



Structure of the Malpighian tubule cells and annual changes in the structure and chemical composition of their spherites in the cave cricket *Troglophilus neglectus* Krauss, 1878 (Rhaphidophoridae, Saltatoria)

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ABSTRACT

Periodical changes in the structure of spherites in the Malpighian tubule cells of the cave cricket *Troglophilus neglectus* were studied to elucidate their role during the cricket's life cycle in natural circumstances. Special interest was given to the dormant overwintering period when we hypothesized that the primary role of spherites is to supply minerals for basic vital processes. The investigation was carried out by light and transmission electron microscopy, energy dispersive X-ray spectroscopy, electron energy-loss spectroscopy and energy-filtering TEM. Spherites are present only in the middle Malpighian tubule segment, consisting of Type 1 cells, characterized, among other features, by a round, apically placed nucleus and numerous spherites, and a few Type 2 cells with an elongated nucleus in the centre and sparse spherites. At the beginning of dormancy in November juveniles, minerals are accumulated in spherites and then decline until March. In one-year-old May larvae, spherites are commonly rich in minerals, and from July onwards they are progressively exploited in the adults. Spherite destruction starts with apoptosis in senile October individuals. The findings suggest that the mineral supply of spherites in Malpighian tubules is crucial to supporting vital processes throughout the life cycle of *T. neglectus*.

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1. Introduction

The Malpighian tubules (MTs) are a type of excretory and osmoregulatory organs in many arachnids, in insects and myriapods (Hickman et al., 2001; Resh and Cardé, 2003). Homonymous organs to the MT with a similar structure and position are the only osmoregulatory organs in tardigrades (Møbjerg and Dahl, 2008), and similar, not homologous organs, are reported in onychophorans (Mayer, 2006). The Malpighian system consists of distally blind-ending tubules extending from the midgut–hindgut junction. These excretory organs maintain the constancy of the internal environment by regulation of electrolyte and water balance via absorption of water and solutes, like mineral salts, from the surrounding haemolymph, and by transfer of purine compounds, damaged proteins, etc. These wastes are involved in the formation of the primary urine (Ballan-Dufrançais, 2002). The urine produced

is emptied into the hindgut (Bradley, 1985; Romoser and Stoffolano, 1998).

Investigation of various insects has revealed a cytologically different structure of the MT (Wigglesworth and Salpeter, 1972; Herbst and Bradley, 1989; Kalender and Kalender, 1996; Kalender et al., 2002). The epithelium forming MT is composed of a single layer of cells transporting fluid and solutes and having a storage role (Martoja and Ballan-Dufrançais, 1984). These cells exhibit a polarity, with numerous infoldings of the basal plasma membrane, and long, tightly packed microvilli apically, which are oriented towards the tubule lumen. This cell layer can consist of diverse cell types. In this respect, there are four major types of MT in arthropods: (1) segmented tubules with a uniform cell type within the segments, (2) non-segmented tubules with diverse cell types, (3) segmented tubules with cellular heterogeneity within segments – all of them round in transect, and (4) non-segmented, bilaterally asymmetric tubules (Bradley, 1985). In most insects, musculature is associated with the MTs (Bradley, 1985), which might play a role in mixing the contents of the tubules and displace the tubules within the haemolymph.

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Most cell types in MT have conspicuous, concentrically layered membrane-bound cytoplasmic structures, called spherites, commonly found in different cell types of various taxa. In spherites of various animals, elements, like C, O, N, Ca, Na, Mg, K, Cl, Si, P, S, Fe, Ba, Ni and Cu have been found (Waku and Sumimoto, 1971, 1974; Becker et al., 1974; Turbeck, 1974; Durfort, 1982; Mason and Simkiss, 1982; Becker and Peters, 1985; Janssen, 1985; Al-Mohanna and Nott, 1989; Alberti and Storch, 1983; Goyffon and Martoja, 1983; Azevedo and Corral, 1987; Ludwig and Alberti, 1988; Triebkorn and Köhler, 1996; Da Cruz-Landim and Serrão, 1997; Luchtel et al., 1997; Lipovšek et al., 2002; Marigómez et al., 2002; Schill and Köhler, 2004; Pigino et al., 2005; Lipovšek Delakorda et al., 2008). In insect digestive tissues, spherites are described as storage excretion sites of toxic and non-toxic waste materials (Jeantet et al., 1977; Sohal et al., 1977; Howard et al., 1981; Bradley, 1985; Maroni and Watson, 1985; Ludwig and Alberti, 1988; Wessing et al., 1992; Triebkorn and Köhler, 1996; Schill and Köhler, 2004). In Mollusca (Angulo and Moya, 1989; Colville and Lim, 2003) and Insecta (Wigglesworth and Salpeter, 1972; Ballan-Dufrançais, 1974; Ryerse, 1979; Krüger et al., 1987; Jeantet et al., 1977; Luchtel et al., 1997; Hazelton et al., 2001; Kalender et al., 2002; Pigino et al., 2005) they have also been studied in the cells of excretory tissues.

Cave crickets of the family Rhabdiphoridae belong to a phylogenetically old group of Ensifera (Jost and Shaw, 2005). Rhabdiphorids inhabit mostly tropical and subtropical regions, as well as temperate and boreal ones (Remy, 1931; Chopard, 1938; Di Russo and Sbordoni, 1998; Sbordoni and Cobolli, 2004). More than half of the over 300 described species are cavernicolous (Di Russo and Sbordoni, 1998; Sbordoni and Cobolli, 2004), and four dozens of them live in Europe (Heller, 2004). Little has been published on the functional anatomy of rhabdiphorids (Lavoie et al., 2007). *Troglophilus neglectus* and *Troglophilus cavicola* are the most widely distributed European species. In *T. neglectus*, different aspects of biology, ecology and physiology, and selected biochemical topics have been studied (Gogala, 1964; Novak and Kuštor, 1983; Us, 1992; Čokl et al., 1995; Jeram et al., 1995; Pehani et al., 1997; Raspotnig et al., 1998; Schrader et al., 2002; Christian, 2008). The life cycle of *T. neglectus* spans two years. Larvae hatch in May and spend their epigean ecophase until autumn. Then, individuals spend an inactive 4–6 months overwintering in caves, usually from November until March, and then again an active epigean ecophase from April until October. They mature in July and die in October. *T. neglectus* is omnivorous feeder eating small quantities of plant material, fruits, small animals, carcass, and it is also a cannibal (Novak and Kuštor, 1983; Christian, 2008).

In this contribution, we investigate the cave crickets *T. neglectus* as an appropriate experimental species from which to obtain preliminary information on the structure and chemical composition of spherites in the excretory system of rhabdiphorids. The main goal was to get an insight into possible physiological roles of spherites in the Malpighian tubule cells (MC) during the crickets' life cycle through the analytical electron microscopy in natural conditions, irrespective of the crickets' diet. In particular, we focused on their role during the winter quiescence, representing a natural starvation period, which is being studied for the first time in insects.

2. Materials and methods

The specimens of the cave cricket *T. neglectus* were collected in four caves in central northern Slovenia (locality centroid 46°24'55"N, 15°10'31"E, altitudes 600–740 m). A preliminary study of five specimens collected in November 2006 showed spherites exclusively in a middle segment of the tubule. All the observations were thus made on this MT segment. The spherites were in general

of a quite comparable number, size and structure and elemental composition; thus, three individuals were analysed during each time frame. The hypogean ecophase lasted from November until March of the following year, the epigean from March until November. Juveniles were analysed at the beginning (November 2006), the middle (February 2007) and the end (March 2007) of overwintering. Juveniles in May 2007 and adults in July 2007 and October 2007 represented the epigean ecophase. The structure of spherites was studied by light microscopy (n per individual = 50) and conventional transmission electron microscopy (TEM), and their chemical composition by a combination of electron energy-loss spectroscopy (EELS), energy-filtering TEM (EFTEM) and energy dispersive X-ray spectroscopy (EDXS) (n per individual = 10–15).

The MTs were fixed in a mixture of 2.45% glutaraldehyde and 2.45% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at room temperature for 3 h and then at 4 °C for 12 h. Afterwards, the tissue was washed in 0.1 M sodium cacodylate buffer at room temperature for 3 h and postfixed in 2% OsO₄ at room temperature for 2 h. Finally, the tissue was washed in 0.1 M sodium cacodylate buffer (pH 7.4) at room temperature for 3 h, dehydrated in a graded ethanol series and embedded in TAAB epoxy resin (Agar Scientific Ltd.) for both light and electron microscopy studies. Semithin sections were stained with 0.5% toluidine blue in aqueous solution. For electron microscopy, ultra-thin sections were stained with uranyl acetate and lead citrate. The preparations were examined in a Nikon Eclipse E800 compound microscope with a mounted digital Net camera DN100, and processed with Eclipse Net software, and in a Zeiss EM 902 transmission electron microscope.

2.1. Elemental analysis

The elemental analysis of the spherites was carried out on unstained, ultra-thin sections of the MT. The sections, with thicknesses of 75 nm, were collected on copper grids covered with a perforated carbon support film and studied in a Philips CM 20/STEM electron microscope operated at 200 kV (LaB₆ cathode), equipped with a Gatan imaging filter (GIF) and a Noran EDX system with an HPGe-detector.

EEL-spectra as well as the elemental distribution images were recorded with the slow-scan CCD camera integrated into the GIF. To collect information on light elements, EEL-spectra were acquired with the GIF operated in spectrum mode. The elemental maps were obtained by recording an energy-filtered TEM (EFTEM) image at the energy of an element-specific ionization edge. This image contains a non-specific background, which must be removed in order to get the true elemental map (Hofer et al., 1995). For the Three Window Method, two energy-filtered background images in front of the edge and one image at the ionization edge of the element of interest were acquired. An extrapolated background image was calculated using the power-law model $I = A \times E^{-r}$, where I is the intensity, E is the energy loss, and A and r are two fitting parameters (Egerton, 1996). The background image was subtracted from the ionization-edge image, thus giving a net image, which is the elemental map. This method was used for the elements P and O. For Ca, the Two Window Method resulted in a better signal-to-noise ratio. To get what is called a jump ratio image, the ionization edge was divided by a pre-edge image.

3. Results

3.1. Malpighian tubules

In the investigated *T. neglectus* there is an average of 50–60 slender, simply structured MTs, which extend from the digestive

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