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# Ultrastructural alterations of the hepatopancreas in *Porcellio scaber* under stress

Nada Žnidaršič\*, Jasna Štrus, Damjana Drobne

Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, 1000 Ljubljana, Slovenia

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# Abstract

Cellular ultrastructure varies in accordance with physiological processes, also reflecting responses to environmental stress factors. Ultrastructural changes of the hepatopancreatic cells in the terrestrial isopod *Porcellio scaber* exposed to sublethal concentrations of zinc or cadmium in their food were identified by transmission electron microscopy. The exclusive structural characteristic of the hepatopancreas of animals exposed to metal-dosed food was grain-like electrondense deposits (EDD) observed in the intercellular spaces and in vesicles of B cells. In addition, hepatopancreatic cells of metal-exposed animals displayed non-specific, stress-indicating alterations such as cellular disintegration, the reduction of energetic reserves (lipid droplets, glycogen), electron dense cytoplasm, ultrastructural alterations of granular endoplasmic reticulum (GER), the Golgi complex and mitochondria. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Metals; Porcellio scaber; Hepatopancreas; Cytopathology; Microscopy; Sublethal effects

#### 1. Introduction

Cellular structure and function normally vary within the fairly narrow range defined by cellular genetic programs of metabolism, differentiation, and specialisation as well as by constraints of neighbouring cells and by the availability of metabolic substrates (Cotran et al., 1999). Exposure of cells to stressful factors can evoke protective cellular adaptations, or/and pathological changes, which can eventually lead to cell death. Cellular changes can translate into alterations at higher biological levels, although the relationship between the different levels of biological organisation is neither straightforward nor deterministic (Segner and Braunbeck, 1998). Cellular responses are suitable tools for the early and sensitive detection of chemical exposure, useful also in ecotoxicology, due to the assumption that cellular change can ultimately develop into ecological change (Moore, 1985; Segner and Braunbeck, 1998; Wester et al., 2002).

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In our study we aimed to identify ultrastructural changes of the hepatopancreatic cells in the terrestrial isopod *Porcellio scaber* (Crustacea: Isopoda) fed on zinc or cadmium-contaminated food. The selection of this model was based on the following grounds: (i) terrestrial isopods are widely spread organisms, participating in decomposition of organic material in the leaf-litter layer, which is an indispensable process for ecosystem function (Hassall et al., 1987; Van Wensem, 1989). In metal pollution of the terrestrial environment terrestrial isopods are likely to be exposed to the highest concentrations of these pollutants, which might affect their activity. The highest concentrations of metals in contaminated deciduous woodland were found in litter (Martin et al., 1982) and in polluted coniferous forest in the organic layer (litter and humus) of the topsoil (Tyler, 1984). (ii) The hepatopancreas is the central metabolic organ of these animals and also has an important role in handling both essential metals involved in normal physiological processes (Szyfter, 1966; Wägele, 1992), as well as nonessential metals (Hopkin, 1990, 1989). (iii) The structure of hepatopancreatic cells of isopods, including P. scaber, is known to reflect influences of internal and external factors, namely moulting (Szyfter, 1966; Štrus and Blejec, 2001), the

<sup>\*</sup> Corresponding author. Tel.: +386-1-423-3388; fax: +386-1-257-3390.

E-mail address: nada.znidarsic@uni-lj.si (N. Žnidaršič).

Table 1

| Experimental groups | Number of an-<br>imals | Concentration of metal in solution | Concentration of zinc in food (µg Zn/<br>g dry food) | Concentration of cadmium in food (µg<br>Cd/g dry food) |
|---------------------|------------------------|------------------------------------|--|--|
| C2                  | 10                     | Not applied                        | 27   | < 0.2  |
| Znl                 | 5                      | 667 mg Zn/l                        | 1070   | < 0.2  |
| Zn2                 | 5                      | 1333 mg Zn/l                       | 1950   | < 0.2  |
| Cd1                 | 5                      | 33 mg Cd/l                         | 36   | 52   |
| Cd2                 | 5                      | 167 mg Cd/l                        | 26   | 300  |

Concentrations of zinc and cadmium in solutions applied to food and final concentrations of zinc and cadmium in the food\* of animals in different experimental groups

\*Concentrations of metals in food were determined by ICP-AES.

daily cycle of secretion (Hames and Hopkin, 1991), starvation (Storch, 1984; Strus et al., 1985; Strus, 1987), food quality (Storch, 1984; Strus et al., 1985; Strus, 1987) and the presence of metals in food (Prosi and Dallinger, 1988; Köhler et al., 1996). (iv) Hepatopancreatic cells are directly exposed to substances in partly digested food, filtered from the proventriculus into the lumen of the hepatopancreas. (v) Knowledge acquired in our laboratory on the responses of *P. scaber*, such as food consumption and moulting, to elevated concentrations of zinc or cadmium in their food in almost equal experimental conditions (Drobne and Hopkin, 1995; Drobne and Štrus, 1996a; Zidar, 1998). In these experiments it was also shown that there were considerable differences regarding the accumulation of zinc and cadmium. (vi) Alterations of cellular ultrastructure were used by several authors for assessing the effects of organic chemicals (Vogt, 1987; Segner and Braunbeck, 1998) and metals (Pawert et al., 1996; De Nicola et al., 1996; Köhler et al., 1996; Bay et al., 1996; Liu et al., 1996; Kazacos and Van Vleet, 1989; Köhler and Triebskorn, 1998) on cells. Changes in cellular ultrastructure seem to be an appropriate indicator of the metal's effect, due to many potential target sites for metals in cells.

# 2. Materials and methods

# 2.1. Animals

The specimens of *P. scaber* Latreille (Crustacea: Isopoda) were collected in the gardens of the village of Mali otok in southeastern Slovenia. Animals representing the field control group and animals included in the exposure experiment were collected in October and in August 1998, respectively. Animals were acclimatised in the laboratory under conditions proposed for culturing *P. scaber* (Hornung et al., 1998). They were kept in glass containers at 22–24 °C and fed on hazeltree (*Corylus avellana*) leaves, fresh carrots and material from their natural environment. The animals of the field control group were maintained in the laboratory for 7 days and

the animals of the experimental groups for 10 days before the experiment was initiated. We selected males weighing between 50 and 100 mg in different phases of the moult cycle for the experiment. Females were excluded because of the possible impact of gravidity on the structure and function of hepatopancreatic cells.

# 2.2. Preparation of food

Partly degraded hazeltree leaves (C. avellana) of approximately 200 mg dry weight were selected. Metal-dosed leaves were prepared by applying 0.3 ml of ZnCl<sub>2</sub> or CdCl<sub>2</sub> solutions of appropriate concentrations over the entire leaf's surface to yield the needed metal concentrations in food (Table 1). The final concentrations of zinc and cadmium in leaves were determined by inductively coupled plasma atomic emission spectrometry (ICP-AES). The concentrations of zinc and cadmium chosen for the experiment are no-observedeffect concentrations (NOEC) for food consumption in experiments under almost equal experimental conditions (Drobne and Hopkin, 1995; Zidar, 1998)<sup>1</sup> and are in the range of concentrations known to produce sublethal effects on P. scaber (reviewed in Drobne, 1997). The concentrations of zinc (1070 µg/g dry food and 1950 µg/ g dry food) and the lower concentration of cadmium (52  $\mu g/g$  dry food) employed in the experiment are in the range of concentrations encountered in litter of contaminated woodland (Martin et al., 1982), whereas, a higher experimental concentration of cadmium (300 µg/ g dry food) was recorded in highly industrially polluted environments (Bengtsson and Tranvik, 1989). Metaldosed as well as non-contaminated leaves were cut into five to six pieces and randomly selected pieces of different leaves in each treatment of approximately 200 mg total weight were put in individual Petri dishes.

 $<sup>^1</sup>$  The data for 2000  $\mu g$  zinc per g dry weight of leaves does not match.

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#### 2.3. Experimental set-up

Ten animals of the field control group (C1) were dissected after a week of acclimatisation in the laboratory and their digestive glands were prepared for microscopic analysis.

Thirty animals for the exposure experiment were randomly assigned to experimental groups C2, Zn1, Zn2, Cd1 and Cd2 (Table 1). They were maintained individually in plastic Petri dishes with perforated side walls (2r = 9 cm) for 5 weeks (one moult cycle) and fed on non-contaminated hazeltree leaves and zinc or cadmium contaminated leaves (Table 1). Petri dishes with animals were held in glass containers at a temperature of 20–23 °C and relative humidity of 90–95%.

# 2.4. Food consumption

As the structure of the hepatopancreatic cells reflects dietary conditions (Storch, 1984; Štrus et al., 1985; Štrus, 1987), the food consumption of the experimental animals was determined. Food consumption was measured as mg of consumed leaves in the entire experimental period per mg wet body mass. Food consumptions of control animals (C2) and metal-exposed animals (Zn1, Zn2, Cd1, Cd2) were compared with the Mann–Whitney test. The content of the gut was also inspected macroscopically during dissection.

#### 2.5. Exuviation

Moulting was monitored particularly because of the described relation between the phase of the moult cycle and structural features of hepatopancreatic cells (Szyfter, 1966; Štrus and Blejec, 2001). The moulting phase of each animal was determined on the basis of sternal deposits (Zidar et al., 1998) at the beginning and at the end of the experimental period. Animals were inspected every 2-3 days during the 5-week experiment and exuviations were recorded.

# 2.6. Light microscopy and transmission electron microscopy

Animals were sacrificed at 09:00–12:00 h, because of differences in cell structure occurring in the daily cycle of secretion (Hames and Hopkin, 1991). After decapitation all four gland tubes were isolated and transferred to the appropriate fixative solution.

One of the gland tubes of every animal was prepared for TEM. It was cut transversely in the median region for better penetration of chemicals into the tissue. Due to differences in the cellular structure along the gland tubes (Bettica et al., 1984), the ultrastructural characteristics of hepatopancreatic cells in the median region of the gland tubes were always compared. Three gland tubes were prepared for light microscopy, two were embedded in Paraplast and one in Spurr's resin (Spurr, 1969). Median regions of tubes of each animal were inspected with a light microscope and in several animals cells along the entire tube were screened.

Tubes for TEM analysis were fixed in 3.5% glutaraldehyde in 0.1 M Na-phosphate buffer for 3–4.5 h at 4 °C, washed in 0.1 M Na-phosphate buffer and postfixed in 1% OsO<sub>4</sub> in 0.1 M Na-phosphate buffer for 1 h. After washing in the same buffer and dehydration in a graded series of ethanols, samples were embedded in Spurr's resin. Ultrathin sections were cut with a diamond knife and contrasted with uranyl acetate and lead citrate. The sections were analysed with a JEOL 1200 EX electron microscope.

Light microscopic analysis was performed on semithin sections of tubes embedded in Spurr's resin and on histological sections of tubes embedded in Paraplast with an Axioskop Opton light microscope. Semi-thin sections were cut with a glass knife and stained with Azur II.-methylene blue. Histological sections were prepared from tubes fixed in Carnoy fixative for 3-4.5h at 4 °C, dehydrated in a graded series of ethanols, cleared in xylene, embedded in Paraplast and stained with Weigert haematoxylin and eosin.

# 3. Results

# 3.1. Food consumption

The food consumption of control animals (C2) and metal-exposed animals of groups Zn1, Cd1 and Cd2 was between 1.0 and 2.5 mg consumed leaves per mg wet body mass and according to the Mann–Whitney test, differences among the groups were not statistically significant (Fig. 1). Food consumption of the animals in group Zn2 was lower, namely between 0.8 and 1.2 mg consumed leaves per mg wet body mass. The guts of most dissected animals were full.

# 3.2. Exuviation

In the experimental group C2 all but one animal moulted during the 5 week experiment (Table 2), in the experimental groups Zn1 and Cd1 only one animal moulted out of five in each group and in the groups Zn2 and Cd2 three and four animals moulted out of five in each group, respectively. The majority of animals in all groups exuviated in the first 3 weeks of the experiment (Table 2). At dissection all the animals but two premoult animals from groups C1 and Zn1 were in the intermoult phase.



Fig. 1. Mass of consumed leaves in a 5 week experiment (each point represents data for food consumption of an individual animal). \* Significantly lower food consumption in comparison with the groups C2 and Zn1 according to the Mann–Whitney test.

#### 3.3. Structural characteristics of hepatopancreatic cells

The hepatopancreas is the endodermal part of the digestive system, consisting of four blind-ending tubules, which open into the stomach and are composed of onelayered epithelium comprising B and S cells, surrounded by the neuromuscular network (Wägele, 1992; Hames and Hopkin, 1989).

Ultrastructural characteristics of the cells in the median regions of the gland tubes were analysed in field animals and in animals submitted to the laboratory exposure experiment.

The hepatopancreatic epithelia of all ten field animals (group C1) were structurally uniform, composed of dome-shaped B cells and wedge-shaped or cylindrical S cells, both with finger-like microvilli, basal labyrinths and spherical nuclei (Fig. 2a).

The main ultrastructural characteristics of B cells were numerous homogeneous lipid droplets (Fig. 2a); large glycogen fields (Fig. 2b and c); short cisternae of granular endoplasmic reticulum (GER) in the apical parts of the cells and long cisternae, frequently set in parallel stacks, in the basal parts of the cells (Fig. 2c); many Golgi stacks composed of cisternae (Fig. 2d); numerous oval and some elongated mitochondria with moderately dense matrices; vesicles containing myelin figures and a small amount of electron dense material. Grain-like electrondense deposits (EDD) along the cytoplasmic surface of plasma membranes were observed in six animals (Fig. 2e; Table 3).

The main ultrastructural characteristics of S cells were a few homogeneous (Fig. 2a) and single non-homogeneous lipid droplets; different amounts of glycogen; frequently branched GER cisternae of different lengths

Table 2

Exuviation of animals during the 5-week experiment

| Experimental group              | Number of exuviated animals/number of all animals in group | Number of exuviated animals during each week of experiment |        |       |        |       |
|---------------------------------|--|--|--------|-------|--------|-------|
|                                 |  | First  | Second | Third | Fourth | Fifth |
| C2                              | 9/10   | 3  | 1      | 3     | 1      | 1     |
| Zn1 (1070 µg Zn/g dry food)     | 1/5  | 1  |        |       |        |       |
| Zn2 (1950 µg Zn/g dry food) 3/5 |  |  | 2      | 1     |        |       |
| Cd1 (52 µg Cd/g dry food) 1/5   |  | 1  |        |       |        |       |
| Cd2 (300 µg Cd/g dry food) 4/5  |  | 1  |        | 3     |        |       |



Fig. 2. Animals of the group C1. (a) Transverse section of the hepatopancreatic tubule, consisting of dome-shaped B cells (B) and wedge-shaped or cylindrical S cells (S), lipid droplets  $(\rightarrow)$ , bar, 80 µm. (b) Apical part of the B cell, glycogen  $(\rightarrow)$ , bar, 500 nm. (c) Basal part of the B cell, lipid droplets (l), stacks of parallely arranged GER cisternae  $(\rightarrow)$ , mitochondria ( $\triangleright$ ), glycogen ( $\triangleright$ ), basal lamina (bl), bar, 2 µm. (d) Central part of the B cell, Golgi stack  $(\rightarrow)$ , GER (GER), glycogen ( $\triangleright$ ), bar, 1 µm. (e) Apical part of the B cell, grain-like electron dense deposits (EDD)  $(\rightarrow)$  along the cytoplasmic surface of the plasma membrane, bar, 200 nm. (f) S cell, Golgi stack  $(\rightarrow)$ , GER (GER), mitochondria (m), bar, 500 nm.

(Fig. 2f); a few Golgi stacks, frequently in close associations with GER (Fig. 2f); numerous moderately dense oval mitochondria; vesicles containing membranes and in some of them also a small amount of electrondense material. EDD along the cytoplasmic surface of the plasma membranes were observed in a few cells of four animals (Table 3).

Hepatopancreatic cells of animals submitted to the laboratory exposure experiment displayed numerous structural alterations as compared with the cells of control field animals. The majority of these alterations were identical in the control and in the metal-exposed animals. Some of the alterations were more prominent in the metal-exposed animals and some were observed exclusively in the metal-exposed animals. Considerable differences were observed among individual animals of the same group and among the cells of the same gland. The ultrastructural characteristics of hepatopancreatic cells of animals in the group Zn2, did not differ from those of animals in the group Zn1, although the food

Table 3

|   | C1   | C2  | Zn1 and Zn2 | Cd1 and Cd 2 |
|---|------|-----|-------------|--------------|
| EDD in intercellular spaces                                     | 0/10 | 0/6 | 2/7         | 2/7          |
| EDD in basal lamina and basal labyrinth                         | 0/10 | 0/6 | 2/7         | 4/7          |
| EDD in the cytosol of B cells                                   | 0/10 | 0/6 | 5/7         | 5/7          |
| EDD in the cytosol of S cells                                   | 1/10 | 1/6 | 4/7         | 1/7          |
| EDD along the cytoplasmic surface of plasma membrane in B cells | 6/10 | 3/6 | 5/7         | 7/7          |
| EDD along the cytoplasmic surface of plasma membrane in S cells | 4/10 | 2/6 | 7/7         | 7/7          |
| EDD in vesicles in B cells                                      | 0/10 | 0/6 | 0/7         | 4/7          |
| EDD in vesicles in S cells                                      | 1/10 | 2/6 | 6/7         | 2/7          |

Occurrence grain-like EDD in hepatopancreatic epithelia expressed as number of animals with EDD/number of all animals with dome-shaped B cells and wedge-shaped or cylindrical S cells

consumption of animals in Zn2 group was lower than that in Zn1 group.

In one third of the animals submitted to the exposure experiment (four out of ten animals in the control group C2, three out of ten animals exposed to zinc contaminated food and three out of ten animals exposed to cadmium contaminated food) low or completely flat disintegrated cells prevailed (Fig. 3a and b).

In the other two thirds all or the majority of B cells were dome-shaped (Fig. 3c) and all or the majority of S cells were wedge-shaped or cylindrical (Fig. 3c). Compared with the cells of animals from the field, the main structural alterations observed in cells of these animals were a reduction in the number of lipid droplets (Fig. 3c); an increased incidence of non-homogeneous lipid droplets with electron-lucent areas of completely clear or flocculent appearance (Fig. 3d) in the animals exposed to metal-dosed food; the absence of glycogen (Fig. 3e), with the exception of a few cells in two animals from the group C2; electrondense cytoplasm (Fig. 3e); reduction of the number of GER cisternae stacks in B cells; reduction of the amount of long GER cisternae in B cells, which was more prominent in animals fed on metal-dosed food; slightly increased vesiculation and dilatation of GER cisternae (Fig. 3f); individual concentric whorls of GER cisternae in a few S cells of some animals fed on metal-dosed food and in a few B cells of one animal from group C2 (Fig. 4a); increased incidence of clusters of vesicles, presumably representing transformed Golgi cisternae in B cells (Fig. 4b); compressed Golgi stacks in B cells observed in some animals fed on metal-dosed food; reduction of the number of Golgi stacks in S cells, which was more prominent in animals exposed to metal-dosed food; and long, branched mitochondria in S cells observed in some animals fed on metal-dosed food (Fig. 4c). Grain-like EDD in intercellular spaces (Fig. 4d), parts of the basal lamina and basal labyrinth (Fig. 4e) and in the cytosol of some B cells (Fig. 4f) were observed in animals fed on metaldosed food (Table 3). EDD in vesicles of some B cells (Fig. 5a) were observed only in animals exposed to cadmium-dosed food. EDD along the cytoplasmic surface of plasma membranes in B (Fig. 4f; Fig. 5b) and S cells (Fig. 5c) were present in all but two animals exposed to metal-dosed food and all metal-exposed animals, respectively, whereas in control animals these deposits were less frequently detected. EDD in the cytosol and in vesicles of some S cells (Fig. 5d) were observed in control animals, as well as in animals exposed to metal-dosed food.

In addition to these alterations, two ultrastructural features were recorded in animals of the exposure experiment, which were not fully identified and were observed only rarely. These were structured spherical formations in nuclei (Fig. 5e) and bundles of filamentous or lamellar structures arranged in parallel in the cytoplasm (Fig. 5f).

# 4. Discussion

Food consumption of metal-exposed animals in the experimental groups Zn1, Cd1 and Cd2 did not differ from the food consumption of the control animals (C2), as was expected on the basis of literature data (Drobne and Hopkin, 1995; Zidar, 1998). Food consumption of the animals in group Zn2 was significantly lower than that of control and Zn1 group, which corroborates the observations of Drobne and Hopkin (1995).

All but one control animal of the exposure experiment moulted during 5-weeks, whereas only roughly half of the animals exposed to metal-dosed food moulted during the same period. This is in concert with the observation that in *P. scaber* fed on zinc-contaminated leaf litter the moulting cycle is prolonged (Drobne and Štrus, 1996a). Differences in the ultrastructure of hepatopancreatic cells observed among animals from different groups cannot be ascribed to variability due to moulting, as at the time of dissection almost all animals were in the intermoult phase.

The ultrastructural analysis of hepatopancreatic cells of field animals, control animals of the laboratory exposure experiment and animals exposed to sublethal concentrations of zinc or cadmium in food revealed



Fig. 3. (a) Group Zn2, transverse section of the hepatopancreatic tubule composed of low or completely flat disintegrated cells, bar, 80  $\mu$ m. (b) group Zn2, disintegrated B cell, autophagic vacuoles ( $\rightarrow$ ), net-like pattern of chromatin ( $\triangleright$ ), bar, 2  $\mu$ m. (c) group Zn2, transverse section of the hepatopancreatic tubule composed of dome-shaped B cells (B) and wedge-shaped S cells (S), note the reduction of the amount of lipid droplets, bar, 80  $\mu$ m. (d) group Cd1, apical part of the B cell, non-homogeneous lipid droplet, bar, 2  $\mu$ m. (e) group C2, apical parts of the B cell (B) and S cell (S), note the electron-dense cytoplasm and the absence of glycogen and lipid droplets, bar, 1  $\mu$ m. (f) group Cd1, B cell, vesiculated GER ( $\rightarrow$ ), bar, 500 nm.

numerous differences among the groups. The observed ultrastructural differences are discussed with respect to stress and include disintegration of cells, the amount and appearance of lipid droplets, the amount of glycogen, electron density of the cytoplasm, ultrastructural alterations of GER, the Golgi complex and mitochondria and the incidence of grain-like EDD. Low or completely flat hepatopancreatic cells prevailed in one third of the animals submitted to the exposure experiment in our study.

Hames and Hopkin (1991) described thin and flattened B cells and flattened S cells in *Oniscus asellus* and *P. scaber* as normal stages in the daily cycle of apocrine secretion. Very thin glandular epithelium was observed



Fig. 4. (a) Group Cd1, S cell, concentrically arranged GER cisternae ( $\blacktriangleright$ ), bar, 1 µm. (b) Group Zn1, B cell, cluster of vesicles presumably representing transformed Golgi cisternae ( $\rightarrow$ ), bar, 500 nm. (c) Group Cd1, S cell, long, branched mitochondria ( $\rightarrow$ ), bar, 1 µm. (d) Group Cd2, EDD ( $\rightarrow$ ) in the intercellular space, bar, 200 nm. (e) Group Cd2, B cell, EDD ( $\rightarrow$ ) in the basal lamina and basal labyrinth, bar: 500 nm. (f) Group Zn2, B cell, EDD ( $\rightarrow$ ) in the cytosol and along the cytoplasmic surface of the plasma membrane, bar, 500 nm.

also in *Ligia italica* after 4 weeks of starvation (Štrus, 1987) and in *P. scaber* exposed to 5000 and 10 000  $\mu$ g Zn per g dry weight of food (Drobne and Štrus, 1996b).

As the low or completely flat cells in our experiment were disintegrated, we cannot ascribe their altered shape to physiological variability of the cells, but most probably to stressful conditions.

In animals of the laboratory exposure experiment the number of lipid droplets in hepatopancreatic cells was reduced as compared with the field animals. The size and number of lipid droplets in cells of various tissues vary markedly in different physiological and pathological situations (Ghadially, 1997). The absence or reduction of the number of lipid droplets in hepatopancreatic cells of isopod crustaceans was described in starved animals (Storch, 1984; Štrus, 1987) and in postmoult *P. scaber* (Szyfter, 1966) and *L. italica* (Štrus and Blejec, 2001).

The smaller number of lipid droplets we observed in hepatopancreatic cells in animals of the exposure



Fig. 5. (a) Group Cd1, B cell, EDD ( $\rightarrow$ ) in the vesicle, bar, 1 µm. (b) Group Cd2, B cell, EDD ( $\rightarrow$ ) along the cytoplasmic surface of the plasma membrane, bar, 50 nm. (c) Group Zn1, S cell, EDD ( $\rightarrow$ ) along the cytoplasmic surface of the plasma membrane, bar, 200 nm. (d) Group Zn1, S cell, EDD ( $\rightarrow$ ) in vesicle, bar, 200 nm. (e) Group Zn1, B cell, structured spherical formations ( $\rightarrow$ ) in the nucleus, bar, 1 µm. (f) group Zn2, B cell, bundle of filamentous or lamellar structures ( $\rightarrow$ ), bar, 200 nm.

experiment could be explained by enhanced utilisation of energetic reserves during the experimental period due to stress and/or inadequate nutrition. The reduced number of lipid droplets in our experiment cannot be explained as variability related to moulting, as no animal was in the postmoult phase when dissected.

Non-homogeneous lipid droplets were observed in roughly half of the animals exposed to zinc or cadmium in their food and only rarely in either control groups. The appearance of intracytoplasmic lipid in an ultrathin section depends on the size of the droplet, the method of tissue preparation, the fatty acid content of the lipid, and the degree of unsaturation of the fatty acids present (Ghadially, 1997). Vogt (1996) described lipid droplets with electron-lucent spherical areas in the hepatopancreas of the starved crustacean *Troglocaris anophthalmus* and interpreted this as mobilisation of lipids.

As all specimens in our study were processed in the same way, it seems probable that the observed nonhomogeneous lipid droplets with electron-lucent areas indicate the mobilisation of lipid reserves, but the possibility of artificial lipid extraction cannot be excluded. If the latter is the case, the more frequent recording of non-homogeneous lipid droplets in metalexposed animals could be explained as the consequence of changed chemical characteristics of the lipids.

The absence of glycogen was a general feature of the hepatopancreatic cells in the animals of the exposure experiment.

The absence or reduction of glycogen in isopod hepatopancreatic cells was described in starved animals of different species (Storch, 1984; Štrus, 1987). Szyfter (1966) related the glycogen content of hepatopancreatic cells in *P. scaber* to particular stages of the moult cycle, with accumulation of glycogen during premoult and a gradual decrease to complete disappearance in the first days after ecdysis. From studies on other animal species and cell cultures it is known that organic chemicals and metals can influence the tissue glycogen content (Srivastava, 1982; Rana et al., 1985; Toury et al., 1985; Segner and Braunbeck, 1998).

The absence of glycogen in hepatopancreatic cells in animals of the exposure experiment in our study indicates enhanced utilisation of energetic reserves due to stress and/or inadequate nutrition. As all but two of the animals we analysed were in the intermoult when dissected, the observed differences in glycogen content of hepatopancreatic cells could not be explained by physiological variability due to different phases of the moult cycle.

The cytoplasm of B and S cells in animals of the exposure experiment was electron denser than the cytoplasm of cells in field animals.

Köhler et al. (1996) observed condensed cytoplasm in hepatopancreatic cells of P. scaber exposed to cadmium, lead or zinc in a contaminated substrate/food for 3 weeks. They concluded that most probably the 'condensation' is artificial, due to changes of cell osmolarity and consequently due to an inadequate fixation of the cytoplasm. Pawert et al. (1996) described condensation of the cytoplasm in the midgut cells of collembolans exposed either to lead, cadmium or zinc, and Bay et al. (1996) in the hepatocytes of zinc-treated mice. In discussion of the dark and light variants of apparently the same cell type in a tissue preparation Ghadially (1997) concluded that at least in some instances, dark cells are dead or dying cells. According to Ghadially (1997) the common factor explaining the dark cell/light cell phenomenon is excessive cellular dehydration, which could occur in vivo, engendered by physiological or pathological states, or could be produced in normal cells due to tissue preparation.

As hepatopancreatic tissue is one-layered epithelium and as all specimens were processed in the same way, the observed electrondense cytoplasm in our study is most probably the result of stressful factors and is not an artefact of tissue preparation procedures.

Ultrastructural alterations of GER observed in animals of the exposure experiment comprised reduction of the number of GER cisternae stacks, reduction of the number of long cisternae, slightly increased vesiculation and dilatation of cisternae, and concentric whorls of cisternae.

Reports about alterations of GER in response to various factors are numerous. Ultrastructural alterations of GER as observed in our study were reported to occur in different cell types of different organisms after exposure to organic chemicals (Braunbeck and Völkl, 1991; Okazaki et al., 1992; Triebskorn and Köhler, 1992; Arnold et al., 1996; Abrami et al., 1998; Segner and Braunbeck, 1998) and metals (Kazacos and Van Vleet, 1989; Köhler et al., 1996; Pawert et al., 1996). A detailed explanation of two GER alterations, namely dilatation/vesiculation and concentric arrays, was presented by Ghadially (1997). Dilatation and vesiculation of GER can be due to an ingress of water, which occurs in cells subjected to various noxious influences, or to storage of secretory products. Cellular swelling appears whenever cells are incapable of maintaining ionic and fluid homeostasis and dilatation of the endoplasmic reticulum is considered as one of the ultrastructural changes of reversible cell injury (Cotran et al., 1999). Ghadially (1997) cited various conditions, from starvation to different pathological conditions and administration of various drugs, in which vesiculation of hepatocyte ER has been reported to occur. Dilatation of GER can also be an artefact caused by poor techniques of tissue sampling and processing. Ghadially (1997) listed a variety of normal and pathologically altered cells in which concentric membranous bodies (composed of either GER, SER or membranes in association with glycogen) were present. He presented two views regarding the nature of concentric membranous bodies: (1) they represent a degenerative change or an elaborate autophagic vacuole; and (2) they represent a regenerative change leading to a specialised type of hypertrophy of the endoplasmic reticulum that may have a functional significance.

The dilated GER observed in our study was electron lucent and is probably related to water ingression and not to storage of secretory products. As the dilated and/ or vesiculated GER was found in company with normal cisternae in the same cell or other cells of the same hepatopancreatic tubule, artificial dilatation does not seem to have occurred. On the basis of the discussion presented we can conclude that ultrastructural alterations of GER recorded in our study were the consequence of stressful conditions, but could not be ascribed solely to elevated concentrations of metals in food.

Structural alterations of the Golgi complex in animals of the exposure experiment comprised the presence of clusters of vesicles presumably representing transformed Golgi cisternae and reduction of the number of Golgi stacks. In some animals fed on metal-dosed food compressed Golgi stacks were observed.

A variety of changes, including hypertrophy, atrophy and dilatation has been reported to occur in the Golgi complex in normal and pathological tissues. Such differences can be accounted for in terms of cell differentiation, physiological activity, and pathological or toxic influences (Ghadially, 1997). Marked atrophy or destruction, and the disappearance of recognisable Golgi complex elements, have been noted to occur in hepatocytes subjected to a variety of toxic influences. The transformation of the Golgi complex into a cluster of vesicles was described for different cell types after the administration of different organic chemicals (Renau-Piqueras et al., 1985; Veit et al., 1993; Neises et al., 1997; Tanaka et al., 1998).

In concert with the discussion presented we ascribe the ultrastructural alterations of the Golgi complex observed in our study to stress.

In the cells of some animals fed on metal-dosed food long, branched mitochondria were observed.

Various alterations in the number, size, and shape of mitochondria occur in different pathological conditions. Mitochondria may assume extremely large and abnormal shapes, as can be seen in the liver in alcoholic liver disease and in certain nutritional deficiencies (Cotran et al., 1999). Ghadially (1997) described cup-shaped mitochondria, which have been seen in normal and pathological tissues, and discussed this as a degenerative phenomenon or an adaptive change. Deformation of mitochondria was also described by Segner and Braunbeck (1998) in isolated rainbow trout (*Oncorhynchus mykiss*) hepatocytes after in vitro exposure to different organic chemicals. Zinc and cadmium were reported to alter mitochondrial function (Dineley et al., 2002; Al-Nasser, 2000).

The presence of long, branched mitochondria in the cells of some animals exposed to zinc or cadmium in our study could reflect altered mitochondrial function due to metal exposition.

Grain-like EDD were recorded in our study in different parts of the hepatopancreatic epithelia and their incidence differed considerably among the experimental groups (Table 3).

It is well known that hepatopancreatic cells of isopod crustaceans are involved in the metabolism of essential and non-essential metals. The dynamics of calcium with respect to the moult cycle have been investigated in hepatopancreatic cells (Štrus and Blejec, 2001; Szyfter, 1966). The hepatopancreas is involved in the metabolism

of copper (Wieser, 1968), which is a part of hemocyanin, the respiratory pigment. In numerous studies isopods from uncontaminated and metal-contaminated environments were analysed and the role of hepatopancreatic cells in the accumulation and/or detoxification of metals was discussed (Hopkin and Martin, 1982; Prosi and Dallinger, 1988; Hopkin, 1989, 1990; Drobne, 1996). Accumulation of copper, calcium, zinc, cadmium, lead and iron in the granules in B and/or S cells is a well known feature of isopodian hepatopancreatic cells (Prosi and Dallinger, 1988; Hopkin, 1989). Hopkin (1990) also described the deposition of calcium, zinc and lead on the cytoplasmic side of the cell membranes of B and S cells in P. scaber. Köhler et al. (1996) observed precipitation of electron-dense material at the intracellular membranes in hepatopancreatic cells of P. scaber exposed to cadmium, lead or zinc. They interpreted this as an artefact due to the fixation process.

In our study, EDD in the intercellular spaces were observed exclusively in some animals fed on zinc or cadmium contaminated food. So the presence of these electrondense granules could be related to the metabolism of zinc or cadmium originating from metal-dosed food (exogenous zinc and cadmium), or to disturbances in the metabolism of other metals present in hepatopancreatic cells (endogenous metals). EDD in the basal lamina and basal labyrinth and in the cytosol of B cells were observed in several animals exposed to metal-dosed food, but also in one animal with disintegrated epithelium from the group C2. The presence of these EDD could not be related solely to the metal exposition, but evidently higher incidence of these EDD in metalexposed animals could indicate some connection between these EDD and metal metabolism. As EDD in the membrane vesicles of B cells were found only in animals exposed to cadmium and as it was shown that in similar experimental conditions cadmium was intensively accumulated in the hepatopancreas (Zidar, 1998), it is tempting to propose that these granules could be related to accumulation, but further investigations would be necessary to answer this question. The presence of EDD along the cytoplasmic surface of plasma membranes in B and S cells in all but two and in all of the metalexposed animals, respectively, but in roughly half of control animals, is in concert with the assumption that the observed electrondense granules in hepatopancreatic cells are somehow related to the metabolism of exogenous and/or endogenous metals. For EDD in vesicles and in the cytosol of S cells a distinction between control and metal-fed animals was not apparent, but we have to stress that these EDD were most frequently recorded in zinc-exposed animals. In order to confirm the presumption that the grain-like EDD observed in hepatopancreatic epithelia are related to the metabolism of endogenous and/or exogenous metals, further investigations should be performed, including X-ray microanalysis of these deposits.

The structured spherical formations in nuclei observed infrequently in animals of the exposure experiment could be viral inclusions. Several viruses are known to infect crustacean hepatopancreatic cells (Vogt, 1992; Vogt and Štrus, 1998).

The bundles of filamentous or lamellar structures arranged in parallel in the cytoplasm observed in our study most resemble the ribosome-lamella complex described by Ghadially (1997, 1999). He described the ribosome-lamella complex as a hollow cylindrical structure composed of spiral or concentric lamellae studded with ribosomes.

# 5. Conclusions

The ultrastructural characteristics of hepatopancreatic cells of *P. scaber* from the field were generally uniform and indicated well-performing tissue.

The ultrastructural analysis of hepatopancreatic cells of animals in the laboratory exposure experiment, which were fed on non-contaminated food or exposed to sublethal concentrations of zinc or cadmium in food, revealed three general features:

(a) Considerable differences in the ultrastructure were recorded among animals of the same experimental group and among cells of the same organ.

It is well known that individuals in a population vary in their ability to function successfully during exposure to an environmental stress (Forbes and Forbes, 1994) and variations of the structure of the hepatopancreas observed among animals of the same experimental group in our study corraborate this. Ultrastructural differences observed among the cells of the same organ indicate the variability of the response to environmental stress at the cellular level.

(b) Hepatopancreatic cells of animals in the exposure experiment displayed a complex pattern of numerous structural alterations as compared with the cells of field animals, which indicated the influence of the network of stressful conditions on the network of cellular processes. The majority of the observed alterations were identical in all experimental groups, with some of them more prominent in the metal-exposed animals.

The observed changes could be ascribed mainly to nutritional stress, although other factors with respect to the experimental set-up could also be involved. Apart from metal-dosed food, an inadequate nutrient supply most probably contributed to the stressful conditions. Terrestrial isopods are omnivores, although they primarily feed on plant material (Storch, 1984). Coprophagy was also documented (Warburg, 1987). The partly decayed leaves used in our experiment as food were probably an inadequate nutrient source. Diverse

food of higher energetic value would be more appropriate. The importance of microflora in food should also not be neglected, as it is known that individual fitness of P. scaber depends on the microbial colonisation of the litter (Zimmer and Topp, 1997). The disturbance of water balance could be another factor contributing to stressful conditions. Edney (1968) reported that in the natural environment P. scaber migrates to places of different humidities during the day. In our experiment humidity was maintained by spraying the lids of the Petri dishes with distilled water regularly. The animals did not have the possibility to choose places of significantly different humudities. It would probably be more appropriate to design the experimental set-up in such a way that animals would have access to substrata of different moisture contents.

(c) Concerning the ultrastructural alterations which were observed exclusively in metal-exposed animals, the incidence of grain-like EDD could be related to disturbances in metal metabolism. The analysis of their distribution and X-ray microanalysis of electrondense granules are under way in our laboratory.

We would like to point out two additional issues of the discussion: (1) the problem of 'control' animals in experimental procedures, and (2) the problem of the specificity of the changes in cellular ultrastructure due to exogenous disturbances. The response of an animal reflects all exogenous and endogenous influences. Cellular responses are early and sensitive indicators of disturbances. Thus, when we define and evaluate the 'control state' comprising control animals and control conditions and interpret the responses observed, as many aspects as possible should be carefully considered. Our results are in concert with research performed on the marine crustacean Penaeus monodon by Vogt (1987). He investigated the influence of environmental pollutants on the midgut glands of P. monodon postlarvae and stressed that structural alterations in the midgut gland represent a highly sensitive overall response of these animals to the synergistic effect of their nutritional and physiological conditions and the quality of water. The ultrastructural alterations observed in hepatopancreatic cells of *P. scaber* in our study indicate the impact of stressful conditions, but only the incidence of grainlike EDD could be specifically related to disturbances in metal metabolism.

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