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Ultrastructure and morphometry of eggs of *Triatoma rubrovaria* (Blanchard, 1843), *Triatoma carcavalloi* Juberg, Rocha & Lent, 1998 and *Triatoma circummaculata* (Stål, 1859) (Hemiptera-Reduviidae-Triatominae)

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Abstract

This study analyzed the body and the operculum of eggs of *Triatoma rubrovaria*, *T. carcavalloi* and *T. circummaculata*, considered sylvatic species that live in sympatry.*Triatoma rubrovaria* is currently considered the most important vector of *Trypanosoma cruzi* in the rural areas of the state of Rio Grande do Sul, followed by *T.circummaculata*. Significant differences other than morphometry have been observed in the egg structures of the three species using traditional microscopy and scanning electron microscopy. *Triatoma circummaculata* eggs are smaller than those of *T.rubrovaria* and *T. carcavalloi*. The average number of perforations in corionic cells in the egg body is higher for the *T. rubrovaria*. The average number of perforations in the operculum cell is higher in *T. circummaculata*. This is the first morpho-structural description of *T. carcavalloi* eggs. These results widen the concept of these three species and create new subsidies for the entomological monitoring in areas in which these vectors may infest human living quarters.

Key words: Scanning electron microscopy, eggs, Triatominae, *Triatoma rubrovaria*, *Triatoma carcavalloi*, *Triatoma circummaculata*

Introduction

The historic context of Chagas disease in Rio Grande do Sul mentions eleven species recognized as vectors, including *Triatoma infestans* (Klug, 1834), as the main vector of the disease's etiological agent, *Trypanosoma cruzi*. This triatomine has been targeted by control measures and attempts at eradication, according to the campaign of Cone Sul Initiative (Vinhaes & Dias 2000, WHO 2002, Martins *et al.* 2006, Ceballos *et al.* 2011). After eradication measures against *T. infestans* in the state of Rio Grande do Sul, this vector was considered eliminated in June 2006, when the Brazilian Ministry of Health was granted the International Certification of Elimination of Chagas' Disease Transmission for this triatomine by the Pan-American Health Organization (Ferreira & Silva 2006, Schofield *et al.* 2006, Sonoda *et al.* 2010). Barata (1998) mentioned that while the vector is now eliminated, it is still necessary to continue studying these insects, in particular relatively little-known stages including the eggs. According to data obtained by the Brazilian National Health Foundation (Funasa) over the last 20 years during the Chagas Disease Control Program (PCDCH), *Triatoma rubrovaria* (Blanchard, 1843) (Fig.1–1) is now the most commonly captured triatomine in southern Brazil (Almeida *et al.* 2000, 2005). In spite of all efforts to control it, *T. infestans* still occurs in some towns in the northwestern, central, and southern areas of the state (Ruas-Neto & Corseuil 2002, Dias 2007, Sonoda *et al.* 2009).

According to Lent & Wygodzinsky (1979), the geographical distribution of *T. rubrovaria* in Brazil used to be concentrated in the states of Paraná and Rio Grande do Sul, but is currently restricted to the endemic area of

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Chagas disease in Rio Grande do Sul (Rosa, 1995). In its natural environment this species lives in rocky fissures and invades human living quarters when its own environment is intensely altered by man and also when *T.infestans* is eliminated from a household, leaving an empty available niche to be filled (Salvatella *et al.* 1995, Almeida *et al.* 2000, Oscherov *et al.* 2004, Santos *et al.* 2009).*Triatoma rubrovaria* gradually adapts to the peri-household and colonizes the intra-household, becoming an important secondary vector (Salvatella *et al.* 1994, Almeida *et al.* 2002). This species feeds on a variety of hosts including man. The period of time between feeding and the first defecation is short, typical of an efficient vector of the etiologic agent of Chagas disease (Bar *et al.* 2003).

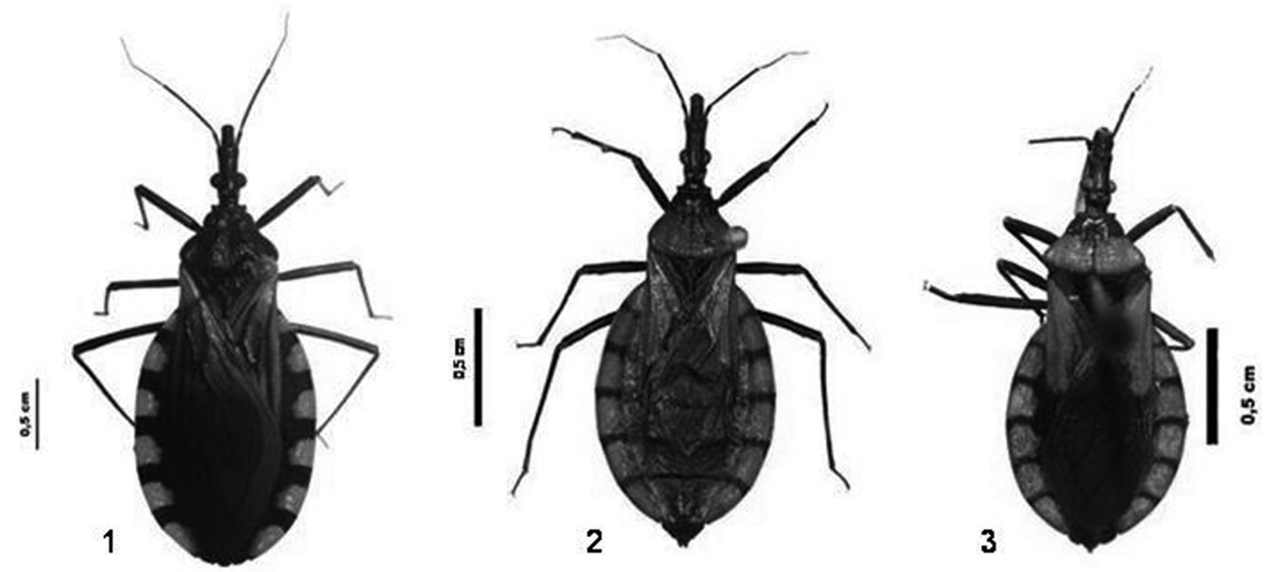

FIGURE 1. *Triatoma rubrovaria*, *Triatoma carcavalloi* and *Triatoma circummaculata*.

Triatoma carcavalloi Jurberg, Rocha & Lent 1998 (Fig.1–2) was described based on four females collected in the municipalities of Santana do Livramento, Canguçu, Jaguarão and Dom Feliciano (RS). This species is sympatric with *T. rubrovaria* and *T. circummaculata*,lives in the peri-household and in the living quarters themselves. For a long time was considered similar to *T. rubrovaria* but can be distinguishedby the intense orange color of the posterior lobe of the pronotum, the chorion and the sharp angle anterior aspect of pronotum, besides themorphologic characteristics regarding length and width of head, the rostrum, eye size and smaller abdomen, giving it a rounded shape, and of the gonocoxite of the ninth segment. After its 1998 description this species was recorded in the municipality of Encruzilhada do Sul (RS); a specimen in the nymphal stage was captured and reached its adult phase in the laboratory (Almeida *et al.* 2002).

Triatoma circummaculata (Stål, 1859) (Fig. 1–3) is the most important species in Uruguay after *T. infestans*. Its geographical distribution in Brazil is restricted to Rio Grande do Sul. This species occupies ecotopes similar to those of *T. rubrovaria* and the two can sometimes be found together (Lent & Wygodzinsky 1979, Rosa *et al.* 2000). Phylogenetic studies with *T. circummaculata* based on DNA sequencing included this species in the *T. infestans* complex, although that would not be the case if only the morphologic classification were taken into consideration (Garcia *et al.* 2001). Sainz *et al.* (2004) also analyzed sequences of *T. circummaculata* mitochondrial DNA based on morphology and confirmed the inclusion of this species, but as a member of a different complex within the *T. infestans* complex.

The aim of this study was to make a comparative analysis of the morphologic and morphometric aspects of the exochorion of bodies and opercula of eggs of *T. rubrovaria*, *T. carcavalloi* and *T. circummaculata* usinglight microscopy (LM) and scanning electron microscopy (SEM). We also studied the homogeneity of the diameter of the egg operculum.

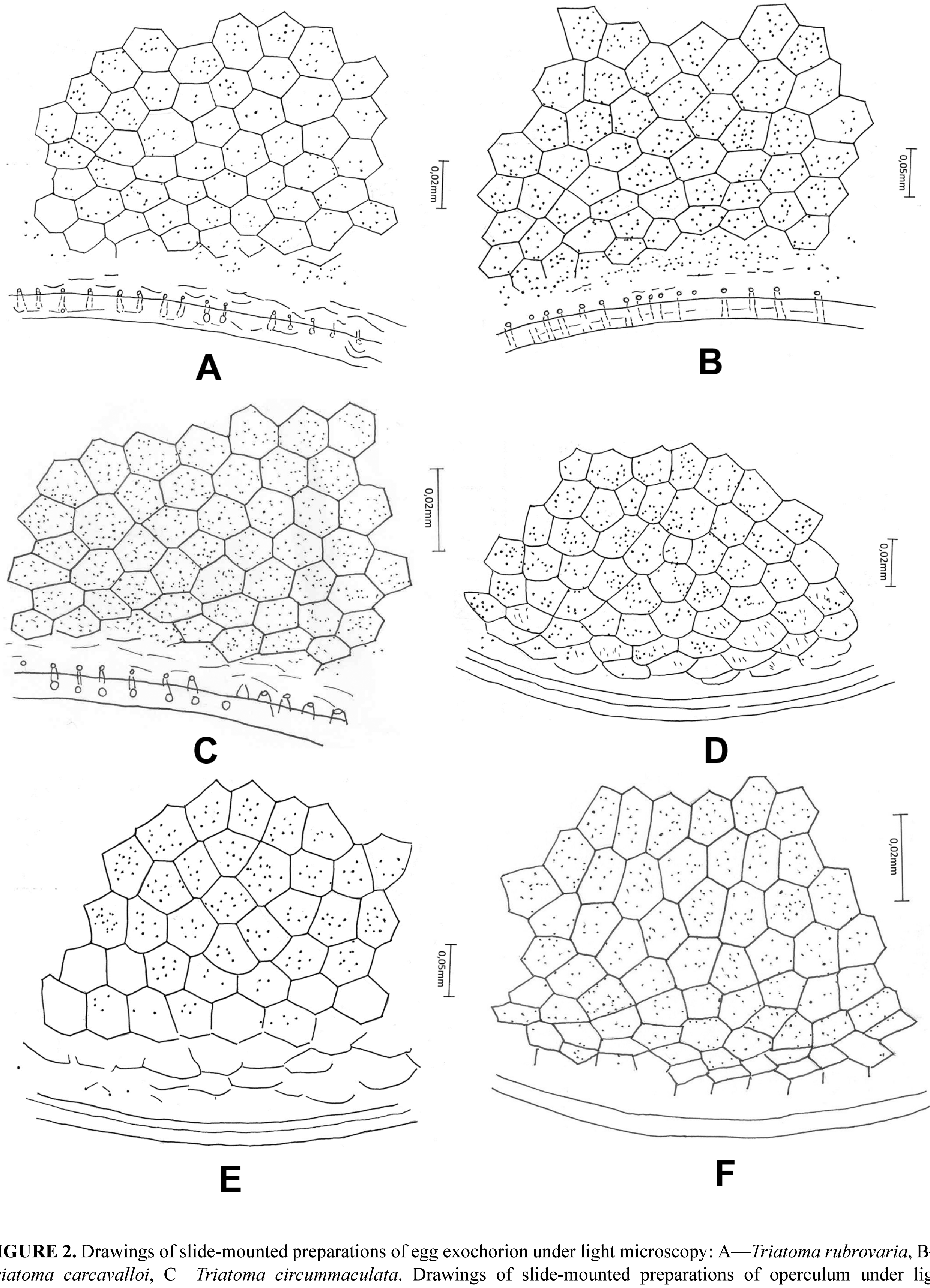

FIGURE 2. Drawings of slide-mounted preparations of egg exochorion under light microscopy: A—*Triatoma rubrovaria*, B—*Triatoma carcavalloi*, C—*Triatoma circummaculata*. Drawings of slide-mounted preparations of operculum under light microscopy: D—*Triatoma rubrovaria*, E—*Triatoma carcavalloi*, F—*Triatoma circummaculata*.

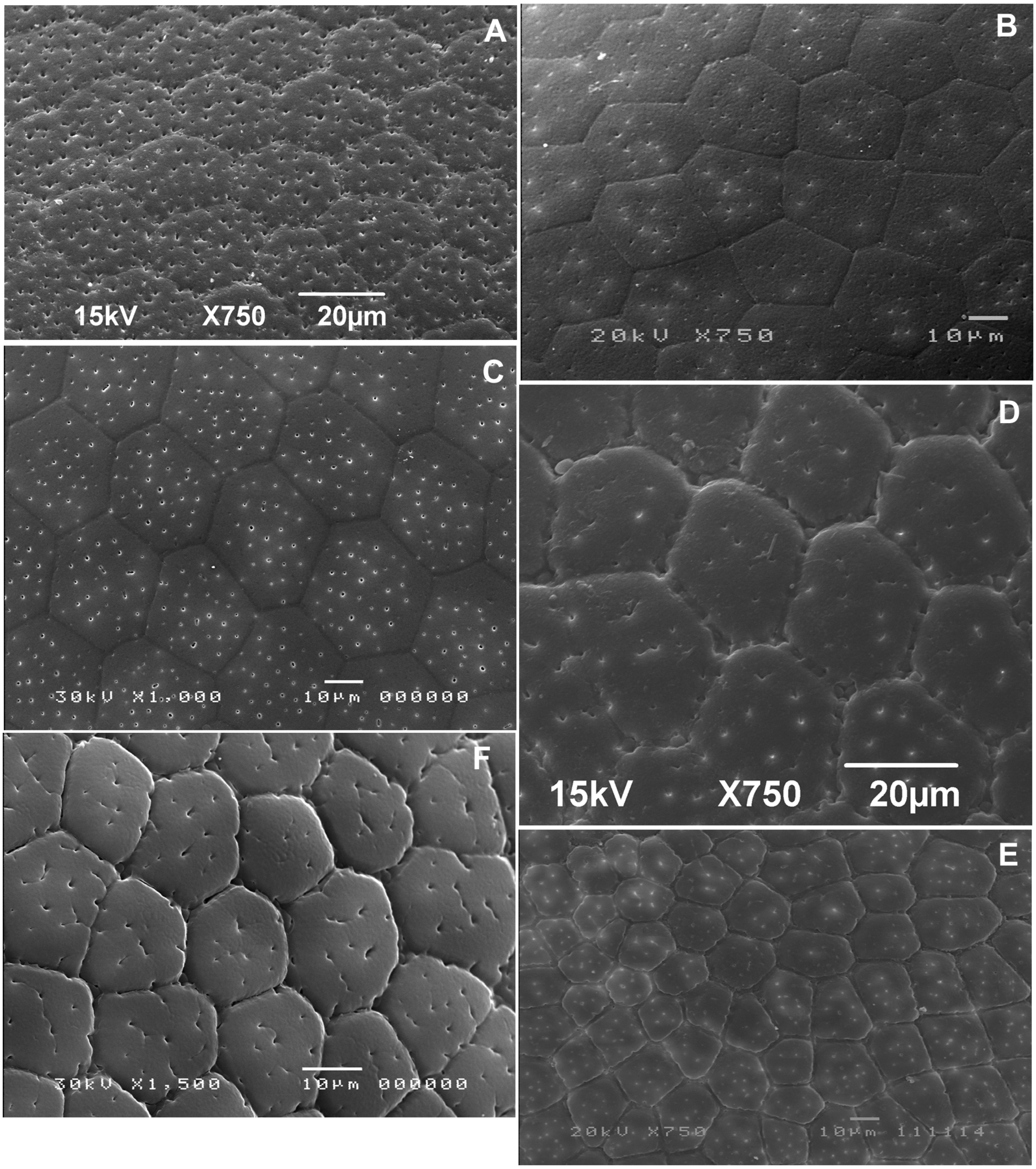

FIGURE 3. Scanning electron microscopy of exochorion of the eggs: A—*Triatoma rubrovaria*, B—*Triatoma carcavalloi*, C—*Triatoma circummaculata*.Scanning electron microscopy of exochorion of the operculum: D—*Triatoma rubrovaria*, E—*Triatoma carcavalloi*, F—*Triatoma circummaculata*.

Material and methods

The eggs of *T. rubrovaria*, *T. carcavalloi* and *T. circummaculata*were obtained from colonies maintained in Setor de Entomologia Médica e Forense, Laboratório de Transmissores de Leishmanioses, Instituto Oswaldo Cruz. The field specimens had been collected in the natural ecotope in October 2005 and October 2011 in the municipality of Encruzilhada do Sul (30°32'38"S; 52°31'19"W).

Light microscopy. For morphometric analysis 30 eggs (body and operculum)were placed on double-sided adhesive tape that was stuck onto a slide for subsequent viewing under a stereoscopic microscope to measure lengthand width.

Drawings of slide-mounted preparations of the entire operculum and egg body fragments were made using a camera lucida on a Leitz-Dialuz 20EB light microscope at 40x enlargement.

Morphometric data were submitted to variance analysis (ANOVA) at a significance of 5% and SNK (Student-Newman-Keuls test) to compare all pairs of means following one-way ANOVA. In all tests SPSS- Windows, version 10 was used.

Scanning electron microscopy. For the morphological analysis 10 egg bodies and 10 opercula were placed on small pieces of double-sided adhesive tape that were adhered onto scanning electron microscopy (SEM) stubs. They were sputtered with gold and observed in a JEOL 5310 scanning electron microscope at 750x, 1000x and 1500x enlargement. The pictures were made in the central zone of the exochorion of body and lid of the eggs and thirty cells of these regions were counted in determining the number of perforations.

Results

The exochorion of *T. rubrovaria, T. carcavalloi* and *T. circummaculata* eggs are similar, with hexagonal rough areas divided by dots and demarcated with short intercepted lines or not by dots (Fig. 2A, 2B, 2C). The operculum of all three species is externally convex, formed by hexagonal cells, mostly with an "upholstered" aspect (Fig. 2D, 2E, 2F). The limiting opercular line of the three species consists of a cementing mass called thesealing band, which fixes the operculum to the chorial edge of the egg.

Average width and length measurements of the egg exochorium are provided in Table 1. Average operculum diameter is shown in Table 2.

TABLE 1. Morphometry of egg bodies of *Triatoma rubrovaria*, *Triatoma carcavalloi* and *Triatoma circummaculata*.

Species		*T. rubrovaria*	*T. carcavalloi*	*T. circummaculata*
Measures (mm)				
Length	maximum	1,77	1,81	1,54
	minimum	1,54	1,38	1,40
	average	1,65	1,59	1,47
Standard Deviation		0,059	0,116	0,042
Width	maximum	1,23	1,31	0,94
	minimum	1,11	1,15	0,82
	average	1,17	1,23	0,87
Standard Deviation		0,039	0,055	0,039

TABLE 2. Morphometry of operculum of *Triatoma rubrovaria*, *Triatoma carcavalloi* and *Triatoma circummaculata*.

Species		*T. rubrovaria*	*T. carcavalloi*	*T. circummaculata*
Measures (mm)				
Diameter	maximum	0,76	0,70	0,64
	minimum	0,66	0,58	0,52
	average	0,71	0,64	0,56
Standard Deviation		0,031	0,034	0,039

The statistic test (ANOVA) highlighted a significant difference between the three populations in terms of egg body size as well as opercular diameter ($p<0,05$, $p=0,0$). We applied the test Student-Newman-Keuls (SNK) test, obtaining a pattern of separation in groups according to the averages obtained for each sample. Each character was analyzed separately (width, length and diameter). The final results show that there were three separate groups for egg width and operculum diameter, and only two for length (Table 3).

TABLE 3. Homogeneity of egg width of *Triatoma rubrovaria*, *Triatoma carcavalloi* and *Triatoma circummaculata.* Student-Newman-Keuls (SNK) test.

		Set		
Population	N	1	2	3
T. circummaculata	30	0,8773		
T. carcavalloi	30		1,1673	
T. rubrovaria	30			1,2313
Significance	30	1,000	1,000	1,000

N= number of eggs.

Thirty exochorium cells from the central region of the egg and from the operculum of *T. rubrovaria*, *T. carcavalloi* and *T. circummaculata* were analyzed using MEV to quantify the number of perforations per cell. The average number of perforations per egg cell and operculum cell respectively was 44.3 (Fig. 3A) and 8,2 (Fig. 3D) for *T. rubrovaria*, 20.8 (Fig. 3B) and 7.5 (Fig. 3E) for *T.carcavalloi*,and 35.0 (Fig. 3C) and 13.3 (Fig. 3F) for *T.circummaculata*. These punctures in *T. rubrovaria* and *T. circummaculata* are distributed on the entire surface of the cells, including on the edges, unlike *T. carcavalloi* which has very few perforations on the edges (they are mostly concentrated on the central region of each cell). The eggs of *T. circummaculata* are smaller than those of *T. rubrovaria* and *T. carcavalloi*. The average number of perforations per egg cell is higher in *T. rubrovaria* and the average per operculum cell is higher in *T. circummaculata*.

TABLE 4. Homogeneity of egg length of *Triatoma rubrovaria*, *Triatoma carcavalloi* and *Triatoma circummaculata.* Student-Newman-Keuls (SNK) test.

		Set	
Population	N	1	2
T. circummaculata	30	1,4673	
T. carcavalloi	30		1,6347
T. rubrovaria	30		1,6483
Significance	30	1,000	0,504

N= number of eggs.

TABLE 5. Homogeneity of operculum diameter of *Triatoma rubrovaria*, *Triatoma carcavalloi* and *Triatoma circummaculata.* Student-Newman-Keuls (SNK) test.

		Set		
Population	N	1	2	3
T. circummaculata	30	0,5773		
T. carcavalloi	30		0,6337	
T. rubrovaria	30			0,7047
Significance	30	1,000	1,000	1,000

N= number of eggs.

Discussion

The eggs of the Triatominae subfamily have been studied for decades. Pinto (1924) described the eggs of *Triatoma brasiliensis* Neiva, 1911; Galliard (1935) showed the importance of the exochorium sculpture for taxonomy, stating that the shells of the eggs of the Triatominae presented a characteristic constant ornament on their outer surfaces, distinguishing one species from another. Galliard also used other macroscopic characters, such as size, shape, color, type of shell and fixation, in his study of triatomine eggs. However only few authors have used the morphological structures of eggs to distinguish species(Barata, 1981, Gonçalves *et al.* 1985, Rosa *et al.* 2000).

The *Triatoma* genus presents high variability in the features of the eggs, "both at a macroscopic level as exochorial", characterized by hexagonal cells with indeterminate limiting lines and perforations of variable size and shape. This perforations is typically detected in ten species, including *Triatoma rubrovaria* (Barata, 1995).

This study confirms the general appearance of *T. rubrovaria*, *T. carcavalloi* and *T. circummaculata* eggs as typical for the *Triatoma* genus, and shows the existence of morphologic features which are quite different among the three species. This is the first morphologic and morphometric description of *T.carcavalloi* eggs.

In addition to the size difference among eggs of *T. rubrovaria*, *T. carcavalloi* and *T. circummaculata,* we observed two other morphologic differences among these three species. The number of perforations per egg cell of *T. rubrovaria* is higher than for *T. carcavalloi* and *circummaculata*, and these perforations on the exochorium of eggs and operculum of *T. rubrovaria* and *T. circummaculata* are distributed all over the cells, while in the case of *T. carcavalloi* the perforations are mostly concentrated in the central region of each cell.The texture of the exochorium of the *T. rubrovaria* and *T. carcavalloi* egg and operculum is similar to that of *T. maculata* and *T. brasiliensis*, as reported by Gonçalves *et al.* (1985) and Jurberg *et al.* (1986). The observations on the opercular limiting line of the three species assessed in this study are in accordance with the data obtained by Rosa *et al.* 2000 for *T. rubrovaria* and *T. circummaculata*.

According to Costa *et al.* (1997), different environmental conditions, such as available source of food, temperature and humidity may affect egg morphology directly or indirectly. However, this study did not consider different environmental conditions, as *T.rubrovaria* was found living in sympatry with *T. carcavalloi* and *T. circummaculata*.

Other than the chromatic and morphologic differences between *T. rubrovaria* and *T. carcavalloi* described by Jurberg *et al.* (1998) and of *T. rubrovaria* described by Rosa *et al.* (1999), this study identifies morphologic differences among the eggs that can be used to show that *T. rubrovaria*, *T. carcavalloi* and *T. circummaculata* are distinct species.

These results widen the specific concept of *T. carcavalloi* and provide another means for more specific monitoring of species that invade domiciliary ecotopes during the vector control campaigns run by the Health Agencies in the south of Brazil.

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