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THE USE OF BIOACCUMULATION, BIOMARKERS AND HISTOPATHOLOGY DISEASES IN *PROCAMBARUS CLARKII* TO ESTABLISH BIOAVAILABILITY OF Cd AND Zn AFTER A MINING SPILL

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Abstract. Individuals of the crayfish *Procambarus clarkii* (males and females) were exposed simultaneously to cadmium and zinc during 21 days. Exposure concentrations were those determined at the Guadiamar river after the Aznalcóllar mining spill (SW, Spain): 10 and 30 $\mu g\,L^{-1}$ of cadmium and 1000 and 3000 $\mu g\,L^{-1}$ of zinc. Three biomarkers (MT: metallothioneins like proteins, VTG: vitellogenin/vitellin like proteins and histopathology) together with heavy metal bioaccumulation were determined in soft tissues of male and female *P. clarkii*. At the concentrations tested, increasing cadmium exposure resulted in increasing cadmium bioaccumulation and increasing subletal effects (induction of MT, VTG and histopathological damage in tissues). Nevertheless, although increasing zinc exposure showed increasing VTG induction and histopathological damages, not a positive relationship was determined with MT induction. Concerning to responses determined in male and female crayfishes only differences were found between sexes at the highest cadmium exposure concentration related to bioaccumulation in hepatopancreas tissues. Biomarkers responses to heavy metal contamination in this crayfish, even VTG induction not before tested in heavy metal contamination assessment in crustaceans resulted potential tools for the monitoring of heavy metal environmental contamination.

Keywords: metallothionein, vitellogenin, reproduction, heavy metals

1. Introduction

In recent years a major emphasis has been placed on the toxic effects of the heavy metals that have accumulated in aquatic ecosystems (Fingerman *et al*., 1998). An extensive literature list is already available on heavy metals exposure and their effects on *Procambarus clarkii*, the red swamp crayfish. These reports include studies of toxicity, accumulation and effects on reproduction (Reddy *et al*., 1997; Martínez *et al*., 1993). The red swamp crayfish *P. clarkii* can live in a wide range of environmental conditions that includes highly polluted waters. It was artificially

introduced into the marshes of the lower Guadalquivir river (SW, Spain) in 1974 (Habsburgo-Lorena, 1979). Since then, this species has extended quickly across Guadalquivir river marshes reaching the Doñana National Park freshwater marsh (SW, Spain) (Gutiérrez-Yurrita *et al*., 1998).

The Aznalcóllar mining spill that poured into the nearby Guadiamar river and then flowed into the Guadalquivir Estuary, produced almost 6 Hm^3 of mud and acidic water with high concentrations of metals in solution. *P. clarkii* was chosen as a representative species from the affected zone to analyze the effect of the spill. The development of sensitive indicators of detrimental but sublethal effects from heavy metal exposure would aid lexicological assessments. These sublethal effects could be determined through biomarker analysis together with a precise chemical determination of the present contaminants in the environment. Biomarkers allow assessment of the effects of sublethal stress to sentinel organisms and they constitute a group of parameters that could change at the molecular, histological, immunological, physiological, organismal, and population or ecosystem levels (López-Barea and Pueyo, 1997).

This ongoing research involves the investigation of the effects associated with of environmental concentrations of dissolved cadmium and zinc on *P. clarkii* males and females. Consequently, several different approaches were assessed as biomarkers: metallothionein and vitellogenin/vitellin levels, histopathology, and heavy metal bioaccumulation. The opportunity was taken to assess the potential use of these biomarkers as indicators of exposure to environmental heavy metal contamination episodes, especially for the study of VTG response not before tested in heavy metal contamination, as well as to analyze possible differences in the responses between males and females of the red swamp crayfish, *P. clarkii*.

2. Materials and Methods

2.1. General approach

The present study was performed using concentrations of dissolved Cd and Zn previously determined during the monitoring of the impact of the spill in the Guadiamar river and surrounding areas during the first months after the spill (http://www.juntadeandalucia.es/medioambiente/guadiamar/indguadiamar.html). Intermoult female and male *Procambarus clarkii* were obtained from the Department of Wildlife and Fisheries, Mississippi State University, MS, USA. All specimens used in this study were of a similar size, length and caught on the same day at the same location and at the same stage of reproduction.

The specimens were placed in laminar flow holding tank with 6 cm of filtered and dechlorinated freshwater and allowed to acclimate for two weeks prior to exposures. The tank conditions were maintained at 25 °C and a 12:12 h light-dark cycle. The material used for the present experiment was previously washed

with nitric acid 10% and cleaned with distilled water (Milli-Q) to avoid metal contamination.

Eight female and eight male, both intermoult *P. clarkii* were placed randomly in 30 L tanks with 5 L of exposure solutions (filtered dechlrorinated freshwater and metal stock solution). Crayfishes were exposed to 1000 $\mu g\,L^{-1}$ and 3000 $\mu g\,L^{-1}$ of zinc (Zinc chloride), and 10 $\mu g\,L^{-1}$ and 30 $\mu g\,L^{-1}$ of cadmium (Cadmium chloride) (Sigma-Aldrich, St. Louis, MO, USA) during 21 days. A set of reference individuals was also performed. Chelipeds were removed in order to reduce antagonistic behavior and cannibalism. The exposure solutions were replaced every three days and the specimens were fed on a diet of frozen brine shrimp just prior to the water change to reduce fouling of the exposure solutions. Exposure conditions were as described for acclimation period. Water samples were taken randomly before and after water exchange in order to perform analyses of the dissolved heavy metals.

Haemolymph samples (200 μl) were collected on days 0, 7, 14, and 21 from the female crayfishes in order to determine vitellogenin/vitellin concentration. On day 21 all specimens were dissected and samples from gill, hepatopancreas, muscle and ovary were taken for metal concentration and histopathological diseases determinations of each tissue. Metallothioneins were determined only from hepatopancreas tissues.

2.2. Water monitoring

Treatments were checked for pH and dissolved oxygen concentrations continually during the experiment. Dissolved metal concentrations were determined in each tank prior to start of crayfish exposure and during the assay (Gómez-Parra *et al.*, 2000). Briefly, water samples (250 ml) were collected from the tanks, filtered (0.45 μm), acidified to pH 2.5 with 2 ml of HCl, and stored in the dark at 4 °C. Samples were treated with UV radiation and metal concentration determined through Anodic Stripping Voltammetry (ASV).

2.3. Metal concentration in gill, hepatopancreas and muscle tissues

Metal determinations were performed on lyophilised dissected samples of gill, hepatopancreas and muscle from male and female *P. clarkii* ($n = 8$ per treatment). Samples were digested with nitric acid (Suprapur) and hydrogen peroxide (Suprapur) for 1 h at 95 °C. Then they were brought to a total volume of 5 ml with distilled water and analysed by atomic absorption spectrophotometry (flame: Zn; graphite furnace: Cd) (Amiard *et al.*, 1987). The analysed concentrations were validated by performing metal analyses on reference material TORT II lobster hepatopancreas (National Research Council Ottawa, ON, Canada) (Table I). Metal concentrations in soft tissues are expressed as $\mu g\,kg^{-1}$ dry weight.

TABLE I
Average and standard deviations of certified (TORT-II) and measured values of cadmium and zinc (mg·kg^{-1} dry weight)

Metal	Certificate values	Our values
	TORT-II	
Cd	26.7 ± 0.6	24.8 ± 1.1
Zn	180 ± 6.0	178.6 ± 4.2

2.4. METALLOTHIONEIN CONCENTRATION IN HEPATOPANCREAS

Metallothionein (MT) concentration in hepatopancreas tissues of *P. clarkii* male and female were analysed through Anodic Stripped Voltammetry in the heat stable fraction of samples based on the protocol developed by Olafson *et al.* (1979). It relies on the detection of SH-groups in a specific electrolyte by differential pulse polarography (DPP). It has been improved by Thompson and Cosson (1984) by the use of a thermostated cell and a new model of static mercury drop electrode. Eight hepatopancreas from each treatment and sex were homogenized in ultraturrax at a ratio (1:4) w/v with Trizma-HCl/Trizma-Base 0.1 M (pH 8) buffer in ice bath (4 °C); 1 ml of the homogenate was centrifuged at $50{,}000 \times g$ for 1 h at 4 °C. The supernant (cytosol) was separated from the pellet; 0.1 mL of the supernant was added to 0.9 mL of NaCl (0.9%), heated to 95 °C for 4 min, and centrifugated at $10{,}000 \times g$ for 15 min at 4 °C. Supernatant was stored at −80 °C prior to MT concentration determinations by ASV (Olsson and Olafsson, 1987) using purified rabbit metallothionein (Sigma-Aldrich). MT concentrations were expressed as μg MT/mg total proteins. Total protein determinations from hepatopancreas were determined in the cytosol by Bradford method (1976). Bovine serum albumin (Sigma-Aldrich), was used as standard.

2.5. VITELLOGENIN/VITELLIN CONCENTRATION IN HAEMOLYMPH

Vitellogenin/vitellin (VTG) was measured in the haemolymph of intermoult female *P. clarkii* using an indirect enzyme-linked immunosorbent assay (ELISA) adapted from Pateraki and Stratakis (1997). VTG concentration was identified using a polyclonal antibody raised in rabbits against purified *P. clarkii* VTG and made monospecific by the addition of male hemolymph (Tuberty, 1998). In brief, the sample of interest (100 μl) and a serial dilution of vitellin (standard curve) ranging from (0, 2, 10, 20, 50, 75 and 100 ng · 100 μl^{-1}) in dilution buffer were allowed to bind to the well of the microtitre plate, either overnight at 4 °C or at room temperature for 2 h (all samples were run in triplicate). Excess protein in the well was removed with washing (PBS-T). Any sites on the wall of the plate well not covered by protein were then blocked using a general "bland" protein, in this case dried milk powder was reconstituted. This sloped antibody binding direct to the polystyrene wall and

giving false positives. After further washing the primary antibody was added to the wells and incubated for one hour at 37 °C. If vitellogenin/vitellin was present in the sample this primary antibody would bind with it. After one hour the plate was again washed out removing antibody. Then the secondary antibody was added. This second antibody was a goat anti-rabbit monoclonal preparation which will bind with any antibody produced in a rabbit. This antibody is bought commercially from Sigma. The plate was incubated for a further hour at room temperature, and washed out to remove out secondary antibody. Then, the ABTS was allowed to react at room temperature in the dark for 10–30 min. The color change was quantified by an automated plate reader at 405 nm and the VTG standards were fit to a linear regression. The standard curve was linear over the range of standards.

No non-specificity of the antibody to male haemolymph was found. Vitellogenin/vitellin concentrations were expressed as μg of protein per ml of haemolymph.

2.6. Histological damage in gill, hepatopancreas, muscle and ovary tissues

Portions of hepatopancreas, gill, muscle from males and females, and ovary tissues of each animal were fixed for 24 h in Bouin's fixative. After dehydration in graded concentrations of ethanol, the samples were embedded in paraffin wax and sagittal sections of 6 to 8 μm thickness were stained with haematoxylin-VOF (Gutiérrez, 1967). Histological sections were reviewed by light microscopy and photographed (Olympus CH20).

2.7. Statistical analysis

The data obtained for heavy metal bioaccumulation, metallothioneins and vitellogenin/vitellin concentrations from both males and females exposed to different concentrations of cadmium and zinc were compared to those determined in control specimens by use of Student's t-test.

3. Results

3.1. Water parameters

The results of the water monitoring tests (dissolved oxygen and pH) did not show any significant differences among the exposure groups, or between the exposure groups and the control aquaria. Ranges of these parameters over each day in aquaria water were as follows: dissolved oxygen: 6.8 ± 0.4 mg L^{-1} and pH: 7.5 ± 0.2.

Concentrations of dissolved heavy metals analyzed from aquarium water samples varied between 90–97% of the nominal concentrations for all treatments.

3.2. Cadmium and zinc concentrations in gill, hepatopancreas and muscle tissues

3.2.1. *Cadmium Exposure*

In general, cadmium bioaccumulation after 21 days of exposure was highest in gills ($81.01 \pm 6.65\,\mu g\,kg^{-1}$), followed by hepatopancreas ($7.18 \pm 0.23\,\mu g\,kg^{-1}$), and then muscle ($0.30 \pm 0.07\,\mu g\,kg^{-1}$) where the cadmium values were almost inappreciable (Figure 1).

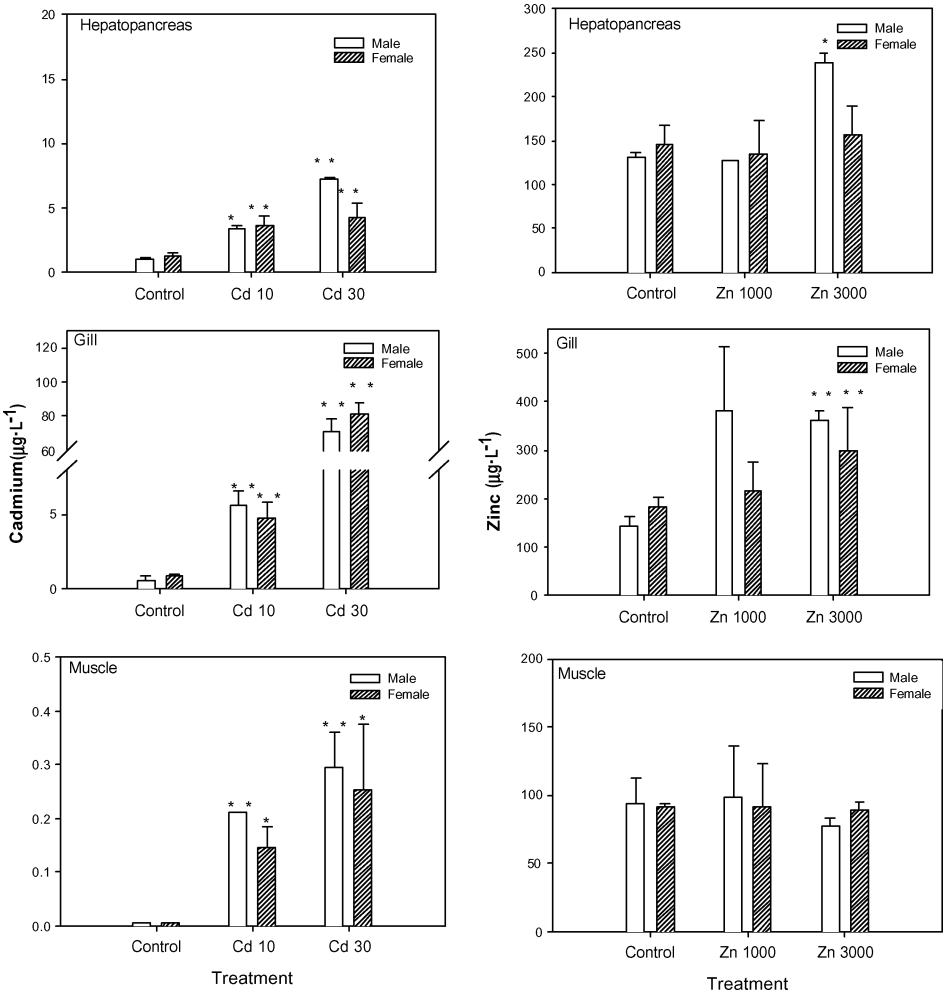

Figure 1. Summarized results of metal concentrations (Cd and Zn) in gill, hepatopancreas and muscle tissues of males and females *P. clarkii* after 21 days of exposure to dissolved cadmium and zinc. Asterisks indicate significant differences between heavy metal concentrations in exposed specimens and control ($^{**}p < 0.01$, $^{*}p < 0.05$).

An exposure-dependent increase in cadmium concentration was found in each tissue, although different cadmium bioaccumulation trends were indicated. Cadmium concentrations determined in hepatopancreas and gill tissues of crayfish exposed to 10 and 30 $\mu g\,L^{-1}$ of Cd was significantly higher than those determined in control individuals (males and females) ($p < 0.01$). At the highest Cd (30 $\mu g\,L^{-1}$) concentration tested, significant higher Cd concentration was observed in male hepatopancreas tissues than in female hepatopancreas ($p < 0.01$). Contrary to results obtained in hepatopancreas tissues, gills of males had not significantly more cadmium than did females.

Although Cd concentration in muscle tissues was very low, significant differences were shown for males ($p < 0.01$) and females ($p < 0.05$) at both cadmium exposure concentrations compared with control individuals.

3.2.2. *Zinc Exposure*

In general, zinc concentration in the tissues after the 21 days of exposure was highest in gills (360.25 $\pm$ 22.05 $\mu g\,kg^{-1}$), followed by hepatopancreas (238.46 $\pm$ 10.77 $\mu g\,kg^{-1}$), and muscle (97.97 $\pm$ 38.65 $\mu g\,kg^{-1}$) (Figure 1).

Zinc concentrations determined in hepatopancreas of crayfish exposed to this metal did not show an exposure-dependent increase in values nor were significantly different from controls, except in hepatopancreas tissues from male crayfish exposed to 3000 $\mu g\,L^{-1}$ of Zn, where significant differences were observed compared with control individuals ($p < 0.05$). However, gill tissues did demonstrate an exposure-dependent increase in zinc concentrations, although significant differences were observed only in zinc bioaccumulation between individuals exposed to the higher zinc concentration and controls ($p < 0.01$) for both males and females.

Bioaccumulation in muscle does not significantly differ in either exposure treatment from controls.

3.3. Metallothionein (MT) concentration in hepatopancreas

MT concentrations in *P. clarkii* hepatopancreas following exposure to Cd and Zn are summarized in Figure 2. Both males and females demonstrated an exposure-dependent increase following Cd exposures being significant at 30 $\mu g\,L^{-1}$ of Cd ($p < 0.05$), whereas following exposure to 1000 $\mu g\,L^{-1}$ of Zn, both male and female MT levels increased and while females showed no further increase, males exhibited a decrease at the higher Zn concentration.

Significant differences were observed between individuals exposed to zinc at both concentrations, for males and for females ($p < 0.05$). No differences were observed in metallothionein induction between males and females after exposure to both heavy metals.

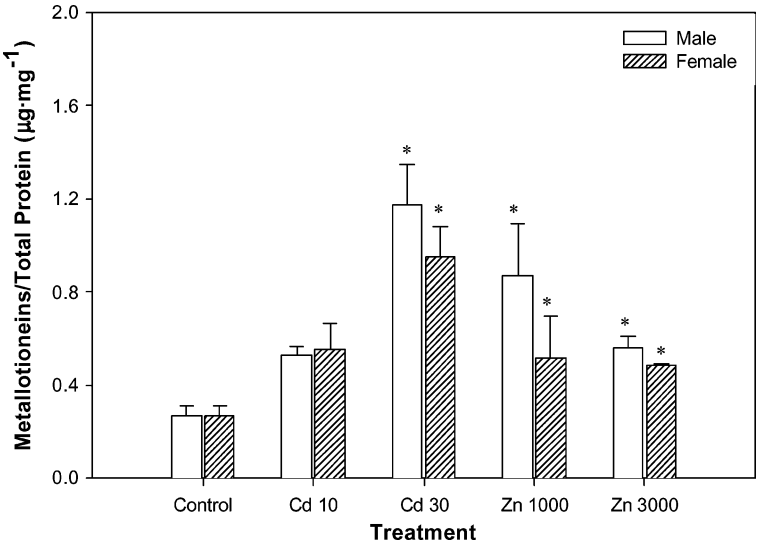

Figure 2. Summarized results of metallothionein concentration in hepatopancreas tissues of male and female *P. clarkii* after 21 days of exposure to dissolved cadmium and zinc. Asterisks indicate significant differences between metallothionein concentrations in exposed specimens and control ($^{*}p < 0.05$).

3.4. Vitelloegenin/vitellin (VTG) concentration in haemolymph

Vitellogenin/vitellin concentrations circulating in haemolymph increased during the 21 days of exposure in all female treatments. At day 21, the highest values determined were in crayfish exposed to 30 μg L^{-1} Cd (529.09 $\pm$ 49.01 μg/ml) and 3000 μg L^{-1} Zn (353.08 $\pm$ 40.51 μg/ml) (Figure 3) which were significantly higher than were controls ($p < 0.01$). On the other hand, because of a lower concentration of VTG, a significant difference was found on day 7 in females exposed to 30 μg L^{-1} Cd when compared with the control.

3.5. Histological damage in gill, hepatopancreas, muscle and ovary tissues

Histological alterations were observed, except in muscle for both contaminants, especially for Cd exposed animals. Exposure-dependent histopathology was seen (Figures 4–6) in female gonads and male and female gill and hepatopancreas vessels following zinc and cadmium treatment. The greatest abnormalities were observed in hepatopancreas of specimens treated with cadmium, which were characterized by disorganized epithelium, degenerating tubules, and necrotic connective tissue. In comparison, the hepatopancreas from the 3000 μg L^{-1} Zn treated individuals only showed swelling and some disorganization of the epithelium.

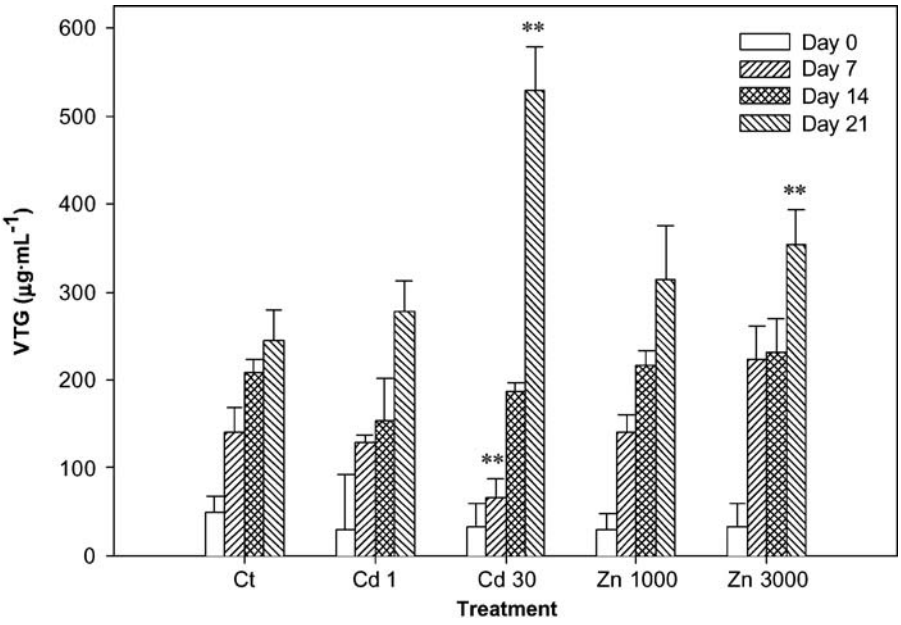

Figure 3. Summarized results of vitelloegenin/vitellin concentration in female *P. clarkii* haemolymph exposed to dissolved cadmium and zinc on days 0, 7, 14 and 21. Asterisks indicate significant differences between vitellogenin/vitellin concentrations in exposed specimens and control ($^{**}p < 0.01$) at each day.

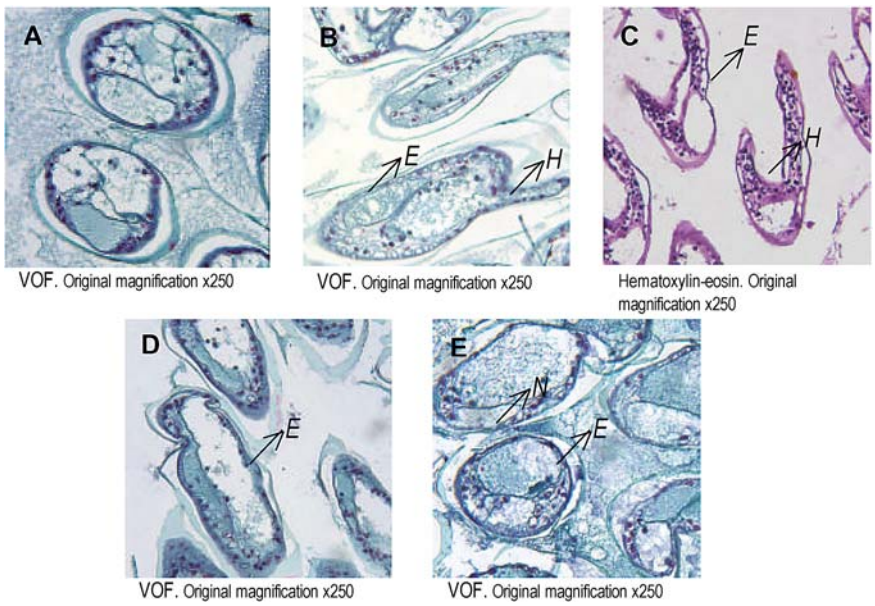

Figure 4. Histopathological alterations in *P. clarkii* gill vessels after 21 days of exposure to dissolved heavy metals: (A) Control; (B) 10 μg L^{-1} Cd; (C) 30 μg L^{-1} Cd; (D) 1000 μg L^{-1} Zn; (E) 3000 μg L^{-1} Zn. The pathologies observed were: E: Disorganized gill epithelium; H: Hyperplasia; N: Necrosis.

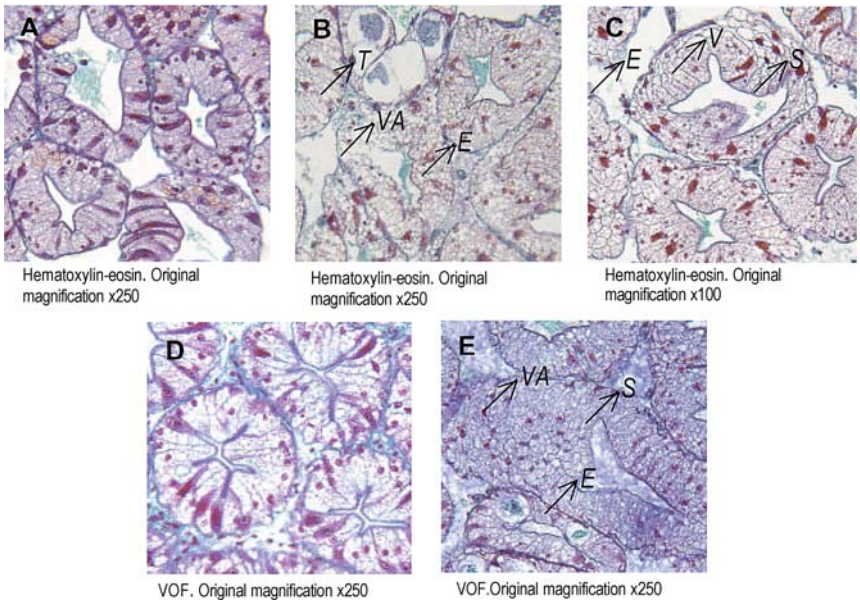

Figure 5. Histopathological alterations in *P. clarkii* hepatopancreas vessels after 21 days of exposure to dissolved heavy metals: (A) Control; (B) 10 μg L^{-1} Cd; (C) 30 μg L^{-1} Cd; (D) 1000 μg L^{-1} Zn; (E) 3000 μg L^{-1} Zn. The pathologies observed were: T: Degenerating hepatopancreas tubules; E: Disorganized hepatopancreas epithelium; VA: Vacuolazitation; S: Sewelling; N: Necrosis.

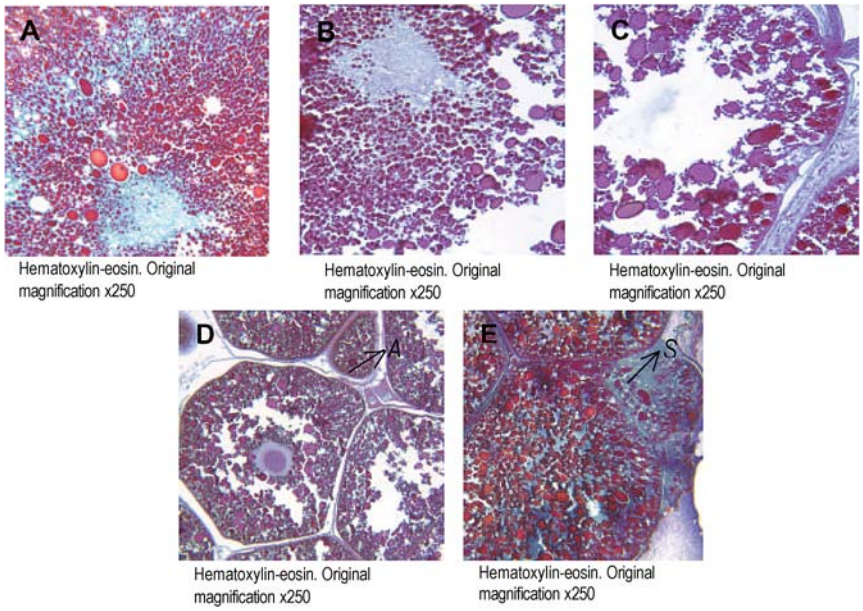

Figure 6. Histopathological alterations in female *P. clarkii* gonad vessels after 21 days of exposure to dissolved heavy metals: (A) Control; (B) 10 μg L^{-1} Cd; (C) 30 μg L^{-1} Cd; (D) 1000 μg L^{-1} Zn; (E) 3000 μg L^{-1} Zn. The pathologies observed were: S: Sewelling; A: Atretic oocytes.

Epithelial cells lining the branchial vessels of the gill filaments were disorganized, and this damage increased with exposure concentration, especially within the cadmium treated individuals.

All histological samples of ovary showed evidence of mature vitellogenic oocytes containing eosinophilic proteic yolk granules, which were enclosed by a single layer of flattened follicle cells. Atypical oocytes intensively stained by haematoxylin and eosin, lacking nuclei and yolk-bodies, and further characterized by rupture of the external follicular layer in some oocytes were detected especially in specimens treated with cadmium. Moreover an increase of lipidic globules (vacuoles in paraffin) present in these vitellogenic oocytes were also observed. With increasing the concentration of dissolved metal exposure, a decrease of vitellin granules and an increase of lipidic ones was observed.

In general no significant differences were observed in regard to histopathological damage between males and females.

4. Discussion

There is no evidence that any decapod regulates the body cadmium concentration to a constant level by balancing uptake and excretion. This should not be surprising since Cd is a nonessential metal and, therefore, most organisms should lack adequate mechanisms to control or eliminate it from the body. Indeed the crab *Carcinus maenas* (Wright, 1977), the prawn *Palaemon elegans* (White and Rainbow, 1986) and the shrimp *Crangon crangon* (Amiard *et al*., 1985) all show increasing body concentrations of cadmium with increased exposure to dissolved cadmium in the laboratory. The results of this study support these reports since it was shown that an increase in cadmium concentration in hepatopancreas, gill and muscle tissues occurred when specimens were exposed to increasing cadmium levels ($10\,\mu g\,L^{-1}$ and $30\,\mu g\,L^{-1}$) (Figure 1). Significant higher cadmium uptake ($p < 0.01$ and $p < 0.05$) was observed in the three tissues mentioned, exposed to cadmium than in control tissues (Figure 1).

Bioaccumulation in individuals exposed to zinc was not as positive correlated with increasing dissolved metal concentration ($1000\ \mu g\,L^{-1}$ and $3000\ \mu g\,L^{-1}$), as in cadmium treated crayfishes (Figure 1). Only hepatopancreas (male) and gill (male and female) tissues exposed at the highest zinc concentration showed significant zinc uptake ($p < 0.05$ and $p < 0.01$ respectively) when compared with controls (Figure 1). It is important to take into account that zinc is an essential metal and that crustaceans regulate body zinc concentrations approximate to the concentrations needed to meet metabolic demands, therefore any increase in zinc uptake may be relatively easy to accommodate by increasing zinc excretion (Rainbow, 1988).

Cadmium and zinc accumulation was higher in gill than in hepatopancreas tissues. Results are in concordance with those reported by White and Rainbow

(1984) that showed heavy metal uptake from solution via permeable surfaces of the body, especially across the gills in most adult aquatic decapods.

In general, higher concentrations of cadmium and zinc were determined in all tissues of males when compared to females, although a significant difference was only observed between cadmium bioaccumulation in hepatopancreas tissues of males and females exposed to $30\,\mu g\,L^{-1}$ of cadmium ($p < 0.01$). It can be explained by a reduction in branchial net cadmium influx associated with females. This hypothesis is supported by the results of Bondgaard *et al.* (2000), who reported that cadmium exposure during ovarian maturation in *Carcinus maenas* also resulted in diminished gill uptake of cadmium. Although gonads were not sampled for cadmium content in this study, there is some indication that females may enjoy another mechanism of detoxification not present in males such as toxins associated with circulating haemolymph lipoproteins that are eventually incorporated into developing oocytes (Lee, 1993). Females would, therefore, sacrifice the health of a single progeny of young but relieve themselves of the burden of toxic heavy metals.

The metallothionein concentrations reported here concur with those of other investigators who reported an increased body burden with increasing cadmium exposure in crustaceans (Olaffson *et al.*, 1979; Pedersen *et al.*, 1994) (Figure 2). Although the experiments carried out here, in cadmium have failed to show increases in MT concentrations upon laboratory exposure of *P. clarkii* crayfishes, this trend was not observed in individuals exposed to zinc at the environmental concentrations tested. Consequently, it was shown a decrease in metallothionein concentration in males when they were exposed to $3000\,\mu g\,L^{-1}$ of zinc. The decrease of MT concentrations with increasing zinc exposure observed at the present study with male *P. clarkii* was also described in some laboratory exposures of invertebrates to dissolved availabilities of trace metals, probably as a result of toxic effect preventing detoxification processes from being fully functional (George *et al.*, 1992; Barka *et al.*, 2001). This hypothesis has been enlarged to other detoxification mechanisms based upon the induction of specific proteins such as cytochrome P450-dependant monooxygenases (Flammarien, 2000). As mentioned by Couillard *et al.* (1995) for the freshwater clam *Pyganodon grandis*, overall changes in MT concentrations integrate both biosynthetic and degradative processes. It may well be that physiological detoxification processes involving MT are more active upon exposure of *P. clarkii* to a metal challenge, but this increase in activity of metallothioneins in the crayfish may be reflected in increased turnover of the proteins as opposed to any increase in the protein concentration. In female crayfishes exposed to $1000\,\mu g\,L^{-1}$ zinc, MT concentrations in hepatopancreas tissues were higher than in control females, yet the MT concentration did not increase further when exposed to $3000\,\mu g\,L^{-1}$ (Figure 2). This might be due to a higher Zn necessity in females, and a declination of MT concentrations to facilitate transfer of the essential metal Zn to cellular component required for vitellogenin/vitellin synthesis in females.

Vitellogenin/vitellin concentration in female crayfish haemolymph during the 21 days of exposure showed an increase, registering significant high concentrations on day 21 in specimens exposed to cadmium and zinc ($p < 0.01$) (Figure 3). No studies have to our knowledge ever investigated how the exposure to heavy metals could affect to the VTG concentration in the haemolymph in crustaceans. Nevertheless it could be affirmed that high VTG in females is therefore a warning of possible disruption to normal reproductive function (Kime *et al.*, 1999). It long been known that atretic eggs that do not mature normally can be reabsorbed and may result in high concentrations of VTG being carried through the haemolymph to the hepatopancreas for digestion (Lee and Walker, 1995). Insult from the metal exposure could have disrupted the normal development of the oocytes and induced this process of reuptake of yolk proteins. There is also evidence that there may be a bimodal effect on vitellogenesis from heavy metal exposure. First, the VTG production may be inhibited indirectly by 30 $\mu g\,L^{-1}$ Cd by interfering with either 5-hydroxytryptamine (5-HT) or gonad stimulating hormone (GSH) release from the brain or thoracic ganglion (Reddy *et al.*, 1997), or directly by inhibition of VTG synthesis, resulting in lowered haemolymph levels following 7 days of exposure (Figure 3). Second, reuptake of VTG from immature and atretic eggs resulting from the extended period (2–3 weeks) of reduced 5-HT or GSH, resulted in higher circulation of VTG later in the study. Future efforts that determine whether the VTG is being synthesized at a greater rate or simply reabsorbed from atretic eggs exposed to Cd would further elucidate the mechanism of toxicity.

Histopathological studies support the hypothesis related to the increase of VTG in the hemolymph due to the reabsobation of atretic eggs. Atretic oocytes were determined specially in cadmium treated individuals with increasing heavy metal concentration (Figure 6).

Histopathological damages are in concordance with bioaccumulation determined in soft tissues. Significant bioaccumulation occurs preferably in gill tissues at both cadmium concentrations, being the histopathological lesions higher and more severe in the same tissues. It is also significant bioaccumulation in *P. clarkii* hepatopancreas exposed to the highest zinc concentration, being the histopathological damage higher at these concentrations.

There are differences in the bioaccumulation of Cd at the cadmium concentrations tested (30 $\mu g\,L^{-1}$) between males and females in hepatopancreas tissues. These differences were not observed in histapathological damages.

The effects occurred at the initial moment that the accident took place could not be measured, but through laboratory simulations could be shown that the contamination due to the mining accident was present in the organisms, specially for cadmium exposure and could have affected to the reproduction affectivity in organisms. Recent studies (Riba *et al.*, 2003) have pointed out the presence of a sequel produced by the spill and associated with the bioaccumulation of Cd provoking histopathological diseases in estuarine field collected organisms.

5. Conclusions

At the environmental cadmium and zinc concentrations tested with *P. clarkii*, the result suggest that significant bioaccumulation of cadmium and zinc occurs principally in gill tissues. Increasing concentration of cadmium exposure resulted in increasing metal bioaccumulation in all the tissues as well as increased metallothioneins and vitellogenin/vitellin and histopathological damage. Nevertheless, increasing zinc exposure showed increasing vitellogenin/vitellin and histopathological damages, although a clear relationship was not found with metallothionein concentrations determined in the hepatopancreas. Only different responses were observed between males and females exposed to $30\,\mathrm{mg\,L^{-1}}$ of Cd.

The concentrations of cadmium in different tissues seem to reflect the exposure of the crabs to this metal, whereas concentrations found in crabs exposed to zinc, maybe due to their essential metal condition, do not reach significant values compared with control, except in gill tissues at the highest zinc concentration. These results were in concordance with histopathological damages in different tissues. Damages were observed preferently in gill and hepatopancreas tissues exposed to cadmium and zinc, and in gill tissues exposed to zinc. Cadmium was found to be a good inducer of MT concentrations in hepatopancreas tissues. Concentrations of this metal in these tissues are directly correlated with metallothionein production in the same tissue. Nevertheless, the other metal assayed, Zinc, did not show a positive correlation with metallothionein induction.

Increasing in heavy metal bioaccumulation resulted in increasing VTG concentration in hemolymph. In cadmium exposure, it could be due to the reabsortion of atretic oocytes, nevertheless, no atretic oocytes were observed in individuals exposed to zinc, so the mechanism could be different, maybe it could be related to the induction of VTG because of the presence of zinc in the hepatopancreas. As this metal takes part of the VTG molecule.

The effects that could occur at the initial moment that the accident took place could not be measured, but through laboratory simulations could be shown that the contamination due to the mining accident was present in the organisms, specially for cadmium exposure and could have affect to the reproduction affectivity in *P. clarkii* organisms. Recent studies (Riba *et al*., 2003) have pointed out the presence of a sequel produced by the spill and associated with the bioaccumulation of Cd provoking histopathological diseases in estuarine organisms. In the present study it has been demonstrated that the simulation of environmental heavy metal concentrations could be used as a potential tool in the evaluation of environmental contamination sublethal effects. However, concerning to metal contamination assessment, metallothioneins in hepatopancreas tissues may be a good biomarker of exposure, especially for cadmium contamination, and VTG and histopathology resulted to provide significant information of effects of dissolved heavy metals, although they are not as specific as metallothioneins, the information that they provide together is very important.

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