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Abstract

Crypt cells - one of the three cell types composing Strombidae digestive tubules - are characterized by the presence of numerous metal-containing phosphate granules termed spherocrystals. We explored the bioaccumulation and detoxification of metals in Strombidae by exposing wild fighting conch *Strombus pugilis* for nine days to waterborne $CuSO_4$ and $ZnSO_4$. The total amount of Cu and Zn was determined in the digestive gland and in the rest of the body by Inductively Coupled Plasma (ICP) analyses. The digestive gland spherocrystal metal content was investigated based on the semi-quantitative energy dispersive X-ray (EDX) elemental analysis. ICP analyses of unexposed individuals revealed that 87.0±5.9% of the Zn is contained in the digestive gland, where its concentration is 36 times higher than in the rest of the body. Regarding Cu, 25.8±16.4% of the metal was located in the digestive gland of the control individuals, increasing to 61.5±16.4% in exposed individuals. Both Cu and Zn concentrations in the digestive gland increased after exposures, pointing to a potential role of this organ in the detoxification of these metals. EDX analysis of spherocrystals revealed the presence of Ca, Cl, Fe, K, Mg, P, and Zn in unexposed individuals. No difference was found in the relative proportion of Zn in spherocrystals of exposed *versus* control individuals. Contrastingly, copper was never detected in the spherocrystals from controls and Zn-exposed individuals, but the relative proportion of Cu in spherocrystals of Cu-exposed individuals varied from 0.3% to 5.7%. Our results show the direct role of spherocrystals in Cu detoxification.

Keywords: exposure; Mollusca; phosphate granule; trace metal; ultrastructure

Introduction

Strombidae are benthic gastropods, some species of which represent a staple food and important trade commodity (Aldana Aranda 2003). The fighting conch *Strombus* (*Lobatus*) *pugilis* (Linnaeus, 1758) is one of these species and is widely fished in the Caribbean region and especially consumed around the Yucatan coasts in Mexico. Strombidae recently received a special focus because their digestive gland hosts putative symbionts belonging to the Sporozoa group (Baqueiro Cárdenas et al. 2007; Gros et al. 2009; Volland et al. 2010). Ultrastructural investigation of the digestive gland highlighted the presence of three cell types in the digestive tubules: digestive cells, vacuolated cells and crypt cells. It is now well-established that digestive cells are mostly involved in intracellular digestion (Volland and Gros 2012) whereas vacuolated cells store lipids and host the Sporozoan symbionts (Gros et al. 2009; Volland and Gros 2012). In contrast, the function of the crypt cells remains unclear.

Crypt cells, also known as basophilic cells, calcium cells or secretory cells, are described in the digestive gland of many mollusks (Fretter and Graham 1962; Merdsoy and Farley 1973; Voltzow 1994). They are characterized by their pyramidal shape and their location in the crypt of digestive tubules (Taïeb and Vicente 1999). Other frequently reported characteristics of this cell type include the presence of a large nucleus with a prominent nucleolus and an abundant rough endoplasmic reticulum (Fretter and Graham 1962; Lutfy and Demain 1967; Merdsoy and Farley 1973; Wigham 1976; Voltzow 1994; Lobo-da-Cunha 1999; Taïeb and Vicente 1999; Volland et al. 2012). Finally, one remarkable component of the crypt cells is spherical mineral inclusions termed spherocrystals. While not always present, they are often characteristic of mollusk crypt cells.

Spherocrystals, also known as phosphate granules or metal-containing granules, are mineral inclusions amorphous to X-ray and electron diffraction (Taylor et al. 1989; Taylor et al. 1990; Mitchell et al. 1996; Masala et al. 2004). They are present in various taxonomic groups and are particularly well described in arthropods (Ballan-Dufrançais 2002). Among mollusks, spherocrystals have been described from bivalves (George et al. 1980; George et al. 1982; George et al. 1984), gastropods (Howard et al. 1981; Greaves et al. 1984; Nott et al. 1993; Triebskorn et al. 1996; Gibbs et al. 1998; Taïeb and Vicente 1999) and cephalopods (Martoja and Marcaillou 1993; Costa et al. 2014). Spherocrystals have been observed in the crypt cells of various Strombidae species including *S. pugilis*, where they occupy a considerable

part of the cytoplasm (Gros et al. 2009; Volland et al. 2010; Volland et al. 2012). These structures store minerals and trace metals and are thus thought to be involved in their detoxification (Marigómez et al. 2002; Delakorda et al. 2008). Nevertheless, their function remains unclear.

A previous exposure experiment of *S. pugilis* to waterborne Cd and Pb suggested that spherocrystals are not involved in detoxifying these two nonessential trace metals in this species (Volland et al. 2012). We did, however, detect Al, Ca, Fe, Mg, Mn, P, and Zn in the spherocrystals of two Strombidae, *S. gigas* and *S. pugilis*, indicating that these structures play a role in regulating minerals and essential trace metals in these gastropods (Volland et al. 2012). Moreover, spherocrystals in barnacles accumulate considerable amounts of metals, whereby Cu and Zn concentrations can reach 3.7 mg g^{-1} and 27.1 mg g^{-1}, respectively (Pullen and Rainbow 1991).

In 2010, reproductive failure in a *Strombus gigas* population from Florida Keys nearshore waters was attributed to heavy metal exposure; the offshore population not exposed to the pollution reproduced normally (Spade et al. 2010). Those authors found an inverse correlation between Cu and Zn concentrations in the digestive gland and the spermatogenic index (which reflects the fecundity level). The nearshore population showed a repression in the expression of many genes related to spermatogenesis and mitochondrial functions. The transcription of small GTPase-related signaling genes was affected and reflected in the lack of testis development they observed histologically. The authors hypothesized that heavy metals contribute to the reproductive failure of the conchs. They proposed that the elevated concentrations of Zn, and possibly Cu, in the digestive gland affect the development of the conch testis.

In this context, the present study investigates the potential role of the digestive gland in storing and detoxifying the two essential metals Cu and Zn. We analyzed unexposed and waterborne-exposed individuals of the fighting conch *Strombus pugilis* maintained in controlled conditions in the laboratory. The total amount of these metals was determined in the digestive gland and in the rest of the body by ICP analyses. This was accompanied by ultrastructural observations of the digestive gland coupled with the EDX-ray elemental analysis of spherocrystals.

Materials and methods

1.1. Sample collection and exposure

Specimens of *Strombus pugilis* were collected by hand on sandy areas in Guadeloupe, French West Indies (F.W.I), and in Campeche bank, Mexico. Ultrastructural observations were performed on the two populations, whereas the exposure experiment with trace metals was performed on the Mexican population only. Once collected, organisms were rapidly brought to the laboratory. Many specimens (n=740) were processed for histological examination and 30 individuals were starved for 24 h and placed in five 40 L aquaria filled with 5µm filtered seawater in order to depurate their gut. Two aquaria each were used for the exposure to Cu and Zn. A stock solution of $CuSO_4.5H_2O$ at 3.20 mmol L^{-1} was used to obtain final concentrations of 0.40 µmol L^{-1} and 0.80 µmol L^{-1}. A stock solution of $ZnSO_4.7H_2O$ at 2.78 mmol L^{-1} was used to obtain final concentrations of 0.35 µmol L^{-1} and 0.70 µmol L^{-1}. A fifth aquarium was used as negative control without addition of Cu and Zn. The water was oxygenated and renewed every three days. Individuals were fed daily with artificial food developed and provided by the CINVESTAV-IPN of Mérida (Nutrition and Aquaculture of Mollusks Laboratories). After nine days of waterborne exposure (and to a lesser degree dietary-borne because food was in contact with the contaminant in the water), the individuals were killed. From each of the five aquaria, the six individuals were killed: three were processed for the ICP quantitative analyses of trace metals and the other three for the EDX-ray analysis of spherocrystals from the digestive gland.

1.2. Histology and transmission electron microscopy

The digestive glands were dissected and fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in cacodylate buffer (0.1 M; 1100 mOsm; pH: 7.2) for 24 h at 4°C and rinsed with an iso-osmotic buffer. For histology, tissue samples were dehydrated in an ascending ethanol series and infiltrated with Paraplast wax before embedding. Wax sections, 7 µm-thick, were obtained from each sample and stained with a modified Goldner trichrome method that included Alcian blue at pH 2.5 in order to stain proteoglycans (Gabe 1968). For TEM, samples were post-fixed for 1 h in 1% osmium (O_sO_4) in the same buffer. The samples were then dehydrated through an ascending ethanol series and embedded in epoxy resin according to Volland et al. (2010). Semi-thin sections were stained with 0.5% toluidine blue in 1% borax for

photonic microscopy observations. Ultra-thin sections (60 nm) were contrasted with uranyl acetate and lead citrate before observation in a Leo 912 transmission electronic microscope.

1.3. Scanning electron microscopy and EDX-ray analysis

Three individuals from each condition (control, exposure to Cu at 0.8 µmol L^{-1}, exposure to Zn at 0.7 mmol L^{-1}) were used for the EDX-ray analysis of spherocrystals. The digestive glands were dissected and fixed as described for TEM. They were then dehydrated through an ascending acetone series, critical point dried, and manually fractured before observation with a Quanta 250 scanning electron microscope (FEI®) at an acceleration voltage of 20 kV under low vacuum (650 Pa). Energy-dispersive X-ray analysis (EDX) of individual spherocrystals was carried out using an M-max 50mm^2 Oxford detector, monitored by an INCA system, in the point mode.

1.4. Trace metal analysis

The digestive gland and remaining parts of the body of *S. pugilis* exposed to Cu (0.40 µmol L^{-1}, n=3 and 0.80 µmol L^{-1}, n=3) and Zn (0.35 µmol L^{-1}, n=3 and 0.70 µmol L^{-1}, n=3) and from the control (n=3) were prepared for Cu and Zn analysis using a Varian Vista-Pro ICP-OES and a Thermo Fisher Scientific X Series 2 ICP-MS (following Kojadinovic et al., 2011). Briefly, the tissues were freeze-dried for several days then ground with a Fritsch® Planetary ball mill. Aliquots of the biological samples (100–300 mg) were digested with 6 ml 67–70% HNO_3 and 2 ml 34–37% HCl (Fisher Scientific, trace element grade quality). Acidic digestion was carried out overnight at room temperature and then in a Milestone microwave (30 min with constantly increasing temperature up to 120°C, and finally 15 min at this maximal temperature). Each sample was completed to 50 ml with milli-Q water. Three control samples (two Certified Reference Materials (CRM), and one blank) treated and analysed in the same way as the samples were included in each analytical batch. CRMs were Dolt-4 dogfish liver (NRC, Canada) and TORT-2 Lobster Hepatopancreas (NRC, Canada). The results were in good agreement with the certified values, with a mean recovery rate of 90-104% for DOLT-4 and 92-102% for TORT-2. Trace element concentrations are expressed in µg g^{-1} dry weight (dw).

2. Results

2.1. *Digestive gland structure*

The digestive gland of individuals from Guadeloupe (F.W.I.) was observed under the light microscope (semi-thin sections) and under the transmission electron microscope. The digestive gland of controls from Mexico was observed under the light microscope after histological staining and under the scanning electron microscope. Based on many observations in recent years (F.W.I: n=40 and Mexico: n=700), digestive gland structure of individuals from the two locations did not differ. The gland of the individuals investigated here represented on average 14.4±3.2% of the whole body mass (n=15). It is composed of an assemblage of digestive tubules and ducts. The digestive tubule epithelium composes three cell types: digestive cells, vacuolated cells and crypt cells (Fig. 1). The vacuolated cells together with the crypt cells form a pseudo-stratified epithelium. The vacuolated cells are on average 30 µm long and 10 µm wide. They are characterized by the presence of an intracellular sporozoan symbiont and their cytoplasm is full of lipid droplets which appear metachromatic (green) on osmificated samples (Figs. 1A, B). The digestive cells are long columnar cells averaging 80 µm in length and 7 µm in width. They are the most abundant cells in the digestive tubules. They are packed together in well-aligned groups of cells which form a uni-stratified epithelium. A basal nucleus is surrounded by lipid droplets. The median third of their cytoplasm usually contains one large granule (~ 7 µm in diameter) with a dense heterogeneous content stained by Alcian blue. The apical third of digestive cells contains vesicles and granules of various diameters from 0.5 µm near the apical pole to 5 µm near the median part of the cells (Figs. 1A-C). The third cell type of the digestive tubules is the crypt cell. These cells are characterized by a pyramidal shape and a cytoplasm full of spherical inclusions identified as spherocrystals (Figs. 1B, C). Crypt cell size varies from 10 to 50 µm long and from 10 to 50 µm wide. The nucleus is large (Fig. 1B) and the rough endoplasmic reticulum abundant (Fig. 1D). The spherocrystals are spherical inclusions about 1 µm in diameter. They are composed by alternating electron-dense and electron-lucent concentric layers organized around a matrix core (Fig. 1D). EDX analysis of spherocrystals revealed the presence of Ca, Cl, Fe, K, Mg, P, and Zn, whereby P, Mg, Zn and Ca were most abundant (Fig. 2). Between-cell variability of Fe, K and Zn was high, within-cell variability low (Fig. 3).

2.2. Zinc

The analysis of Zn in unexposed *Strombus pugilis* (controls) revealed that 87.0±5.9% of the total metal body burden was located in the digestive gland (Fig. 4A). The concentration in the digestive gland (1796±174 µg g^{-1} dw) was 36 times higher than in the rest of the body (50 ±10 µg g^{-1} dw) (Fig. 4B). After nine days of exposure to waterborne Zn, the proportions of the metal in the digestive gland and the rest of the body did not change significantly (Fig. 4A) but the concentration in the digestive gland was higher after the exposure (Fig. 4B). The Zn concentration in the rest of the body remained in the same range at the end of the exposure period for both concentrations and in controls (Fig. 4B). In parallel, the semi-quantitative elemental analysis of spherocrystals in the crypt cells of the digestive gland showed no differences in the relative proportion of Zn in exposed *versus* control individuals.

2.3. Copper

The analysis of Cu in *S. pugilis* tissues revealed that 25.8±16.4% of the total metal body burden was located in the digestive gland of the control individuals (Fig. 4C), with slightly higher concentrations compared to the rest of the body (32±11 µg g^{-1} dw vs 20±4 µg g^{-1} dw, respectively; Fig. 4D). After nine days of exposure to waterborne Cu, the distribution of the metal within the organism changed significantly. The percentage of the total Cu located in the digestive gland was higher after the exposure (Fig. 4C). The digestive gland of individuals exposed to 0.40 µmol L^{-1} of Cu contained 46.8±17.5% of the whole body burden whereas those exposed to 0.80 µmol L^{-1} contained 61.5±16.4% (Fig. 5). At the same time, the digestive gland Cu concentration increased almost nine fold to 316±153 µg g^{-1} dw in exposed individuals (Fig. 4D). Cu concentrations in the rest of the body were not significantly different from the control after the 0.40 µmol l^{-1} exposure to waterborne Cu (16±6 µg g^{-1} dw) but increased to 33±6 µg g^{-1} dw after the 0.80 µmol l^{-1} exposure. In the controls, no Cu was ever detected in any of the spherocrystals (n=42) analyzed by EDX. Likewise, no Cu was detected in any of the spherocrystals (n=29) from the three individuals contaminated with Zn. However, after nine days of exposure to Cu 0.80 µmol L^{-1}, the metal was detectable in spherocrystals (n=22) of all three individuals. The relative proportion of Cu in spherocrystals of exposed individuals varied from 0.3% to 5.7% (average 1.5±1.5%).

3. Discussion

3.1. Digestive gland structure and ultrastructure

The structure and ultrastructure of the digestive gland of mollusks have been extensively studied, especially in bivalves, pulmonate gastropods and cephalopods. While the overall organization (primary and secondary ducts connected to digestive tubules) is the same in all groups, the digestive tubule epithelium composition is group specific and sometime varies within the groups. Bivalves usually exhibit only two cell types: digestive (also termed acidophilic) and crypt cells (also termed basophilic, pyramidal or secretory cells) (Pal 1971; Pal 1972; Henry 1984a; 1984b; Morse et al. 1997). The digestive tubules of cephalopods consist of a single digestive cell type (Purchon 1968; Semmens 2002; Swift et al. 2005). Few studies have examined polyplacophoran digestive tubules, but at least two cell types have been described, digestive and basophilic cells (Lobo-da-Cunha 1997). Among gastropods, the epithelium seems to be more complex, involving three to five cell types in pulmonates (Walker 1970; Luchtel et al. 1997). Three to four cell types are described in opistobranchs (digestive, calcium, excretory, and thin cells) (Coelho et al. 1998; Taïeb and Vicente 1999; Lobo-Da-Cunha 2000; Taïeb 2001). Finally, the caenogastropods, to which the Strombidae, have at least two cell types, the digestive and the pyramidal crypt cells. The latter contains various cell types described and differently named as secretory, excretory, basophilic, and calcium cells (Fretter and Graham 1962; Lutfy and Demain 1967; Purchon 1968; Merdsoy and Farley 1973; Boghen and Farley 1974; Wigham 1976; Devi et al. 1981; Voltzow 1994).

In the digestive gland tubules of *S. pugilis*, three cell types are identified: the digestive, vacuolated, and crypt cells – consistent with those described in other *Strombus* species (Gros et al. 2009; Volland et al. 2010; Volland and Gros 2012). While the digestive and crypt cells present the classic features of the taxonomic group, the vacuolated cells seem to be more specific to this family. To our knowledge only one study has reported similar cells in the digestive tubules of the caenogastropod *Maoricrypta monoxyla* (Nelson and Morton 1979).

Three types of granules are present in the digestive gland of *S. pugilis.* The biggest ones are the brownish spherules previously identified as sporozoan symbionts in the vacuolated cells (Gros et al. 2009; Volland et al. 2010). The digestive cells present proteoglycan-rich granules of ~ 7 µm diameter as shown by Alcian blue specific staining. Those first two types of intracellular granules are also observed free in the lumen of tubules, in the digestive gland

ducts and in the feces of *S. gigas* (Gros et al. 2009). We made similar observations in *S. pugilis* (data not shown). The third type of granules in the digestive gland is the spherocrystals in the crypt cells. These granules are too small to be observed in the feces using the light microscope, but SEM used in other mollusks showed they are expelled with the feces (Nott and Nicolaidou 1990; 1996). Spherocrystals have already been reported in the tissues of mammals (Gallien et al. 2001), arthropods (Ballan-Dufrançais 2002), annelids (Jenkins et al. 2002; Mouneyrac et al. 2003) and many mollusks. A study focusing on prosobranchs reported spherocrystals in 40 species belonging to 20 families (Gibbs et al. 1998). Taylor *et al.* (1988) proposed that spherocrystals are deposits of pyrophosphate salt ($CaMgP_2O_7$), which traps metal ions. Elements detected in the spherocrystals of different mollusk families are Ca, Cl, Cu, Fe, K, Mg, Mn, Na, Ni, S, Ti, V, and Zn (Nott and Langston 1989; Nott et al. 1993; 1996; Gibbs et al. 1998). Nott and Nicolaidou (1989) proposed that metals in cells induce the production of magnesium phosphate in spherocrystals as a source of phosphate ions to bind and detoxify the metals. After analyzing the elemental composition of spherocrystals from clean and polluted sites, they concluded that spherocrystals of marine gastropods do respond to different levels of metals in the environment. A previous study on *S. gigas* and *S. pugilis* highlighted the presence of Al, Ca, Fe, Mg, Mn, P, and Zn in the spherocrystals (Volland et al. 2012). Moreover, analyses of spherocrystals from feces have shown that they still present an important proportion of their trace metal content compared to those analysed from the digestive gland (e.g., 50% for Zn and 33% for Mn) after their passage through the digestive tract (Nott and Nicolaidou 1993). Clearly, these structures represent a detoxification pathway for trace metals in mollusks (Marigómez et al. 2002).

3.2. Exposure to Cu and Zn

No study is available on the levels of Cu and Zn in *S. pugilis*. Among Strombidae, only *S. canarium* (Said et al. 2013; Sabri et al. 2014a; 2014b) and the queen conch *S. gigas* were investigated for these metals (Rizo et al. 2010; Spade et al. 2010; Whitall et al. 2016). Unlike in *S. pugilis, t*he digestive gland of *S. gigas* is removed before human consumption. The digestive gland of *S. gigas* shows by far the highest levels of Zn in the organism (Spade et al. 2010). The digestive gland of *S. pugilis* represents only 14.4±3.2% of the whole body mass but contains most of the total Zn body burden (87.0±5.9%) in unexposed gastropods. The very high concentration of this metal in the digestive gland already points to a potential role in Zn

regulation (and possibly detoxification) through its storage in spherocrystals. Copper is more spread in the body of unexposed individuals but still is more concentrated in the digestive gland. Note that in many mollusks, oxygen is transported by a Cu-based haemocyanin pigment. Each oxygen molecule transported in the blood requires two Cu atoms (Van Holde and Miller 1995).

At the end of the exposure period to waterborne metals, both Cu and Zn concentrations increased drastically in the digestive gland. However, only the distribution of Cu between the digestive gland and the rest of the body was significantly modified, with 61.5±16.4% of the Cu being detected in the digestive gland after the exposure to 0.80 µmol L^{-1} of $CuSO_4$. Accordingly, storage in this organ is implicated in detoxifying Cu and Zn. Ultrastructural analysis is required in order to further identify the cell types and structures involved in scavenging these two metals.

The elemental analysis of the spherocrystals from the digestive gland revealed that they naturally contain Zn, which represents 14.2±6.3% of the elements analyzed in spherocrystals. While Zn concentrations increased in the digestive gland during the exposure, the relative proportion of Zn in spherocrystals remained unchanged. The excess Zn might rather be bound to proteins such as metallothioneins (Amiard et al. 2006). Note that Zn is a transition metal showing properties of class A (affinity for O) and class B (affinity for S) metals (Nieboer and Richardson 1980). It has been proposed that spherocrystals scavenge class A metals (K, Na, Li, Ba, Sr, Ca, Mg) more than class B metals (Ag, Cd, Hg), the latter being associated with metallothionein proteins (Nieboer and Richardson 1980; Marigómez et al. 2002). Other structures may be involved in Zn detoxification, including the proteoglycan-rich granules from digestive cells (not analyzed in this study). EDX-ray analyses have reported Zn in similar digestive cell granules in other mollusks (Nott and Nicolaidou 1989). The intracellular symbionts present in the vacuolated cells might also be involved in Zn detoxification because EDX-ray analyses of such structures have revealed the presence of Zn but also Al, Ca, Fe and S (Volland et al. 2010). Both the digestive cell granules and the symbionts are indeed expelled via the feces (Gros et al. 2009), thus potentially representing an elimination pathway for the excess of metals they contain.

In cephalopods, exposure of young cuttlefish to waterborne Zn disturbs the metabolism of several metals including Cu (Le Pabic et al. 2015). In the present study, the spherocrystals of unexposed and Zn-exposed individuals show no Cu peak in the EDX-ray

spectra: they either lack this element or it is present in amounts below the detection threshold of the technique. In a study focusing on the common cerith, *Cerithium vulgatum* (Caenogastropoda), similar EDX-ray analysis of digestive gland spherocrystals detected the following elements: Ca, Cl, Co, Cr, Fe, K, Mg, Mn, Na, P, S, and Zn but no Cu (Nott and Nicolaidou 1989). Those authors also analyzed these structures with energy filtered transmission electron microscopy, a more sensitive technique. They reported a small peak for Cu, suggesting that spherocrystals scavenge this element too. After Cu exposure, we detected this metal in the spherocrystals of *S. pugilis*, where it represented up to 5.7% of the analyzed elements. The presence of Cu in the spherocrystals, together with the increased Cu concentration in the digestive gland, indicate that spherocrystals are involved in the detoxification of this element. EDX-ray analysis, however, is a semi-quantitative analytical technique and cannot determine the absolute amount of Cu detoxified through spherocrystal scavenging.

3.3. Conclusion

The digestive gland of mollusks is a key organ involved in the digestion but also in lipid storage, mineral osmoregulation, and metal detoxification (Owen 1966; Voltzow 1994; Porcel et al. 1996; Penicaud et al. 2017). Most ecotoxicological studies have investigated the impact of metal exposure on i) the organ metal burden and ii) the subcellular partitioning of metals obtained by differential centrifugation. The resulting fractions contain organelles and other cytoplasmic structures referred to as cellular debris or metal-rich granules (Guo et al. 2013). It is difficult to determine whether the different authors are referring to the same structures. Other studies have focused on the ultrastructure of the digestive gland, analyzing the elemental composition of metal-rich granules with precise cellular localization (Marigómez et al. 2002; Delakorda et al. 2008; Volland et al. 2012).

Combining ICP analyses with microscopy techniques and elemental analyses of subcellular compartments in targeted cell types enables investigating detoxification processes at two very different scales. This approach is well suited to test hypotheses on the role of specific cell structures in regulating and detoxifying metals. Using this approach for *S. pugilis*, we demonstrate that spherocrystals help detoxify Cu. The Zn detoxification pathway, on contrast, may involve metallothioneins. The relative contributions of these two detoxification pathways in Strombidae remain to be investigated.

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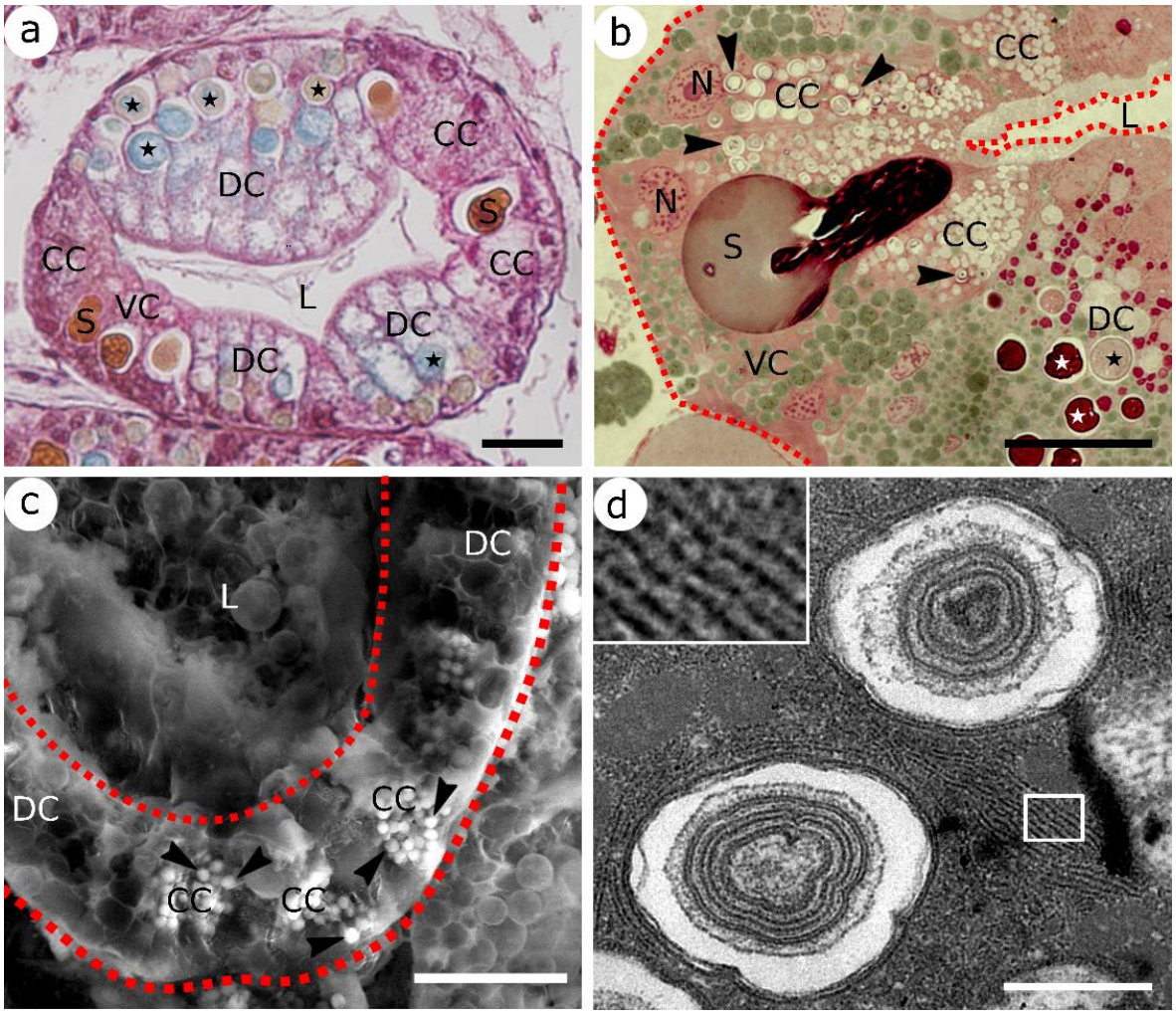

Figure 1: Microscopic images of *Strombus pugilis* digestive gland with ultrastructural details of spherocrystals. **a.** Histology of a digestive tubule transversal section showing the three cell types. The long columnar digestive cell (DC) with characteristic blue granules (stars). Vacuolated cells (VC) host the sporozoan symbiont (S). Groups of crypt cells (CC) are also visible. **b.** Semi-thin section of a digestive tubule showing more details of the digestive cell (DC) granules and the spherocrystals (arrowheads). Lipid droplets in vacuolated and digestive cells appear green on this osmificated sample. **c.** Scanning electron micrograph of a fractured digestive tubule (used for the EDX analysis). Spherocrystals (arrowheads) are readily recognizable. **d.** TEM detail of two spherocrystals surrounded by rough endoplasmic reticulum (insert) from a single crypt cell. Scale bars: **A**, 30 µm; **B**, 40 µm; **C**, 15 µm; **D**, 1 µm.

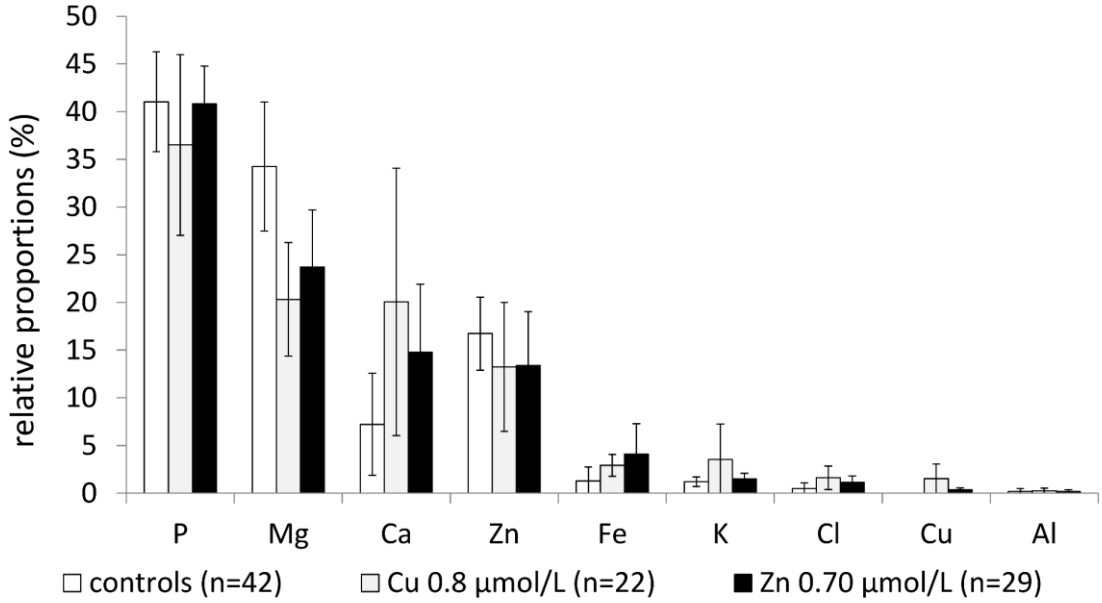

Figure 2 : Relative proportion of the elements Al, Ca, Cl, Cu, Fe, K, Mg, P, and Zn in spherocrystals of control individuals and individuals exposed for nine days to 0.80 µmol L^{-1} of $CuSO_4$ and individuals exposed for nine days to 0.70 µmol L^{-1} of $ZnSO_4$. Number of analyzed spherocrystals in parentheses. P, Mg, Ca, and Zn are the most abundant elements.

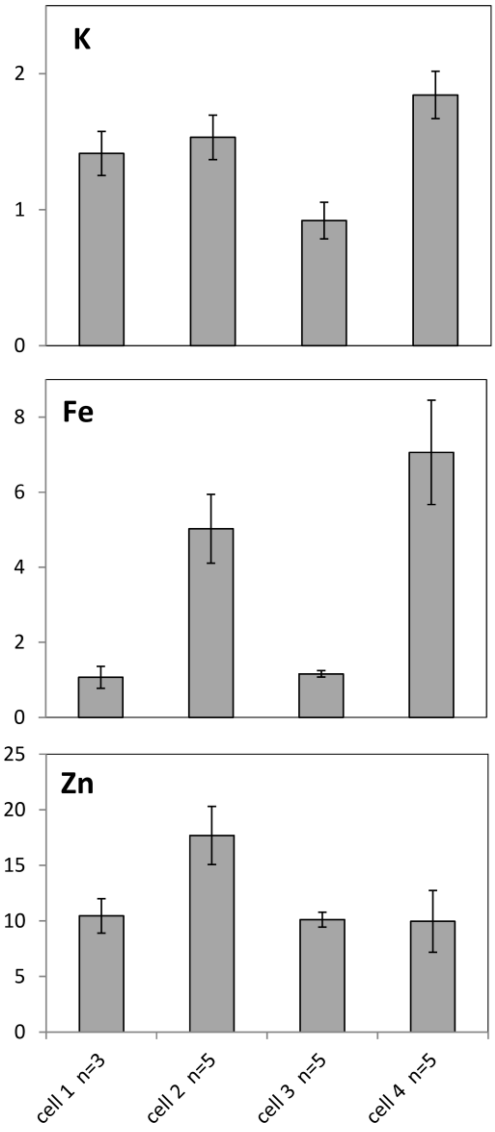

Figure 3: EDX-ray analysis showing the relative proportion of K, Fe and Zn in spherocrystals of *Strombus pugilis* exposed to 0.70 µmol L^{-1} of $ZnSO_4$. The histograms display the average relative proportion of the respective element in four analyzed cells. The number of spherocrystals (*n*) analyzed for each cell varied between 3 and 5. While the relative proportion of an element may vary considerably from one cell to another, all spherocrystals analyzed within one cell display similar relative proportions for the considered element (see low standard deviations).

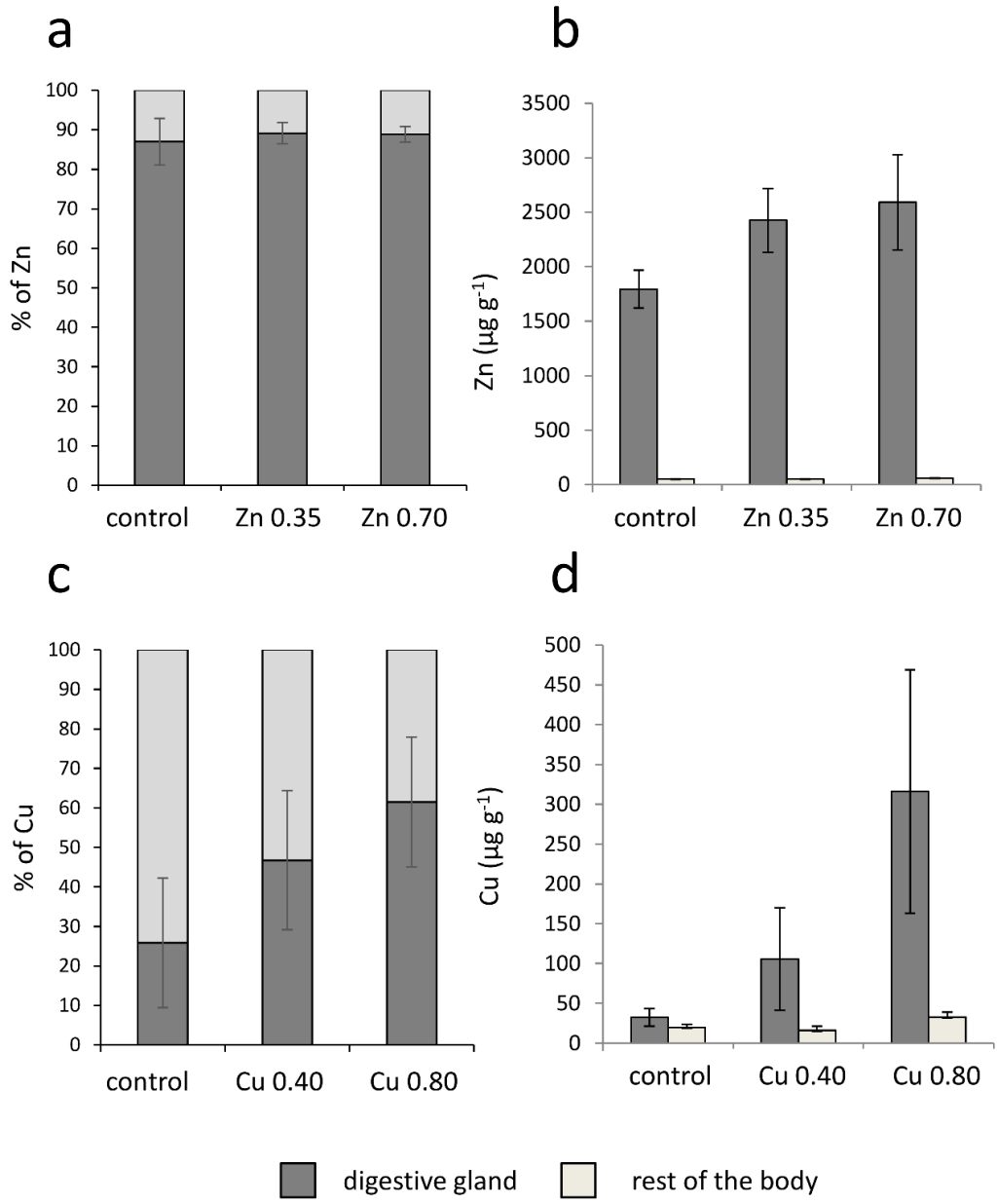

Figure 4: Concentrations (mean ± SD; µg g^{-1} dw) of Cu and Zn and their relative whole body distribution (mean ± SD; %) in the digestive gland and the rest of the body of *Strombus pugilis* individuals (n=3) from control and exposed groups. a: distribution of Zn in the body. Most of the Zn is located in the digestive gland. **b:** Zn concentration in the digestive gland and the rest of the body in control and exposed individuals. **c:** distribution of Cu in the body. In control individuals, most of the Cu is located in the rest of the body. **d:** Cu concentration in the digestive gland and the rest of the body in control and exposed individuals.