 74 species, most of them without information on the morphology of the immature stages. Some species are able to colonize human dwellings, therefore the finding of immature stages inside houses is a key factor to identify colonization processes since nymphs do not move far. So, the identification of immature stages inside houses is important for the design of control strategies (Galvão *et al*. 2005).

Triatoma ryckmani Zeledón & Ponce, 1972, a species from Central America (Honduras, Nicaragua, Guatemala, El Salvador, and Costa Rica) was originally described based on a single female specimen found at an army camp in Salamar, Honduras (Figure 1). Subsequently, Lent & Wygodzinsky (1979) re-described the species using the original female holotype and three other females found on airplanes at the Miami airport, Florida, USA. One of these females was from Honduras and the other two were found in bromeliad freight from Guatemala. The male description was not published until 16 years later (Sherlock & Morera1972) with an allotype from Guanacaste province, Costa Rica.

Triatoma ryckmani shows a variety of habitat preferences, ranging from plants of the families Cactaceae, Mimosaceae and Bromeliaceae, as well as domestic and peridomestic structures (Marroquín *et al*. 2004, Marín *et al*. 2006). Monroy *et al*. (2004) evaluated the potential of dispersal and colonization of this species in an artificial environment and found that, although *T. ryckmani* is mainly found in wild habitats, this species can colonize human dwellings and chicken coops. In addition to the ability to colonize human houses, laboratory experiments found high infection rates with *T. cruzi* (about 90%) making this species of epidemiological concern in Central America (Zeledón *et al*. 2010). However, there are no reports of *T. ryckmani* naturally infected with *T. cruzi* (Marroquín *et al*. 2004).

Although epidemiologically important, currently there are few resources to identify Triatominae species beyond the morphology of adults. The scant resources available on the morphology of the immature stages include information on egg morphology for the *Rhodnius* Stal (1859) genus and a key to the genera of Triatominae based on first and fifth instar nymphs (Lent & Wygodzinsky 1979; Galvão 2014). In this paper, we extend the resources of Chagas epidemiology by

Figure 1. *Triatoma ryckmani*, male adult, deposited at CTIOC (n. 4973).

providing species-level descriptions of the five immature stages of *T. ryckmani* including an ontogenetic morphometric study of head development.

MATERIAL AND METHODS

The analyzed specimens came from a colony maintained at the Universidad Nacional de Costa Rica. A total of 46 specimens were used for geometric morphometric analysis (10, 13, 9, 7 and 7, from 1st through 5th instars respectively, plus 4 male and 3 female adults). All specimens were preserved in 70% alcohol and deposited in the "Coleção de Triatomíneos do Instituto Oswaldo Cruz", (CTIOC), Rio de Janeiro, RJ, Brazil, in a single batch. Another specimens were deposited in the CTIOC under voucher numbers 4973 (male), 3079 (male), 3080 (female), 3397 (female) and 3398 (male). The 12 specimens used for scanning electron microscopy 2(1°), 2 (2°), 2 (3°), 2 (4°), and 4 (5°) were preserved at Faculdade de Ciências Farmacêuticas, Departamento de Ciências Biológicas, Universidade Estadual Paulista Júlio de Mesquita Filho (UNESP), Araraquara, SP, Brazil.

Measurements were made with an ocular micrometer attached to a Zeiss Stemi SV11 stereoscopic microscope and photographs taken with a Nikon Coolpix 990 digital camera. The characters measured were: total length (CT), total length of the head (CTC), synthlipsis (RIO), anteocular region (RAO), postocular region (RPO), visible labium segments (R1, R2, R3) and antennal segments (A1, A2, A3, A4). The morphometric parameters of the nymphs was used according to Oliveira *et al*. (2015).

For ontogenic morphometrics, the dorsal surface of the head of each specimen was assayed from digital images for seven landmarks, as shown in Figure 2, using the TPSdig software, version 1.27 (James Rohlf). Shape variables (*x, y* coordinates) were subjected to generalized Procrustes superimposition and then, using the TPSrelw software 1.35 (James Rohlf), "partial warps" (weight matrix) were computed to investigate the ontogenetic profile. To quantify the variation among instars, relative warps were analyzed by Principal Component Analysis (PCA) and Canonical Variable Analysis using JMP 3.2.6 (SAS Institute). Additionally, the relationship between size and shape was analyzed using the TPSregr software for multivariate regression to compute the ontogenetic profile (thin-plate spline grid deformations) The geometric morphometry methodology was based on Oliveira *et al*. (2015).

For the Scanning electron microscopy (SEM), the specimens were dehydrated in a graded series of ethanol from 70% to 100%, cleaned with ultrasound, dried at 60 °C and then attached to metallic stubs using a double-sided stick tape and coated with gold. Specimens were analyzed using Topcon SM 300 (Topcon Corporation, Hasunuma-Cho, Tokyo Itabashi-ku, Japan) at the Institute of Chemistry of UNESP, Araraquara, São Paulo, Brazil, following Rosa *et al*. (1999). Digital images were edited with Adobe Photoshop 6.0.

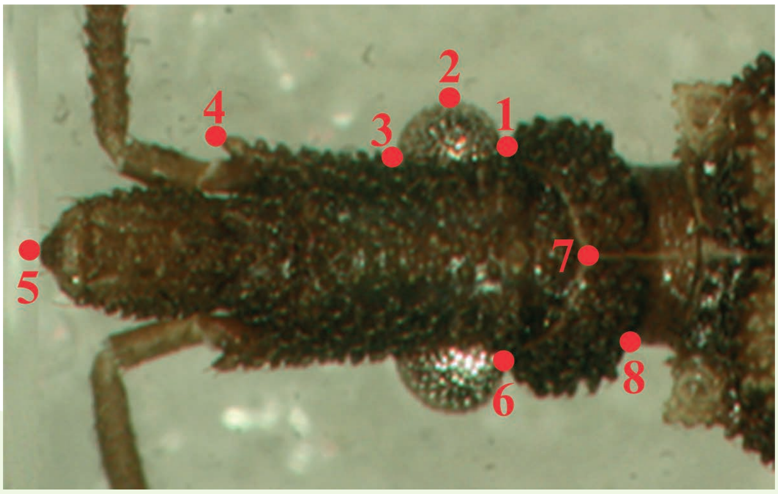


Figure 2. Digital image of dorsal surface of the head of *T. ryckmani* showing the seven landmarks captured at TPSdig software.

RESULTS AND DISCUSSION

Overall size and ratios, along with their standard deviations calculated from the body measurements and landmark head measures showed gradual growth from the 1st to the 5th instar in all the morphological characters. Specific details for each instar are given below.

Description of Nymphs

First instar (Figure 3). Total length of the body (average) 2.7 mm. The overall body color light brown and tegument rugose with bristles in setigerous tubercles. Head light brown-yellow and granulose with setigerous tubercles and light bristles. Rostrum attaining the anterior border of prosternum. Ratio of rostral segments 1: 2.2: 1.2. Clypeus light with rounded genae, reaching the apex of antenniferous tubercles, but not attaining the apex of anteclypeus. Postocular sutures well delimited, converging in a "Y" shape. The ratio between the width of the anteocular region and postocular region 1: 0.5, while the ratio between the width of the head at the level of eyes and the synthlipsis region, 1: 1.2. The postocular sulcus clearly visible. Antennae pillose, yellow. The first antennal segment not attaining the apex of the head. The ratio of the antennal segments: 1: 1.8: 2.3: 2. Eyes lacking ommatidea in the posterior portion. Thorax rugose with setigerous tubercles, overall color brown. Prontonum larger than meso- and metanotum, with 1 + 1 sclerotized plates. Anterolateral angles rounded with setigerous tubercles. Metanotum dark, formed only by 1 + 1 sublateral sclerotized plates. Legs yellow with setigerous tubercles with small golden bristles in all segments. Abdomen brown, lighter than the head and thorax, with several lighter setigerous tubercles. Urotergites with bristles arranged in transverse rows, external border of connexivum presenting bristles, without connexival spots.

Second instar (Figure 3). Similar to the first instar except total length of the body (average) 4.6 mm. Overall color darker. Head longer. Ratio of the anteocular to post-ocular region 1: 0.4 and between the width of the head at the level of eyes and the interocular distance 1:1.6. Antennal segments darker than with light rings between segments. Ratio of antennal segments 1: 2.6: 2.9: 4.0. Rostrum dark brown. Ratio of rostrum segments: 1: 2.4: 1.2 (Tab I). Pronotum trapezoidal with 1 + 1 light stripe presenting tubercles. Mesonotum with 1 + 1 irregular light-colored areas, setiferous tubercles. Metanotum with 1 + 1 light-colored spots in the external border. Legs yellowish brown. Urotergites with two transverse rows of bristles and abdomen with connexival marks in the middle part of the lateral borders.

Third instar (Figure 3). Similar to the second instar except total length of the body (average) 5.7 mm. Overall color dark brown. Head with light areas from the basal portion of the jugae to the anteclypeus. Ratio between the length of postocular and anteocular region 1: 0.5 and between the width of the head at the level of eyes and the synthlipsis 1: 1.5. First and second antennal segments lighter than third and fourth, but fourth antennal segment presenting the apical half lighter-colored. Ratio of antennal segments 1: 2.8: 3: 3.4. Ratio of rostrum segments 1: 2.8: 1.4. Thorax with overall color dark brown with setiferous tubercles randomly distributed and bristles on the lateral border. Pronotum with 1 + 1 light-colored spots on the hind angles, divided by a longitudinal midline in two equal plates. Mesonotum dark brown with 1 + 1 rectangular plates divided by a lighter longitudinal line, with a well defined 1 + 1 yellow spots and bristles inserted in tubercles. Metanotum dark brown to black with 1 + 1 rectangular plates with bristles. Legs presenting numerous bristles, with the coxae and trochanter lighter in color than the tibia and tarsus. Abdomen granulose with more evident connexival spots.

Fourth instar (Figure 4). Similar to the third instar except total length of the body (average) 8.5 mm. Overall color darker. Head presenting a grainy appearance around the eyes. Ratio between the length of anteocular region and postocular region 1: 0.4 and between the width of the head at the level of eyes and the synthlipsis is 1: 0.7. First antennal segment with dark spots. The ratio of antennal segments 1: 5.1: 6.7: 5.0. Ratio of rostrum segments 1: 4.8: 1.5. Pronotum with 1 + 1 lighter stripes on the apex with setiferous tubercles and dark glabrous areas. Mesonotum with 1 + 1 lighter stripes at lateral border and glabrous, dark areas. Abdomen with many sensillas and small lighter spots. Connexival spots bigger.

Fifth instar (Figure 4). Similar to fourth instar except total length of the body (average) 11.0 mm. Head dark brown with borders presenting a lot of tubercles, giving a grainy aspect. Anteocular region, in the ventral view, with brown color. Ratio of the anteocular region to postocular region 1: 0.3, and between the width of the head at the level of the eyes and synthlipsis 1: 1.7. Antennae are dark brown with fourth segment lighter. Ratio of antennal segments 1: 4.1: 3.4: 4. Ratio of rostrum segments 1: 5.1: 1.3. Metanotum with 1 + 1 alar thecae, overall color lighter than pro- and metanotum. Abdomen with connexival spots larger with the presence of scattered bright spots.

Ontogenic morphometrics

The first two Principal Components (PC) together represent 85.7% of the variability (PC1 = 71.3% and PC2 = 14.4%). The factorial map of the first two Principle Components show a clear separation between the first through fourth instars, and strong overlap of the fourth and fifth instars,

The Canonical Analysis, based on principal components of each specimen, shows the changes in shape and strong discrimination among all instars. The 1st, 2nd, 3rd and 4th instars show stronger discrimination, while there is overlap of the 4th and 5th, reflecting the PC results.

The ontogenetic profile highlights the allometry of a particular instar, and the transformation grids compare the changes of the shape from 1st to 5th instars nymphs. The analysis shows differences of length, width, as well as the position of antenniferous tubercles and eye size (Figures 5, 6).

Scanning electron microscopy

It was observed that the setigerous granules increase in number and their relative placement changes during development, forming distinct outlines for each instar (Figure 7).

The tenth abdominal segment is annular with bristles around the anal opening only in males, and presence of granules and bristles in the internal part. The granules become more evident on the third instar; and the number of bristles increases. In females, the anal opening with has depressions and no bristles (Figure 8).

Stridulatory sulcus is delimited by sensilla and setiferous tubercles in all stages. First and second instars present a triangular shape sulcus with few transversal stripes. Third, fourth and fifth nymphal stages have a cone shaped sulcus, elongated and with a gradual increase on the number of stripes. The border gradually becomes elevated during the postembryonic development (Figure 9).

Relative size between rostrum and stridulatory sulcus was distinct in the 4th instar, when the third rostrum segment almost reached the final portion of stridulatory sulcus, for the first through third and fifth instars it was shorter.

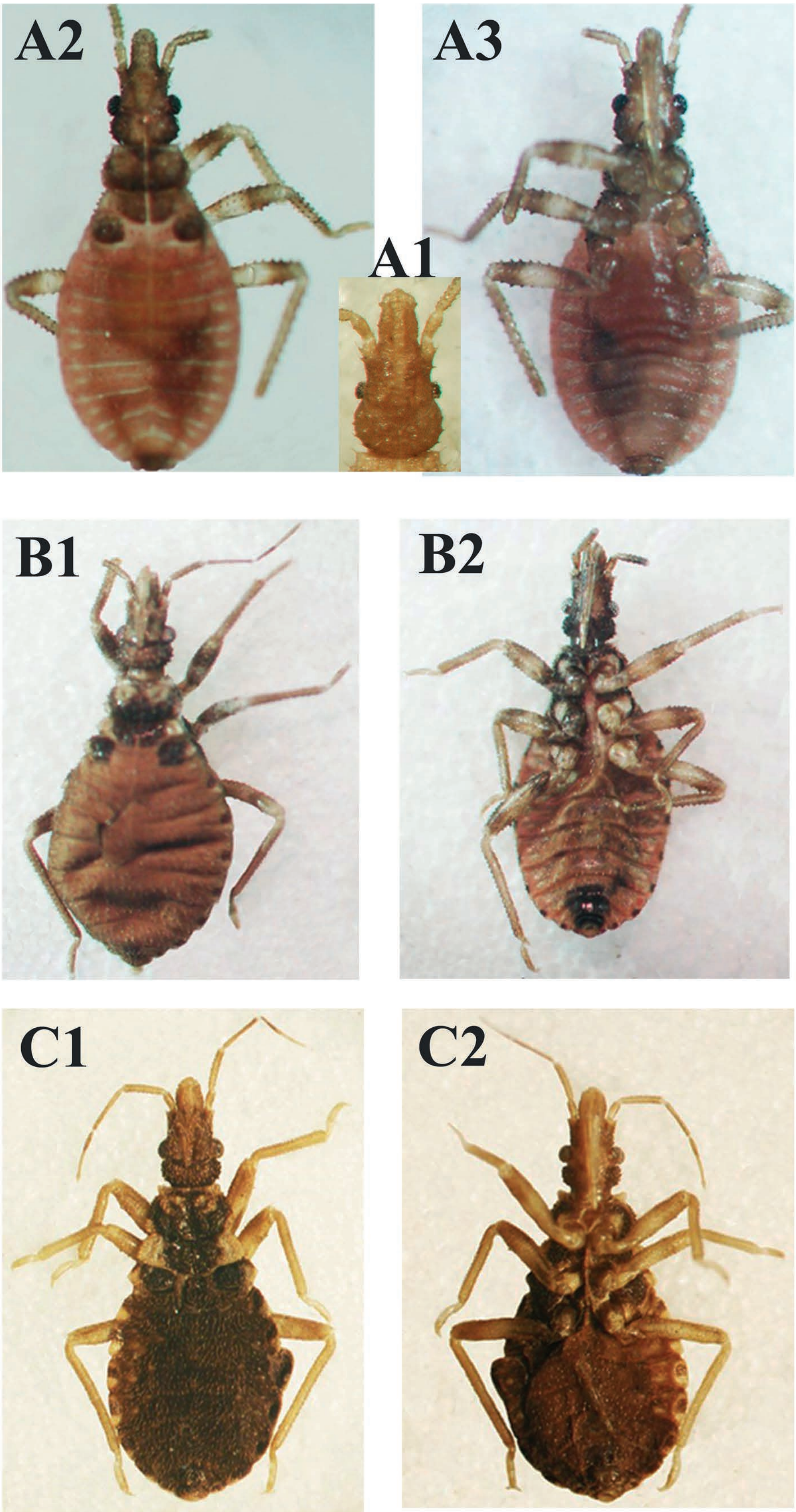


Figure 3. *Triatoma ryckmani* immature stages. First instar (size 2.7mm): A1 - dorsal surface of the head; A2 - dorsal surface of the body; A3 - ventral view. Second instar (size 4.6 mm): B1 - dorsal surface of the body; B2- ventral view. Third instar (size 5.7 mm): C1 - dorsal surface of the body; C2- ventral view.

According to Galvão *et al*. (2005), the study of immature stages is very important because they show epidemiological and systematic importance. Although, the identification of specimens is still centered in morphological traits of the adults and the determination based on nymphs' characters remains a challenge (Oliveira *et al*. 2015). In this study, we describe for the first time the five nymphal instars of *T. ryckmani*, a species previously rare and poorly studied considered as a sylvatic insect capable to move between many habitats (sylvatic, dry forest, peri-domestic and domestic) with high colonization capacity (chicken coops) (Monroy *et al*. 2004; De La Rua *et al*. 2014).

According to Lent & Wygodzinsky (1979), *T. ryckmani* is a "taxonomically isolated species", years later, in a phylogenetic study, Justi *et al.* (2016) showed that it was related to *Triatoma*

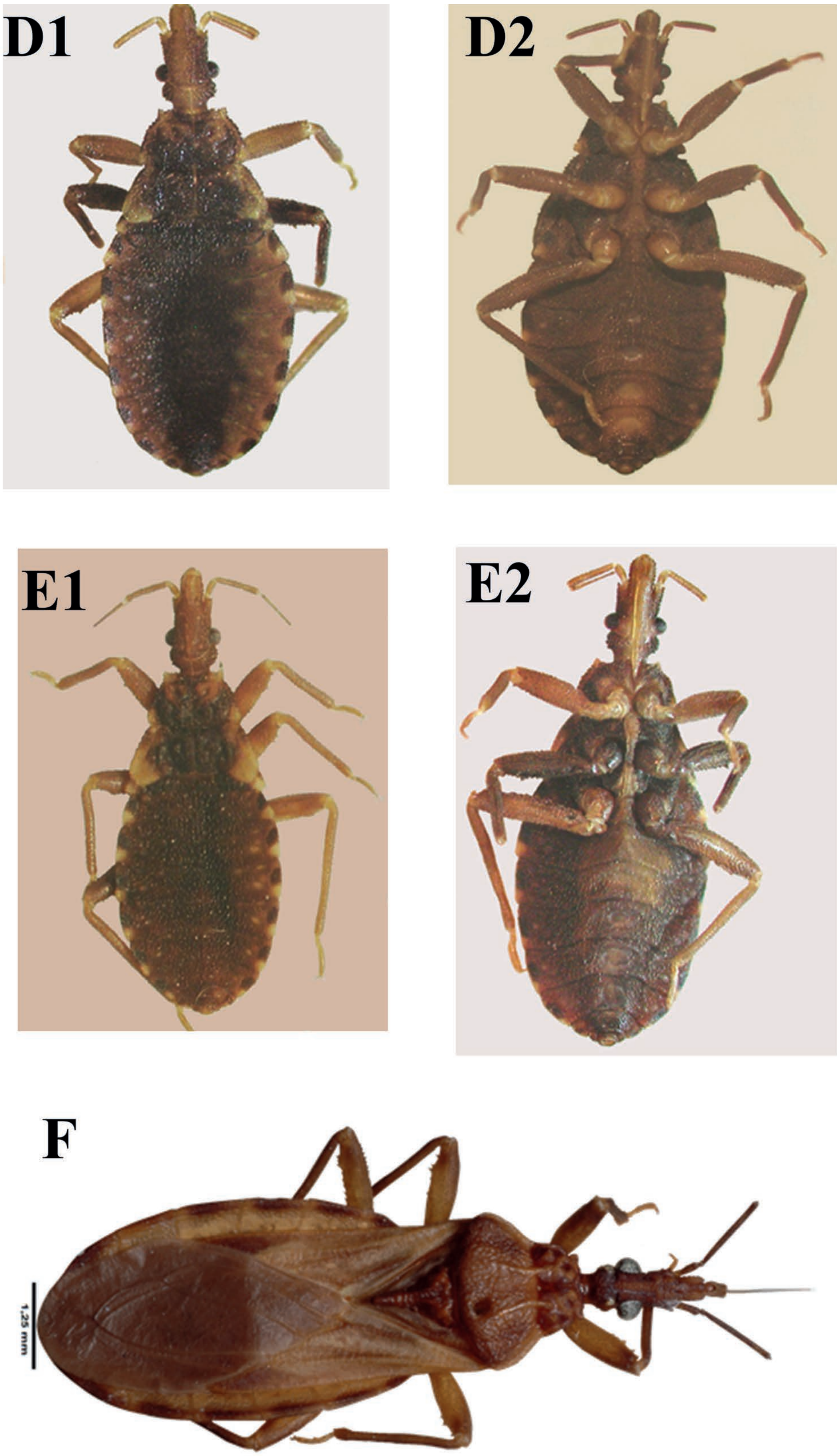


Figure 4. *Triatoma ryckmani* immature stages. Fourth instar (8.5 mm): D1 - dorsal surface of the body; D2- ventral view. Fifth instar (size 11.0 mm): E1 - dorsal surface of the body; E2- ventral view. Adult: F**.**

rubida (Uhler, 1894). One distinguishing nymphal characteristic for this species is the gradual and continuous darkening of the head and thorax, from light brown in the early stages, to dark brown later in the development. Throughout development, the first two antennal segments are darker than the third and fourth antennal segments. The junctions of each segment also have lighter color.

The observation of distinct pilosity patterns in *Triatoma* species (previously studied) suggests this is a taxonomically important character across the genus. The pilosity pattern on the last abdominal segment of *T. ryckmani* can be very useful in the morphological differentiation from other species of *Triatoma* (Jurberg *et al*. 2002; Raigorodschi *et al*. 2011; Rocha *et al*. 2005, 2009; Silva *et al*. 2005).

The stridulatory sulcus of the nymphs acquires a shape of V, with papillae, tubercles and sensilla delimiting it, as

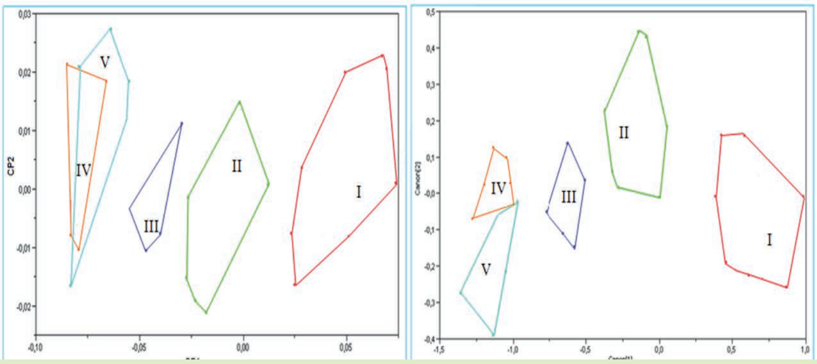


Figure 5. Factorial map of the first two Principal Components (PC1 and PC2) for each nymphal stage (1st-I, 2nd-II, 3rd-III, 4th-IV, 5th-V, M-adult).

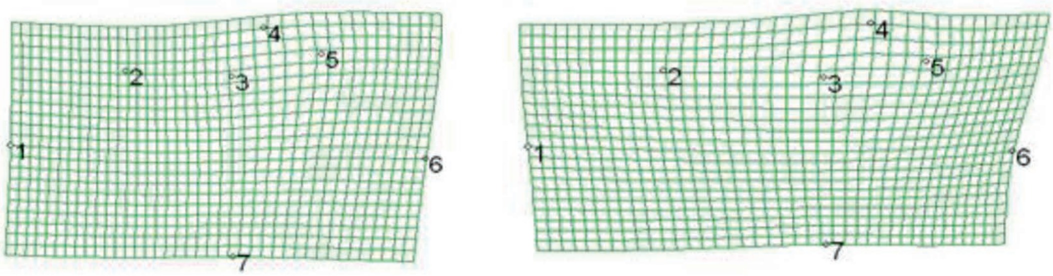
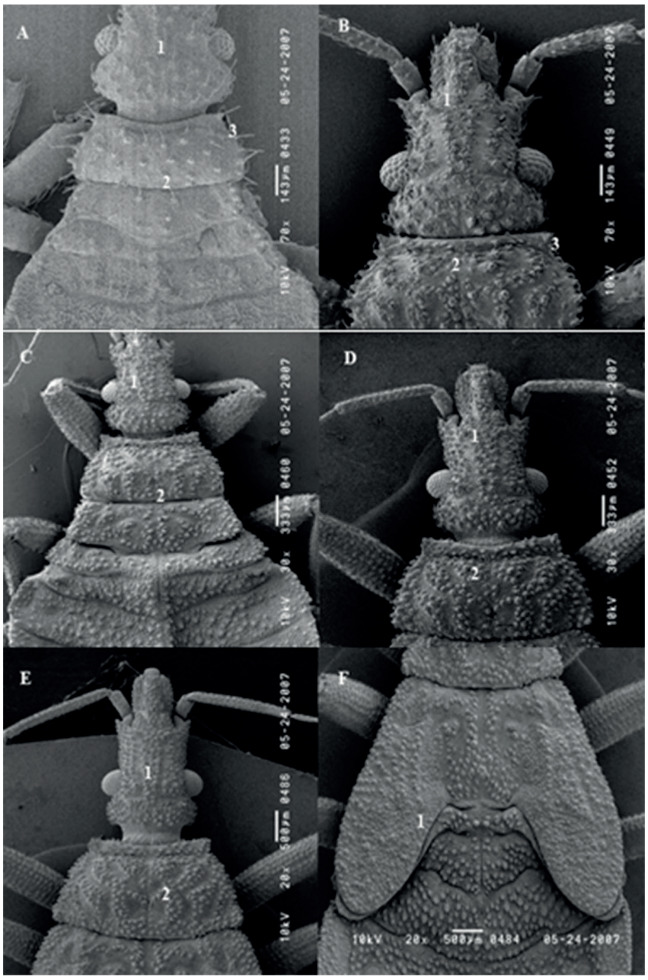


Figure 6. Transformation grids of the ontogenetic profile comparing the development of the deformations with the extreme values of the axis x and y (indicated by narrows).



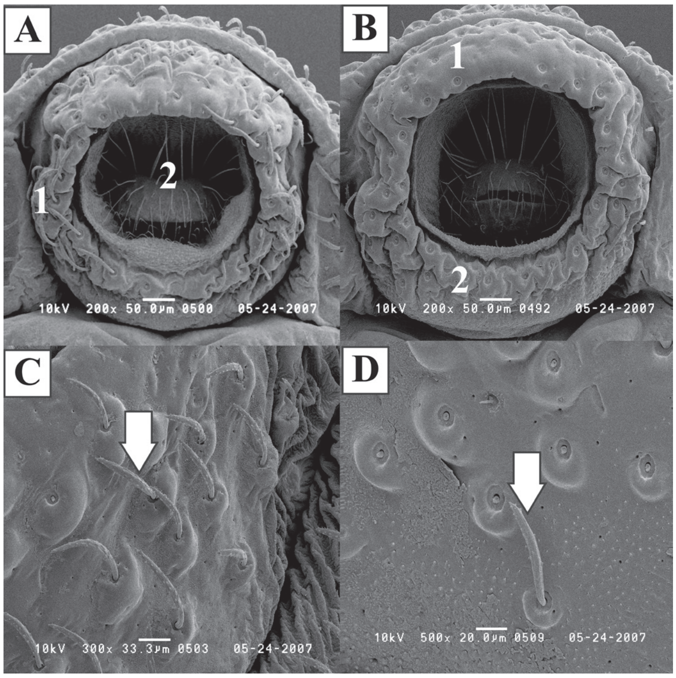


Figure 8. Scanning electron micrographs of 5th instar nymphs: A - fine bristles inside the anal disk 200X magnification: (1) - bristles, (2) fine bristles inside the anal disk. B - X abdominal segment of a nymph 200X magnification: (1) absence of bristles, (2) increased roughness on edge; C - detail of bristles on the 8th abdominal segment 300X magnification; D - detail of single bristle on the 8th abdominal segment 500X magnification.

Figure 7. Scanning electron micrographs showing changes in the integument of the cephalic capsule and thorax at various instars of development. A - 1st instar: (A1) integument with scattered granules, (A2) scattered granules and sensilla in the thorax region, (A3) large and irregular sensilla; B - 2nd instar: (B1) integument head with granules presenting a regularity in positioning, (B2) pronotum with organized granules, (B3) smaller, down-turned sensilla; C - 3rd instar: (C1) granules at the center of the head capsule, (C2) granules forming ornamentation in the thorax; D - 4th instar, (D1) and (D2) integument with granules forming ornamentation; E - 5th instar: (E1) and (E2) ornamentation in the integument resulting from organization of the granules; F - the wing bud (F1) which characterizes the 5th instar.

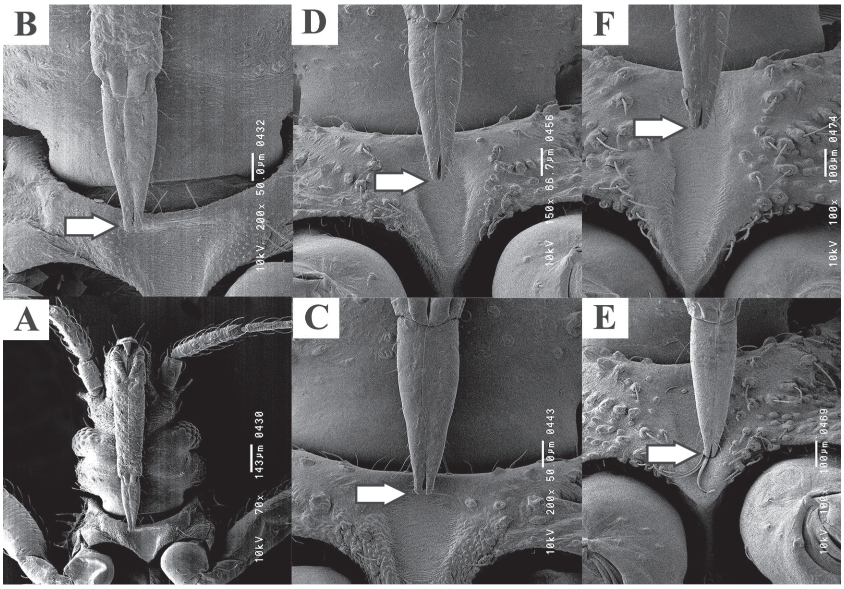


Figure 9. Scanning electron microsgraphs of rostrum and stridulatory sulcus; A - first instar: third rostrum segment reaching the apex of stridulatory sulcus; B - first instar: stridulatory sulcus wide with a few transverse stripes; C - second instar: third rostrum segment in the same position in the previous instar; D - third instar: third rostrum segment in the same position in the previous instar ; E - fourth instar: third rostrum segment reaching the final portion of stridulatory sulcus; F - fifth instar: third rostrum segment in the same position in the first, second and third instars stridulatory sulcus.

previously observed in other nymphs of Triatominae, such as *Linshcosteus karupus* Galvão, Patterson, Rocha & Jurberg, 2002, *Belminus herreri* Len & Wygodzinsky, 1979, *Triatoma vandae* Carcavallo *et al*., 2002, *Triatoma williami* Galvão, Souza & Lima, 1965, *Triatoma jurbergi* Carcavallo, Galvão & Lent, 1998 and *Triatoma guazu* Lent & Wygodzinsky, 1979 (Galvão *et al*. 2002; Galvão *et al*. 2005; Rocha *et al*. 2005; Silva *et al*. 2005). Head shows many setigerous tubercles unevenly distributed in the first instar. From the second to fifth instar there is a gradual organization of such tubercles, resulting in a different ornamentation for each instar (Silva *et al*. 2003; Rocha *et al*. 2005; Silva *et al*. 2005). The rostrum segments shows a unique and curious development in relation to the stridulatory sulcus. The apex of third rostral segment changes the position only in the third instar, reaching the anterior border of stridulatory sulcus (in other studies species of *Triatoma* in the first instar the apex reach the prosternum).

The geometric morphometrics is widely used in different approaches in Triatominae. This tool is useful to sylvatic and domestic differentiation, to discriminate field and laboratory populations, or between geographic regions, and to taxonomic or phylogenetic inferences (Dujardin *et al.* 1997; Jaramillo *et al.* 2002; Monroy *et al.* 2003). Geometric morphometrics studies among nymphal instar, could be a useful tool for the characterization during the species development, however, only two studies, in different genera have been published so far, *L. karupus* (Galvão *et al*. 2005) and *B. herreri* (Rocha *et al.* 2005). Our study agreement with both previous studies that conclude the geometric morphometrics is an informative tool for the detection of anatomical variation, on the other side, the analysis in a comparative way of three unrelated genera is not easy. Our data about geometric morphometry provide information about the ontogenetic profile of each instar, showing that the biggest conformational and size changes are observed in early development. SEM has revealed structures that differentiate each stage, for example: the area of stridulatory sulcus, shape of the pronotum, the distance between setiferous tubercles, and other structures such as the position of the rostrum relative to the stridulatory sulcus or the structure of the abdominal segments and sensilla.

Because important vectors have, amongst other characteristics, the ability to colonize human dwelling, finding immature stages inside houses could be suggestive of the colonization process. In addition, often only immature stages are found inside houses, and being able to rapidly identify the taxon is important for the design of control strategies. For this reason, systematic and phylogenetic studies are necessary for efficient control. The description of morphological and morphometric data on immature stages can be greatly aided if new characters can be found in immature forms, increasing the identification process, the speed and efficiency of control strategies of Chagas' disease vectors.

ACKNOWLEDGMENTS

We are very grateful to Rodrigo Zeledon, Universidad Nacional de Costa Rica, for providing the specimens of *Triatoma ryckmani*. The senior author (CG) thanks "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq) for financial support.

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Suggestion citation:

Rocha, DS, C Dale, JA Da Rosa & C Galvão, 2020. Description of nymphs and ontogenetic morphometry of *Triatoma ryckmani* Zeledón & Ponce, 1972 (Hemiptera: Heteroptera: Reduviidae: Triatominae) . EntomoBrasilis, 13: e899.
Available in: doi: 10.12741/ebrasilis.v13.e899

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