ULTRASTRUCTURE OF THE CUTICLE OF *ECHINISCUS RUFOVIRIDIS* [Du Bois - Raymond Marcus, (1944) HETEROTARDIGRADA]

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ABSTRACT

An ultrastructural analysis of the cuticle of specimens of *Echiniscus rufoviridis* Du Bois-Raymond Marcus, 1944, collected in Santa Rosa City (La Pampa Province, Argentina) was carried out to characterize and compare them with those from other species of *Echiniscus*. The *E. rufoviridis* cuticle exhibits both specific features and other that are common with other tardigrades taxa of different levels. We propose the existence of two dorsal epicuticular ultrastructural patterns within the genus *Echiniscus*, *i.e.* "*testudo* type" and "*viridis* type". *Echiniscus rufoviridis* shows the "*testudo"* type of the dorsal epicuticle: lacunar basal layer clearly different from the rest by its greater thickness. *E. rufoviridis* and *E. testudo* also share the dorsal intracuticle thickness and the presence of a trilaminar layer between the ventral epicuticle and intracuticle. *E. rufoviridis* differs from *E. testudo* by the fewer lacunae layers, by a more abundant interlacunar matrix, and by a more irregular lacunar arrangement in the dorsal epicuticle. On the other hand, the lacunar system in *E. rufoviridis* communicates with the exterior via wide pores opening in the surface depressions. Also peculiar to *E. rufoviridis* is the proportion between the basal and apical portions of the striate layer of the ventral epicuticle. Ultrastructural similarities observed between the cuticles of *E. rufoviridis* and *E. testudo* agree with similarities in the dorsal sculpture. We suggest that these similarities are related to ecological parallel of both species.

RESUMEN

Se llevó a cabo un análisis ultraestructural de la cutícula de muestras de *Echiniscus rufoviridis* Du Bois-Raymond Marcus, 1944, recolectadas en la Ciudad de Santa Rosa (Provincia de La Pampa, Argentina), para caracterizarlas y compararlas con muestras de otras especies de *Echiniscus*. La cutícula de *E. rufoviridis* exhibe las características específicas y otras que son comunes con otras especies taxonómicas tardigrades de diferentes niveles. Se propone la existencia de dos patrones dorsales epicuticulares ultraestructurales en el género *Echiniscus*, por ejemplo el "tipo testudo" y el "tipo viridis". El *Echiniscus rufoviridis* muestra la epicutícula dorsal tipo "testudo": capa basal laminar claramente diferenciada del resto por su mayor espesor. *E. rufoviridis* y *E. testudo* también comparten el espesor de la intracutícula dorsal y la presencia de una capa trilaminar entre la epicutícula ventral e intracutícula. La especie *E. rufoviridis* se diferencia de la *E. testudo* por las pocas capas lagunares, por una matriz interlagunar más abundante y por un arreglo lagunar más irregular en la epicutícula dorsal. Por otro lado, el sistema lagunar en *E. rufoviridis* se comunica con el exterior vía poros anchos que se abren en las depresiones de la superficie. También es peculiar en *E. rufoviridis* la proporción entre las porciones basal y apical de la capa estriada de la epicutícula ventral. Las similaridades ultraestructurales observadas entres las cutículas de *E. rufoviridis* y *E. testudo* concuerdan con las similaridades en la escultura dorsal. Se sugiere que estas similaridades están relacionadas al paralelo ecológico de ambas especies.

KEYWORDS: cuticle - Echiniscidae - *Echiniscus* - Heterotardigrada - ultrastructure

INTRODUCTION

The knowledge of the tardigrade cuticle ultrastructure is important because of its phylogenetic significance and also because of the role that these structure plays in cryptobiosis [1–5].

Since the studies by Baccetti and Rosati [6], the following layers have been recognized in the cuticle of tardigrades: epi-, intra-, and procuticle. From these, the epicuticle shows the greatest variation, and it can be divides into several different sub-layers [5].

The cuticle of the Heterotardigrada has been studied in several species of *Halechiniscus* [3]; *Batillipes* [7] and *Echiniscus* [1,8–11]. These studies allowed the description of the "heterotardigrade" type of cuticle, which is characterized by the presence of pillars in the epicuticle, a feature distinguishing it from that of the eutardigrades [11]. However, the pillars typical of heterotardigrade epicuticles are sometimes also observed in eutardigrade genera [4,5].

Among the almost 140 species of *Echiniscus* [12], the ultrastructure of the cuticle is well known in *E. viridis* [1] and *E. testudo* [8,–11]. It can be added that in [7,13,14] cuticle's photos of *E. trisetosus and E. viridissimus* can be seen.

The Neotropical species *E. rufoviridis* has been recently re-discovered and its cuticle sculpture has been described [15].

E. rufoviridis and *E. viridis* both belong in the green-pigmented cuticle group of Echiniscids. These two species are the most similar to each other within the group by their morphology [16]. However, plate sculpture is very different in the two species [15].

On the other hand and from an ecological point of view, *E. rufoviridis* seems to play – in urban South American environments – a similar role to that of *E. testudo* in European cities [15]. They are the only heterotardigrades found in these environments [17] and both fits in the low tolerance category, according to the ecological classification of urban tardigrades proposed by Séméria [18].

The objective of this work was to study and to characterize the cuticle ultrastructure of *E. rufoviridis*, and compare it with those of other species of the genus, as the ultrastructural pattern seems to be heterogeneous not only within families but within genera [5,9].

MATERIAL AND METHODS

Tardigrades were collected from lichen communities developed on trees of Santa Rosa city (36°39´S 64°17´W) in La Pampa province (Argentina) in November 2001. The samples were selected according to the method previously described by Ramazzoti and Maucci [16].

Specimens were immediately fixed *in toto* in 3% formaldehyde, 3% glutaraldehyde, 1% picric acid in phosphate buffer 0.1M, pH 7.4 during 2 hours at room temperature. Following, the specimens were washed in phosphate buffer saline 0.1M, pH 7.4, and post-fixed in 2% osmium tetroxide aqueous solution during 2 hours at room temperature. Animals were dehydrated in an acetone series and included in an araldite: acetone mixture (1:1) followed by araldite pure. Histological studies were performed choosing the interest zones through 0.5 μm cross, and sagital semithin sections with a Reichert Ultracut S ultramicrotome. Sections were stained with toluidine blue and observed and photographed with an Olympus BX50 microscope provided with a PM20 photograph camera. The selected areas were sectioned at 60 nm with the mentioned ultramicrotome and counterstained with uranyl acetate / lead citrate previous to observation and photography under a Philips EM 201 TEM. Nomenclature for morphological terms involving the cuticle was taken from Bacceti and Rosatti [6] with the modifications introduced by Greven [3].

RESULTS

Outside of dorsal epicuticle there is a discontinuous flocculated material (Fig. 1). Below it lays the so-called outer epicuticle, which is trilaminated and composed of two dark bands and a central light one. The inner or proximal band is the thickest one and is followed by a clear area bounding the inner epicuticular layer (see insert of Fig. 1). Almost the entire thickness of the epicuticle corresponds to the inner epicuticle in which three zones can be differentiated (Fig. 1). I) A distal zone, perforated by fine canals oriented perpendicularly to the body surface; II) a middle zone, the thickest of all three layers, perforated by an anastomosed lacunae system and fine canals that communicate lacunae to each other; III) a third proximal, very dense, zone with numerous canals of circular section between the basal lacunae and the intracuticle (see insert Fig. 2).

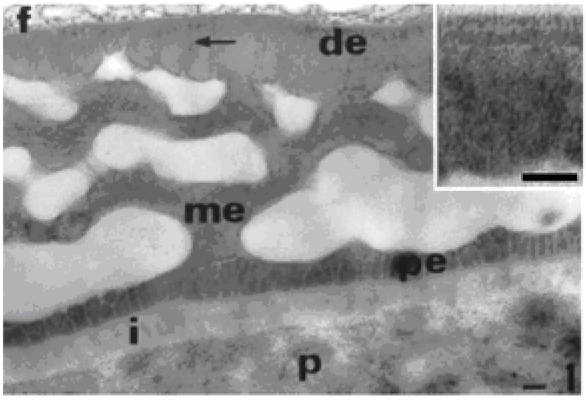

Fig. 1: Sagital section of *Echiniscus rufoviridis* dorsal cuticle. See the flocculated material of the outer cuticle, beneath a outer trilaminar epicuticle (insert, bar = 0.1 μm), a ligh zone and the inner epicuticle. The last present three regions: distal, medial and proximal; all cribbed by channels (arrows). Also, the medial region is crossed by two stratus with lacunae: basal and distal with large and small lacunae, respectively. f: flocculated material; de: distal epicuticle; me: medial epiculticle; pe: proximal epicuticle; i: intracuticle; p: procuticle. Bar = 0.1 μm.

A proximal layer of large lacunae forms the lacunae system parallel to the surface and an overlying distal layer of smaller irregularly placed lacunae (Fig. 1). Lacunae can communicate with the exterior through large pores placed at the bottom of the superficial conical depressions (Fig. 2).

The epicuticle is separated from the intracuticle by a structure that seems to be a trilaminar layer in some regions: a clear lamina between two dark dense ones, the internal one being the thickest (see insert Fig. 3). The intracuticle is thin and composed by a more homogeneous and low electrodensity material than the epicuticle. This layer is thoroughly crossed by filaments (Figs. 1-3). The procuticle is dense, heterogeneous and of very variable thickness. It bears osmophilic granules, randomly placed fibers and lipid droplets (Figs. 1 and 3).

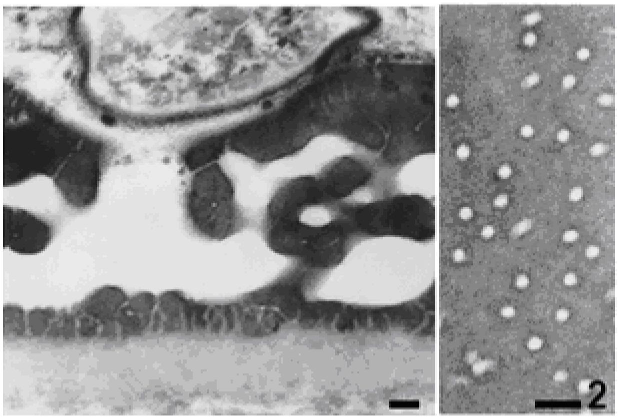

Fig. 2: Sagital section of *Echiniscus rufoviridis* dorsal cuticle, at communication pores level of lacunar system with the exterior. The proximal inner epicuticle, very dense, present numerous canals of circular section (insert, bar =0.1 μm). Bar = 0.1 μm.

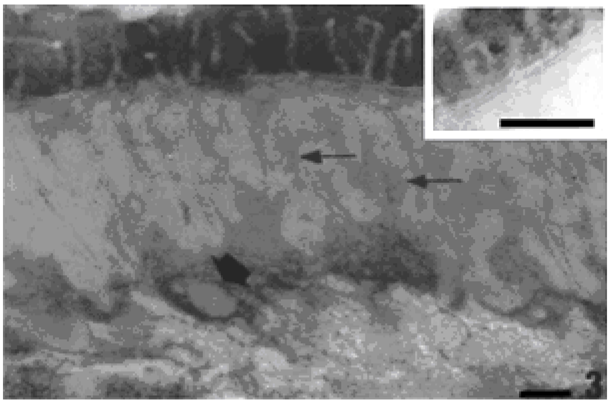

Fig. 3: Underlying to the epicuticle the intracuticle show a apical trilayered structure (insert, bar =). The intracuticle is composed by a more homogeneous and low electrodensity material than the epicuticle and is thoroughly crossed by filaments (small arrows). The procuticle is dense, heterogeneous and of very variable thickness. It presents osmophilic granules, randomly placed fibres and lipid droplets (thick arrow). Bar = 0.1 μm.

Looking at the dorsal surface it is possible to recognize transition regions were the lacunae system is reduced to a simple layer of lacuna and the intracuticle is slightly thickened (Fig. 4).

On the surface of the ventral cuticle there is a layer of discontinuous osmophilic deposits which is more evident than the dorsal surface (Figs. 5 and 7). The external epicuticle is multilaminar, and up to five laminae have been observed. In this case, tree dark layers alternate with two clear ones. From the three dark layers, the basal is the thickest and densest (Fig. 5).

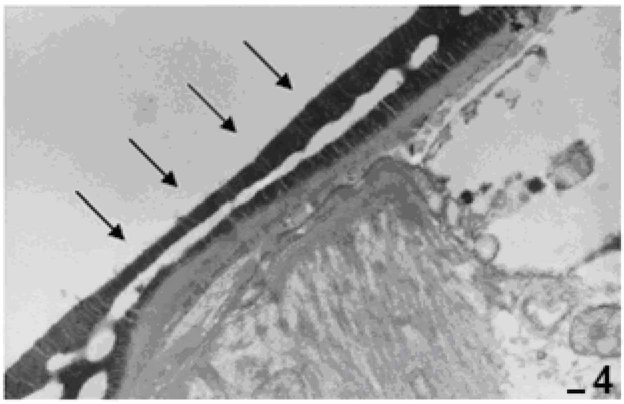

Fig. 4: Sagital section of *Echiniscus rufoviridis* dorsal cuticle showing a transition region (arrows). The lacunar system is reduced to a simple layer of lacuna and the intracuticle is slightly thickened.
Bar = 0.1 µm.

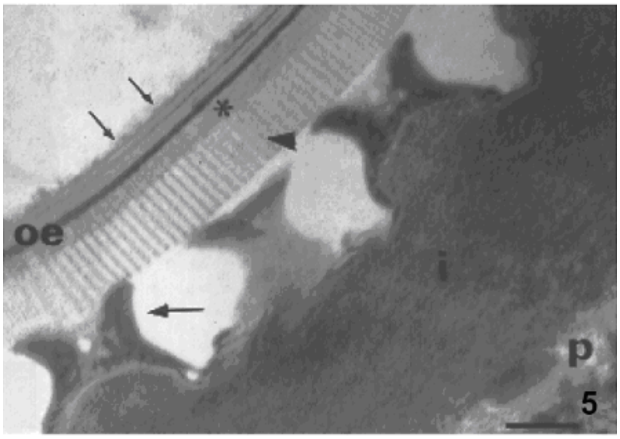

Fig. 5: Sagital section of *Echiniscus rufoviridis* ventral cuticle. Beneath the osmiophilic deposit (small arrows) outer the outer epicuticle present five layers – three dark alternating with two light layers. In contrast, the internal epicuticle have two layers: one striated (apical and basal layers, asterisc and arrow head respectively) and one with pillar (arrow). oe: outer epicuticle; i: intracuticle; p: procuticle. Bar = 0.1 µm.

In the internal epicuticle two zones can be distinguished: one striated and one with pillars. In the first one there is an apical dense zone that takes about a third or a fourth of the total and a basal zone of less density in which striate perpendicular to the surface can be clearly seen (Fig. 5). The striated zone is made up of tubes of hexagonal section with central rods limited to the denser apical zone (Fig. 6). The zone with pillars lies between the striated zone and the intracuticle (Figs.5 and 7). Electron-dense columnar structures run from the intracuticle to the base of the striated layer. These pillars, of an approximately circular section, are oriented perpendicularly to the body surface, with a regular arrangement and leaving electrotransparent spaces between them.

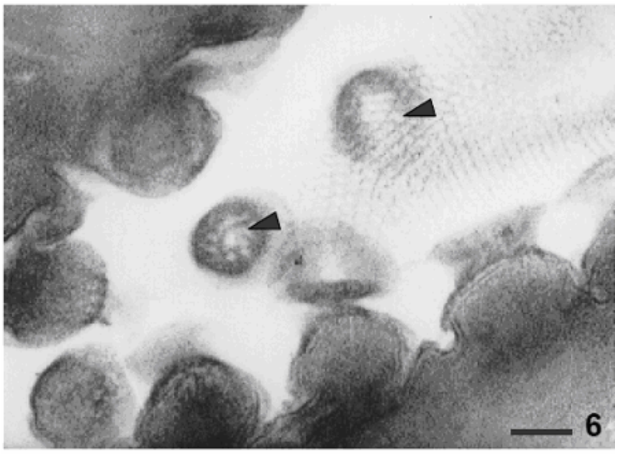

Figure 6: Cross section detail of the striate and pillar regions. The striated zone is made up of tubes of hexagonal section (square red). The pillars (arrow) have a circular section with a central channel accompanied by other narrower canals. The epicuticle is separated from the intracuticle by a wavy layer that appears as trilaminar. Bar = 0.1 µm.

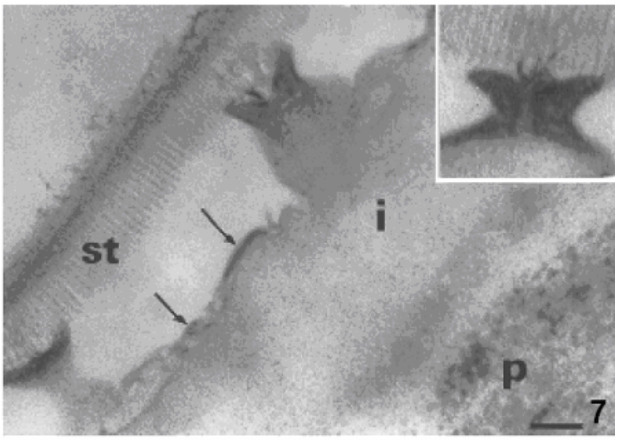

Fig. 7: Sagital section of *Echiniscus rufoviridis* ventral cuticle showing details of the pillar central channel and their apical fibers emerging from them and projecting into the striate layer (insert, bar = 0.1 µm). The intracuticle is thicker than the epicuticle, your surface is wavelet (small arrows) and in your base present a discontinuously layer of granular material followed by a lower electron density zone. st striate region; i: intracuticle; p: procuticle. Bar = 0.1 µm.

They show a constriction in the middle region, becoming wider towards the apical and proximal ends. The apical end shows a concave surface on to which opens a central canal that runs throughout the pillar. The central canal is accompanied by other narrower canals (Fig. 5). Apical fibers reach out from the pillars towards the striated layer (Fig. 7, see insert for details). The epicuticle is separated from the intracuticle by a wavy layer that in some areas appears as trilaminar (Fig. 6). At the base of the pillar the intracuticle is elevated in a dome-like shape (Figs. 6 and 7).

The intracuticle is thicker than the epicuticle and its density is heterogeneous. On its base, a discontinuous, denser, granular and variably thick layer is followed by a zone of low electronic density (Figs. 5-7). The procuticle is similar to the one described for the dorsal cuticle and, in some places, it shows a high electron density (Figs. 5 and 7).

DISCUSSION

The cuticle of *Echiniscus rufoviridis* shows unique, possibly specific features, together with others that it shares with other species in the same genus. The basic ultrastructural characteristics observed are those known for echiniscid heterotardigrades, *i.e.* epicuticle with striate layer and pillars that in the dorsal region form a reticular structure with numerous lacunae [1,3,7,9–12].

As mentioned in the introduction of this paper, the epicuticle is the layer showing the greatest variability in tardigrades. Based on our own results and the analysis of the work carried out by Crowe *et al.*,Greven and Dewel *et al.*[1,7,9], we propose the existence of two dorsal epicuticular ultrastructural patterns within the genus *Echiniscus.* The first one (“*testudo”* type), characterized by a basal layer of large lacunae, significantly larger than in the outer layers; and second (*“viridis”* type), in which the basal lacunae show a thickness similar to that of the upper ones. The latter is observed in *E. viridis* and *E. viridissimus*, while the former occurs in *E. rufoviridis* and *E. testudo*. *E. rufoviridis* differs from *E. testudo* by the fewer lacunar layers, by a more abundant interlacunar matrix and by their more irregular arrangement. On the other hand, in *E. rufoviridis* the lacunar system communicates with the exterior through the wide pores opening at the bottom of the surface depressions observed also under SEM [15]. Contrarily, in *E. testudo* those wide pores are missing and between the lacunae and the bottom of the depressions there is a canal wider than the other epicuticular canals.

Likewise, the intracuticle in the plate zone is similar in *E. rufoviridis* and *E. testudo*, being in both cases thinner than in other members of the genus, as inferred from illustrations by Crowe *et al*, Greven, Wright (1988b) and Dewel *et al*. [1,7,9,11]. In the two mentioned species the intracuticle thickness is always less than 0.25 the thickness of the epicuticle.

The features of the dorsal cuticle in *E. rufoviridis* in the areas between plates agree with those recorded in *E. testudo* by Greven and Wright [9,11].

In the striated layer of the ventral epicuticle of *E. rufoviridis*, the denser apical portion is thinner than in other species of *Echiniscus*: about a third or a fourth of the total thickness of the striate layer. In *E. testudo*, – as in *E. viridis* – this rate is about 0.5, as suggested by the illustrations of Crowe *et al*., Greven and Wright [1,10,11,19].

In the species studied by us, apical fibers reach out from the pillars towards the striated layer, as described previously by Wright [11] for *E. testudo.*

In *E. rufoviridis*, at the boundary between the ventral epicuticle and the intracuticle there is a trilaminar layer similar to that observed in *E. testudo* [11], while in *E. viridis* there is an electro-transparent layer interrupted by a dense band [1].

Ultrastructural similarities observed between the cuticles of *E. rufoviridis* and *E. testudo* agree with similarities in the dorsal sculpture, such as the presence of depressions on the plates [9,15]. Given the importance of the cuticle in the response of tardigrades to changing environmental conditions [10,12,20,21,22], the cuticular similarities shown by these species could be correlated with the ecological similarities mentioned in the introduction to this paper.

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