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MINERALIZED BODIES IN THE FAT BODY OF *Rhinocricus padbergi* (DIPLOPODA)

Carmem Silvia Fontanetti, Bianca Tiritan and Maria Izabel Camargo-Mathias

Department of Biology, Institute of Biosciences, Paulista State University (UNESP), Rio Claro, SP, Brazil

ABSTRACT

The fat body is a loosely packed tissue distributed throughout the body cavities of millipedes. The main function of this tissue is the storage of lipids, glycogen, proteins and uric acid and also serves as a site for the permanent storage for excretion products. In this work, we examined the ultrastructure of the mineralized bodies found in the fat body of the millipede *Rhinocricus padbergi*. The mineralized bodies were spherical bodies that varied in structural organization within a single cell: some consisted of several concentric layers of amorphous material while others were surrounded by a layer of electron-dense material intimately associated with the surrounding membrane. The histochemical and ultrastructural results suggested that these mineralized bodies are involved in the accumulation of calcium and uric acid. The large number of these structures found in the fat body of millipedes may be a consequence of these animals´ diet since they overturn soil rich in large amounts and/or variety of minerals. As in other organisms, uric acid probably accumulates as the metabolic product of the degradation of nucleic acids derived from autophagy of the rough endoplasmic reticulum due an earlier massive protein synthesis, but may also be extracted from the hemolymph.

Key words: Biomineralization, calcium, millipedes, uric acid

INTRODUCTION

Millipedes, popularly known in Brazil as *piolho-de-cobra*, *emboá* or *gongolô*, constitute a class (Diplopoda) of Myriapoda and have a worldwide distribution, although they are particularly abundant in the tropics. Millipedes are predominantly nocturnal animals that protect themselves from the sun by living under rocks or tree trunks, with a few species being commensalists in ant and termite nests. Millipedes play an important role in soil dynamics by promoting the aeration and enrichment of soil. Despite their ecological importance, few studies have examined the survival strategies and detoxification of these animals, for example as for heavy metals present in environment. Diplopods can metabolize and/or accumulate various types of heavy metal ions [13], mainly in midgut and fat body.

Correspondence to: Dr. Carmem Silvia Fontanetti
Departamento de Biologia, Instituto de Biociências, Universidade Estadual Paulista (UNESP), CP 199, CEP 13506-900, Rio Claro, SP, Brazil. Tel: (55) (19) 3526-4135, Fax: (55) (19) 3526-4136. E-mail: fontanet@rc.unesp.br

The fat body of millipedes is a tissue distributed throughout the body cavities of these animals. The main function of the fat body is the storage of lipids, glycogen, proteins and uric acid, but it also serves as a permanent storage site for excreted products [6-8]. The millipede fat body is distributed in two forms: as a peripheral layer referred to as the parietal fat body and as a perivisceral fat body that fills the body cavities and surrounds internal organs such as the digestive tract [6]. The diplopod fat body contains two types of cells (adipocytes and oenocytes) and structural analysis has shown that this tissue is very similar to that found in insects [1].

The aim of this work was to examine the ultrastructure of the mineralized bodies present in the fat body cells of the millipede *Rhinocricus padbergi* in order to understand their composition and function.

MATERIAL AND METHODS

Specimens of *R. padbergi* were collected in February 1999 around the Institute of Biosciences on the campus at UNESP, Rio Claro, and were killed with ethilic ether. For histological analysis, fragments of the parietal and

perivisceral fat body were routinely fixed with 4% paraformaldehyde, dehydrated in a graded series of 70-100% ethanol, and embedded in JB-4 Resin at 4ºC in a dark bottle for 24 h. The material was initially embedded at 4ºC to prevent premature polymerization after which the embedding was completed at room temperature. Sections 5 μm thick were obtained with a dry glass knife and then stained with hematoxylin and eosin (HE).

Some sections were processed by the von Kossa method for the histochemical detection of calcium, as described by Junqueira and Junqueira [12]. In this method, the sections were immersed in $AgNO_3$ solution and then exposed to photographic developer (Dektol, Kodak) and sodium thiosulfate. Other sections were processed to determine the critical electrolyte concentration (CEC) of nucleoli and ribonucleoproteins, as described by Mello *et al.* [17]. For this, the sections were stained with toluidine blue and immersed in an aqueous solution of 0.05 M $MgCl_2$.

For ultrastructural analysis, fragments of parietal and perivisceral fat body were processed for transmission electron microscopy. The material was fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 at 4°C for 2 h. After washing with this buffer, the material was postfixed in 1% osmium tetroxide in the same buffer and block-stained with uranyl acetate for 8 h. Dehydration was done with graded acetone and was embedded in an epon-araldite resin mixture for 12 h at 4°C. Thin sections were stained with uranyl acetate and lead citrate prior to examination and documentation with a PHILLIPS MC 100 transmission electron microscope operated at 80 kV.

RESULTS

Histological and ultrastructural analysis showed that the mineralized bodies varied in structural organization within individual fat body cells (Figs. 1A,B, 2A,B and 3A) in different regions of the body; spherical bodies with different appearances were often seen in the same adipocyte in the parietal body (Fig. 1B) and in the perivisceral fat body surrounding the gonads (Figs. 2A-female and 2B-male) and the digestive tract (Fig. 3A). Each spherical body was surrounded by a membrane (Fig. 1C, details in 1D, 2D, 3C and 4A,B) but was not associated with organelles such as the rough endoplasmic reticulum or Golgi complex.

In some cases, the spherical bodies consisted of several layers of amorphous material organized concentrically (Fig. 1C). Light microscopy showed that most of these structures (1 in Fig. 1B) stained positively in the Von Kossa technique, indicating the presence of calcium. In other cases, the spherical bodies were surrounded by a layer of electron-dense material (arrows in Figs. 2D, 3B,C,D and 4A,B). This layer was clearly seen in histological sections because of its strong staining by hematoxylin (arrows in Figs. 2A,B and 3A), indicating the presence of basophilic compounds. Staining with toluidine blue to determine the CEC indicated that most of these structures contained phosphates, as shown by the reddish staining in many of these structures.

DISCUSSION

The accumulation of minerals in spherocrystals is an important mechanism for maintaining an organism's homeostasis. The importance of biomineralization has been established in several phyla of invertebrates and in various organs. Insects have several structures with this function, including spherocrystals and granules that contain minerals or purine-rich elements [9,10]. Several types of inclusion bodies have also been found in the Mollusca and Crustacea [10]. The accumulation seen in all of these cases reveals a process of ion balance that involves numerous physiological phenomena, particularly the recycling, storage and excretion of minerals. According to Hubert [10], these structures occur as spherites or granules in the Diplopoda. Köhler *et al.* [13] used the term spherites to designate metal inclusions in cells and to distinguish among different types of granules according to their chemical composition. However, the nature of these stuctures in diplopods has not been conclusively determined.

These inclusions are strictly mineral-based and are produced by different organs. However, some have a mixed composition (mineral and purine-based) and, in this case, they are specific for the fat body. Uric acid, one of the waste products eliminated by millipedes, can only be excreted in small amounts in its soluble form, and therefore represents a considerable fraction of the storage products in the fat body, where it forms spherites bound to potassium ions.

Unfortunately, the ultrastructure of these spherites is poorly known because of the technical difficulties inherent to sample fixation and sectioning. Structures with a concentric arrangement such as that seen here in the *R. padbergi* fat body have been described in the midgut [7,11] and mesentery [10] of other millipede species and apparently consist mainly of calcium carbonate. Structures rich in calcium have

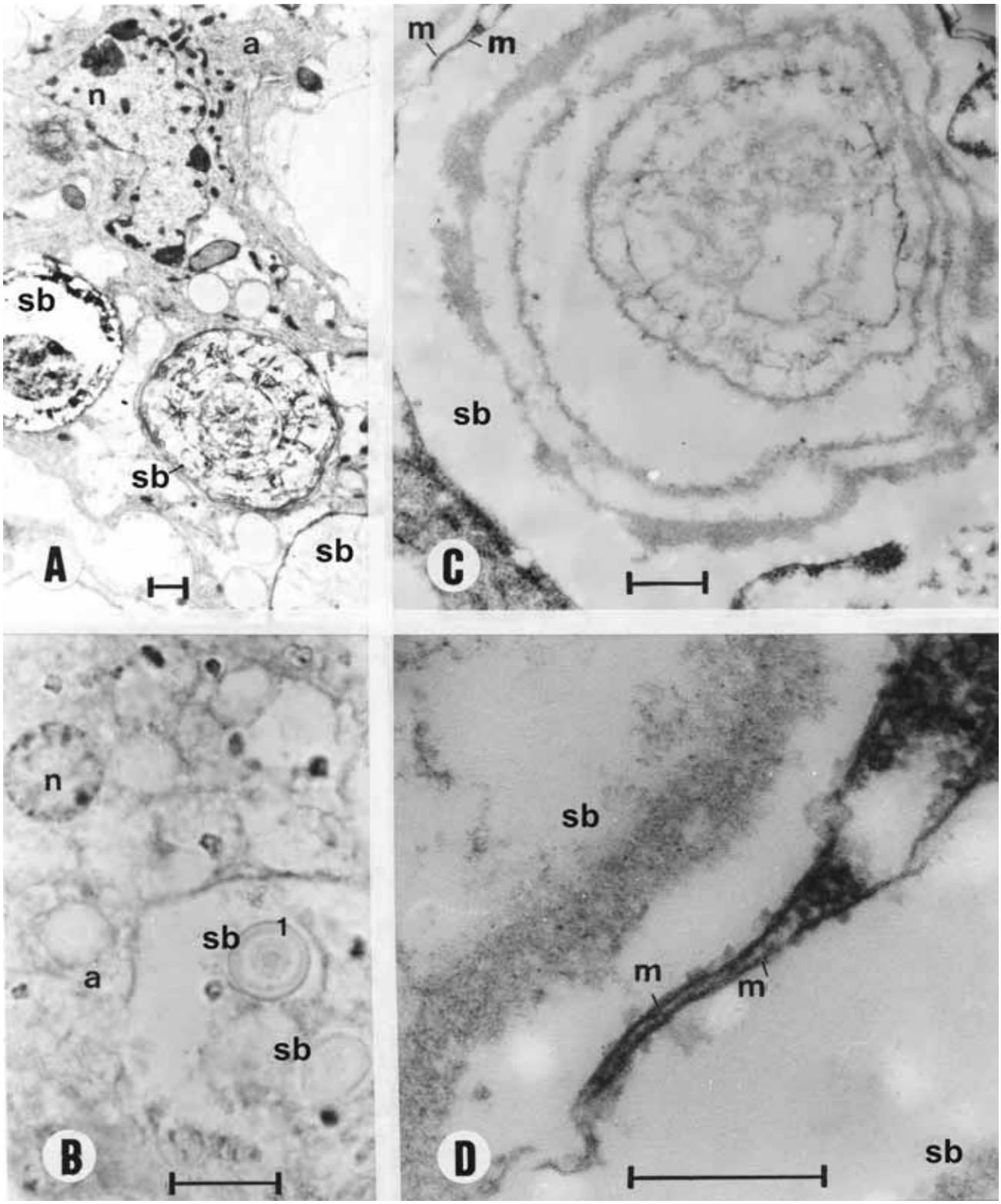

Figure 1. A. Electron micrograph of the perivisceral fat body located around the digestive tract of *R. padbergi*. **B.** Histological section of the parietal fat body of *R. padbergi.* HE staining. **C.** TEM of a spherical body. **D.** Detail of the membrane surrounding the structure shown in **C. a** = adipocytes, **l** = spherical bodies seen in light microscopy and corresponding to that seen in **C** by TEM, **m** = membrane, **n** = nuclei, **sb** = spherical bodies. Bars: **A,C** = 1 μm, **B** = 10 μm, **D** = 0.5 μm.

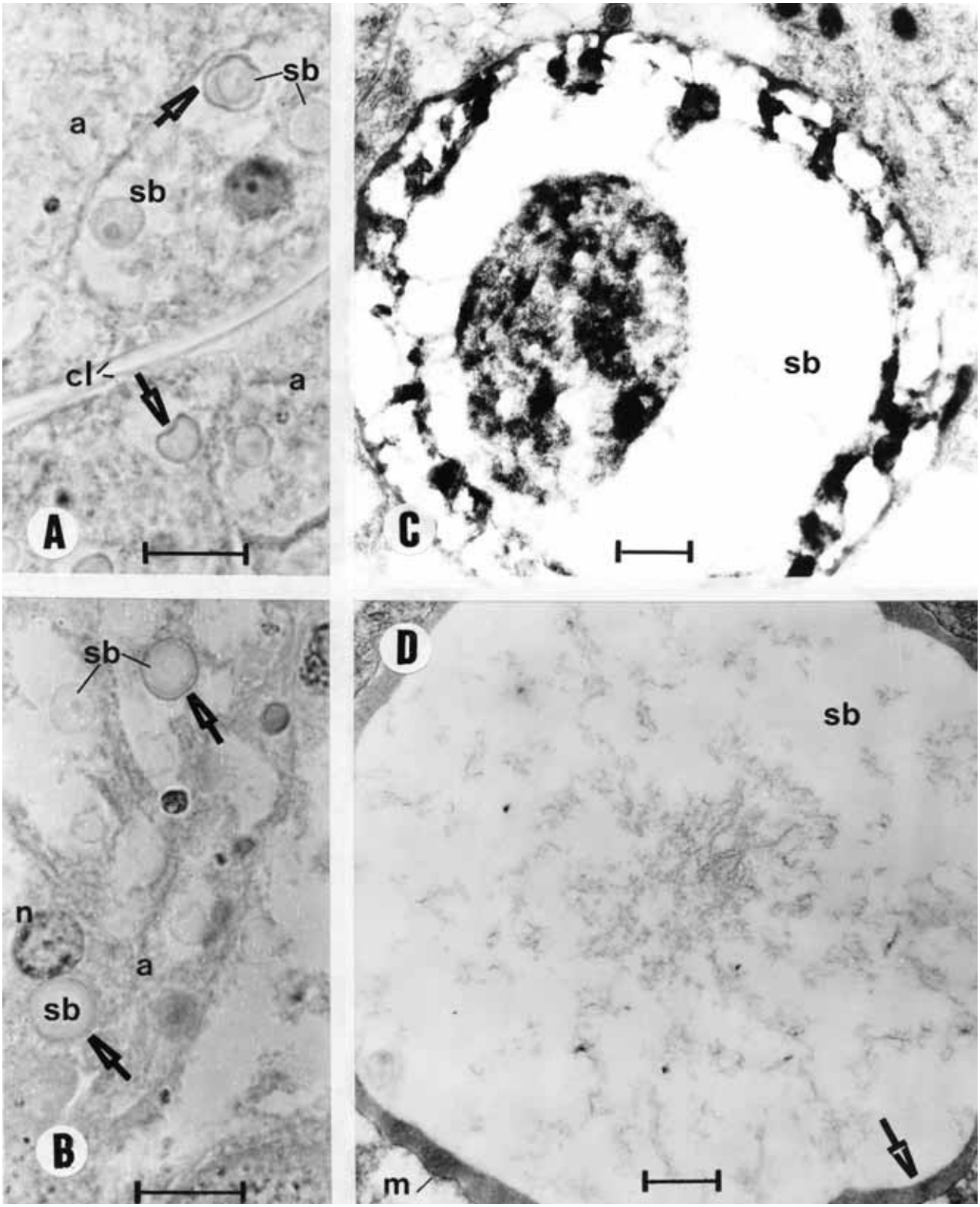

Figure 2. A. Histological section of the perivisceral fat body located around the ovary of *R. padbergi*. HE staining. **B.** Histological section of the perivisceral fat body located around the testes of *Rhinocricus padbergi*. HE staining. **C and D.** TEM of spherical bodies. **a** = adipocytes, **cl** = cellular limits, **m** = membrane; **n** = nucleus, **sb** = spherical bodies. **Arrows** in **A** and **B** = region strongly stained with H&E, and **arrow** in D = electron-dense material surrounding the spherical body. Bars: **A,B** = 10 μm, **C,D** = 1 μm.

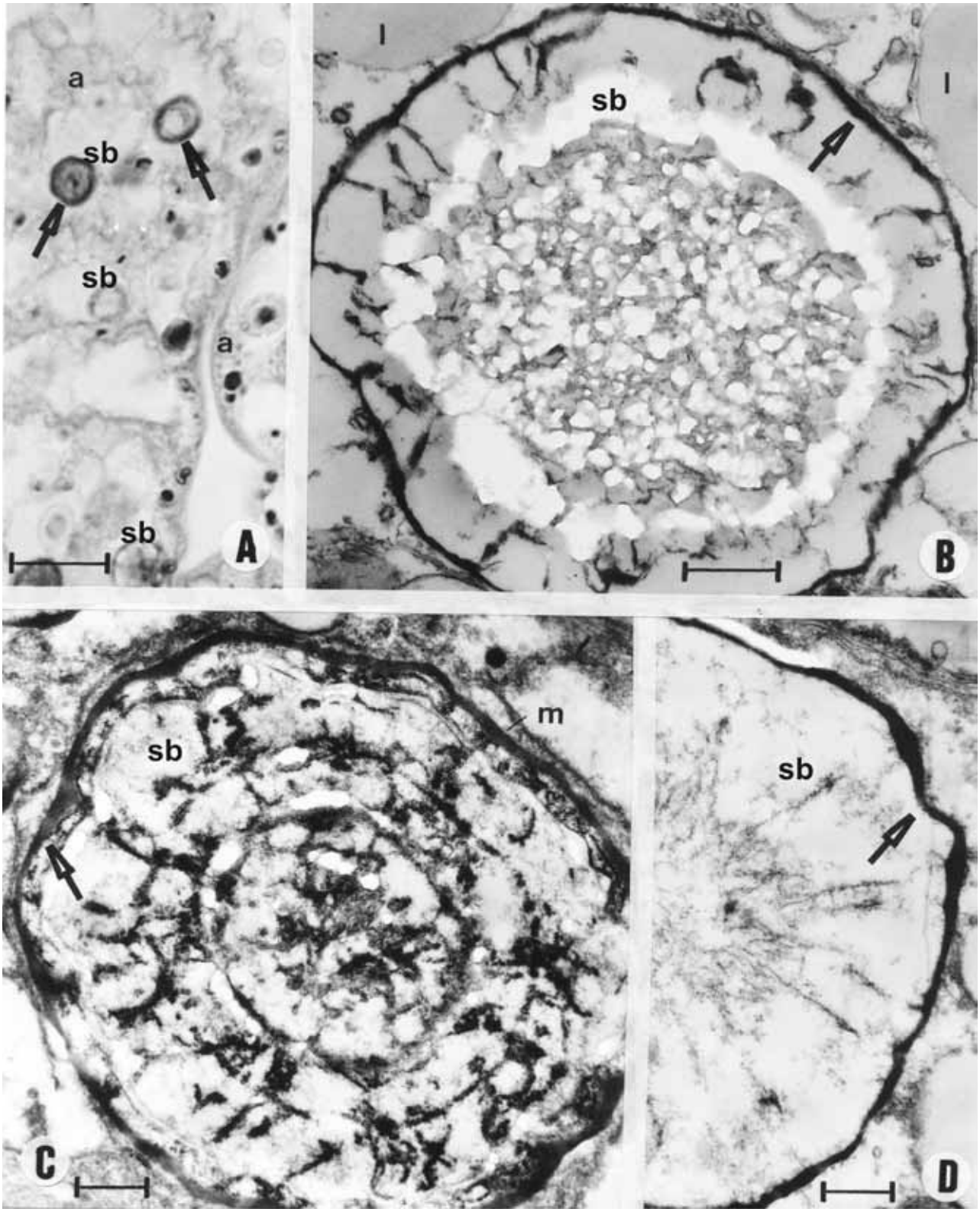

Figure 3. A. Histological section of the perivisceral fat body located around the digestive tract of *R. padbergi*. HE staining. **B**, **C** and **D.** TEM of spherical bodies. **a** = adipocytes, **l** = lipids, **m** = membrane, **sb** = spherical body. **Arrows** in **A** = region strongly stained with HE, and arrows in **B, C,** and **D** = electron-dense material surrounding the spherical body. Bars: **A** = 10 μm, **B, C** and **D** = 1 μm.

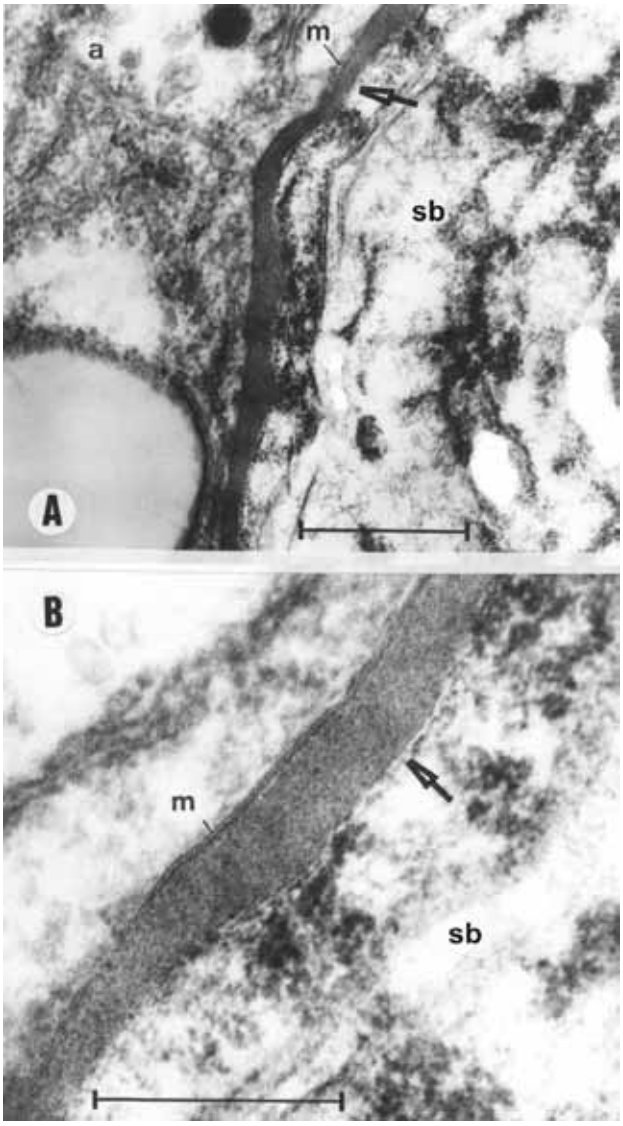

Figure 4. A. TEM of spherical body. **B.** Detail of the membrane surrounding the spherical body. **a** = adipocytes, **m** = membrane, **sb** = spherical body. Arrows in **A** and **B** = electron-dense material around the spherical body. Bars: **A** = 1 μm, **B** = 0.5 μm.

also been observed in the ooplasm of *R. padbergi* [5] and in oocytes of *Amblyomma cajennense* ticks [4]. Martoja and Ballan-Dufrançais [16] reported the presence mineral-rich structures in the columnar cells of the midgut and Malpighian tubule cells of insects. These structures, referred to as mineral concretions or spherocrystals, consist of metals (Ca, Mg, K, Fe, Zn, and Sr) complexed with phosphate, carbonate or chloride. Spherocrystals originate in the cisternae of the rough endoplasmic reticulum and are formed by the conjugation of mineral salts with a polyanionic stroma. However, according to Locke [15], concretions formed in the rough endoplasmic reticulum have not been described in the fat body. In *R. padbergi*, there was no association between these structures and cytoplasmic organelles, despite their membrane-bound organization.

In diplopods, these structures have been referred to as concentric bodies [2,14], and were mistakenly interpreted as a proteinaceous yolk in millipede oocytes by Sharma and Chhotani [19]. Crane and Cowden [2] described these structures as being composed of an organic matrix conjugated with crystallized calcium salts and referred to them as concentric ring bodies (CRBs). Petit [18] subsequently showed that CRBs were composed mainly of phosphates and calcium carbonate associated with a proteinaceous web and that they accumulated in cisternae of the rough endoplasmic reticulum.

Based on their morphology, we suggest that the spherical bodies in *R. padbergi* fat body cells contain calcium and uric acid. Spherical bodies similar to those shown in Figs. 2D and 3D have been found in cockroach urocytes, specialized fat body cells that accumulate and release urate. In urocytes, these structures are known as urate vacuoles. According to Locke [15], these vacuoles have a cortex of fibrous material and a characteristic core composed of material similar to that of the cortex. Urate, which is not preserved during tissue processing for morphological analysis, occupies the lighter area between the core and the cortex. The origin of these vacuoles has not been studied, but all of them are surrounded by thin membranes similar to the plasma membrane [3,15].

Various species of Lepidoptera store urate as granules in their fat body cells [3,15]. These granules contain 75% uric acid and 25% proteins. These granules are initially formed from proteins sequestered into a multivesicular body that may contain different types of protein granules. Tojo *et al.* [20] concluded that these proteins were different from those stored in the fat body. The figure used by Locke [15] to describe this developmental stage in urate granule formation is very similar to that shown in Fig. 3B. Urate begins to be incorporated in the next stage to form the fibrous matrix; most proteins disappear at this stage [15]. Once again, the figure used by Locke to illustrate this latter stage shows structures very similar to those shown in Figs. 2C and 3C. However, there are no other literature reports

of these structures in diplopods to allow comparison with our findings.

Dean *et al.* [3] described the sequence of urate granule development and suggested that these structures originated through the incorporation of endogenous uric acid by endocytosis followed by the addition of this material to vacuoles containing endocytized proteins. These authors suggested that the uric acid may have come from the metabolism of nucleic acids derived from autophagy of the rough endoplasmic reticulum due an earlier massive protein synthesis, but may also have been extracted from the hemolymph. We believe that these same processes also occur in *R. padbergi*. Finally, the large number of spherical bodies seen in the fat body of *R. padbergi* may also be a consequence of these animals´ diet since they overturn soil rich in large amounts of minerals.

In conclusion, this is the first report to describe the ultrastructure of these spherical, mineralized bodies in a diplopod. Our findings show that these structures are morphologically similar to the spherocrystals described in other invertebrates.

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