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Changes in plasma electrolytes and Gill Histopathology in Wild *Liza saliens* from the Esmoriz-Paramos Coastal Lagoon, Portugal

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The Esmoriz-Paramos is a lagoon of great ecological significance located on the Northwest coast of Portugal. The quality of water and sediment within this ecosystem has been gradually degraded due to the discharges of mostly untreated industrial waste and domestic sewage. Contaminants include heavy metals that can be taken up by fish from water, food, sediments, and suspended particulate material. Fish inhabiting polluted water bodies tend to accumulate many chemicals in high concentrations, even when the environmental contamination levels are low (Colombo et al. 1995). The leaping grey mullet (*Liza saliens)* is one of a few dominant species living in this environment. This species may contact xenobiotics in the water column or, when feeding, in the sediments.

Previous studies have analyzed heavy metal concentrations in the water and sediments of this lagoon, and evaluated their bioaccumulation in *L. saliens* (Fernandes et al. 2007a, b). According to these studies, the seasonal range of metal concentrations in surface water was 0.003–0.031 mg $Cu \cdot L^{-1}$, 0.006–0.811 mg $Zn \cdot L^{-1}$ and 0.01–0.026 mg $Pb \cdot L^{-1}$, all mainly found in particulate matter. The main metals found in sediments were Cu, Zn and Pb, respectively 83, 241 and 87 $mg \cdot kg^{-1}$d.w. The Cu and Zn concentrations in *L. saliens* liver were 262 and 89 $mg \cdot kg^{-1}$d.w. and below the detection limit for Pb (0.073 ppm), whereas these concentrations in the gill were 9 mg $Cu \cdot kg^{-1}$ d.w.; 114 mg $Zn \cdot kg^{-1}$ d.w. and 0.6 mg $Pb \cdot kg^{-1}$ d.w. Also, significant age-related increase of metal concentrations in tissues, were noted, for Zn in gill and for Cu in liver and gill (Fernandes et al. 2007a).

The gill is particularly sensitive to physical and chemical changes in the aquatic environment and it is the main target organ in fish for toxic waterborne heavy metals (McDonald and Wood 1993). Effects of metals on ionoregulatory gill functions have been well demonstrated, including regulation of plasma electrolytes (Mazon et al. 2002; Grosell et al. 2003; Martinez et al. 2004). The histological effects of metals on fish gill have also been studied in several fish species (Arellano et al. 1999; De Boeck et al. 2001). The mechanisms of acute Cu toxicity include the osmoregulatory disturbances involving Na^+, Cl^- and K^+ uptake by the gill (Mazon et al. 2002; Grosell et al. 2003). Although the mechanisms of heavy metals toxicity are well known in acute exposure, the process may differ in chronic exposure (Handy 2003).

The objectives of this study were to evaluate the plasma electrolyte concentrations of wild *Liza saliens* from the Esmoriz-Paramos lagoon and to assess the osmoregulatory responses and branchial histopathological changes related to chronic heavy metal exposure in this habitat.

Materials and Methods

Mugilidae specimens were captured from the Esmoriz-Paramos lagoon and from the sea, in April 2004 in post-

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Table 1 Plasma electrolytes concentrations in fish collected from the lagoon and from the sea

Parameters	Lagoon fish (N = 10)	Sea fish (N = 15)
Ca (mg/L)	7.06 (0.98) [5.30–8.20]	6.47 (0.60) [5.50–7.40]
Cl (mEq/L)	129.66 (7.55) [119–148]	134.23 (6.94) [122–146]
K (mEq/L)	5.31 (1.41)* [2.90–6.90]	4.26 (1.15) [2.30–5.90]
Na (mEq/L)	152.28 (9.11) [136–168]	161.27 (12.29) [143–188]
P (mg/dL)	15.88 (2.08)** [12.30–18.50]	12.05 (3.39) [5.80–17.50]

Mean (SD) and [range]

* $p < 0.05$, ** $p < 0.01$

spawning period. The water quality parameters of the lagoon were monitored monthly during 2003–2004: temperature 10–23°C, pH 6.9–7.7 and salinity 0.17–2.60 ppt. Ten fish from the lagoon, with total length of 27–45 cm, and 15 fish from the sea, with total length of 22–47 cm, were randomly collected and quickly euthanized in the field by a sharp blow to the head. Blood was drawn from the caudal vessels with heparinized syringes. The second right gill arch was collected for histopathology according to standard methodology (Pane et al. 2004) and immediately fixed in buffered formalin (10%) for 48 h. Gill and liver samples were collected and frozen at –20°C until metal analyses. Fish age was determined by reading the annual ring structure of scales.

Plasma was obtained by centrifugation (5 min, 10.000 g, 4°C) and electrolyte measurements were carried out for Na^+ (mEq Na/L), Cl^- (mEq Cl/L), K^+ (mEq K/L), Ca^{2+} (mg Ca/L) and inorganic phosphorus (mg P/dL), using a VITROS 950 (Ortho-Clinical Diagnostics, Johnson and Johnson) analyzer (Hrubec et al. 2000). The analyzer uses both dry chemistry slide technology and reflection spectrophotometry. Na^+, Cl^- and K^+ were measured in dry chemistry slide technology by direct ion-selective electrodes (VITROS) using SRM 919[a] and 956 from National Institute for Standards and Technology. The sensitivity for assays was 11 mEq Na/L, 9 mEq Cl/L and 0.16 mEq K/L, while coefficients of variation were less than 3.2%. Colorimetric methods were employed to measure P and Ca by the ammonium molybdate reaction and arsenazo (III) dye formation, respectively, in accordance to National Committee for Clinical Laboratory Standards.

Fish tissues were acid-digested based on the method of Ferreira et al. (1990). Metal concentrations in liver were measured by flame atomic absorption spectrometry (Philips PU9100X). The gill metal concentrations were analyzed in a graphite furnace atomic absorption spectrometer (UNICAMP 939 AA-GF90). Certified reference materials, i.e DOLT-3 (dogfish liver from NRC) were analyzed to check analytical accuracy and precision. Results were expressed in mg kg^{-1} dry weight (Fernandes et al. 2007a).

Gill tissue was dehydrated in graded ethanol concentrations and embedded in paraffin wax for light microscopy analysis. Sagital sections (5 μm of thickness) were prepared and stained with hematoxylin-eosin (HE). Changes observed in gill tissue were analyzed using a Nikon E 200 microscope and photographed using an Olympus UC-MAD3.

To evaluate gill histopathological changes, five filaments and at least 100 lamellae per filament for each fish were evaluated using a scoring system to rank severity and extent. Severity was scored as follows: 0 no pathological alterations, 1 focal mild pathological alterations, 2 moderate pathological alterations, 3 severe pathological alterations (Schwaiger et al. 1997). A mean severity value for each histopathological change was calculated per filament to normalize the results. The extent of histopathological change was quantified, counting the number of lamellae showing alterations in each filament. The extent of lamellae affected was scored as follows: 0 = 0%, 1 ≤ 10%, 2 = 11–49%, 3 = 50–69%, 4 ≥ 70%. The mean extent value of the five filaments was then calculated for each histopathological change. Previous histopathological results, using a larger sample of fish from the lagoon, have shown high prevalence of lesions with high extent.

The relationships between plasma electrolyte concentrations, metal levels, and histopathological evaluation were tested with nonparametric correlations (Kendall's r–p), and differences between metal concentrations in tissues were tested with Mann-Whitney. A 5% significance level was applied throughout. Data are presented as mean values (standard deviation).

Results and Discussion

Table 1 shows the average plasma ion levels in the two fish populations. Plasma concentrations of P and K^+, in fish from the lagoon were significantly higher when compared with plasma from sea fish.

Gill epithelium of fish from the lagoon showed higher prevalence of different types of lesions, as well as higher prevalence of the number of lesions, comparing with mullets caught in the sea. The main lesions were epithelial hyperplasia, occasionally resulting in lamellar fusion, epithelial lifting, vasodilation and lamellar capillary aneurisms (Fig. 1). At a minimum, all the fish analyzed showed hyperplasia of filamentar and lamellar epithelium and vasodilation of the vascular axis. Lifting of lamellar epi-

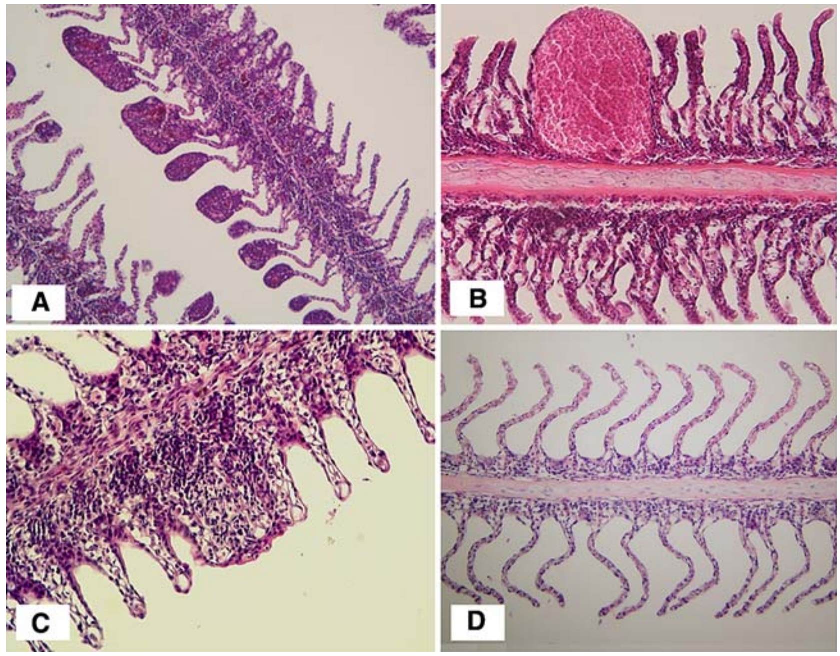

Fig. 1 Gill histopathology of *Liza saliens* from the Esmoriz-Paramos lagoon. **a** High severity of lamellar epithelium hyperplasia with fusion of adjacent lamellae, aneurism with vascular fusion and vasodilation in the lamellar vascular axis (150×); **b** aneurism, with high grade of severity that extends through the entire lamellar vascular axis and lifting of lamellar epithelium (200×); **c** filamentar epithelium hyperplasia with focal lamellar fusion (200×); **d** gill filament of fish collected from the sea with no histopathological changes (150×)

thelium and vascular aneurisms were found in 50% and 80%, respectively of the analyzed fish.

These gill changes were observed at varying degrees of extent and severity. For fish exhibiting aneurism, hyperplasia and vasodilation severity ranged from 1 to 3, whereas severity ranged from 1 to 2 for epithelial lifting. The mean severity of hyperplasia was 2 and for the rest of the changes was 1. The extent of hyperplasia and lifting ranged from 1 to 4 with a mean of 2. The extent of vasodilation, ranged from 1 to 2, and extent was 1 for aneurism; both of these lesions had an extent mean of 1.

Sea mullet presented mainly one or two types of lesions (71%); the main lesions were vasodilation 53%, lifting 43% and hyperplasia 14%. The extent and severity of these gill changes were lower comparing with lagoon mullets (Fernandes et al. 2007c).

L. saliens from the lagoon exhibited the highest Cu content in liver, ranging from 53 to 464 mg kg^{-1} d.w. (mean: 283 mg kg^{-1} d.w.). In gill, Cu content ranged from 7 to 16 mg kg^{-1} d.w. (mean: 10 mg kg^{-1} d.w.). Zn concentration was higher in gill 136 mg kg^{-1} d.w. (range 107–191) than in the liver where 109 mg kg^{-1} d.w. (range 63–190) were obtained.

The plasma electrolyte levels showed no significant correlation with Cu-gill or Zn in tissues. However, K^+ was positively correlated with liver Cu content (Kendall´s $r = 0.556$, $p = 0.037$), and also with gill lifting (Kendall's $r = 1$, $p = 0.042$, for both severity and extent).

Phosphorus levels showed a positive correlation with severity of hyperplasia (Kendall´s $r = 0.568$, $p = 0.041$). Cu-liver content was positively correlated with fish length (Kendall´s $r = 0.556$, $p = 0.025$).

The gill Cu and Zn levels of *L. saliens* found in this work are similar to those observed in laboratory exposures that produced several histopathological changes, such as lifting and hyperplasia of the epithelium in fish species, such as senegales sole, *Solea senegalensis* and rainbow trout, *Oncorhynchus mykiss* (Skidmore and Tovell 1972; Arellano et al. 1999).

Branchial Zn and Cu accumulations result from the chronic metal exposure (Taylor et al. 2004). Lagoon mullets showed highest Zn concentration in the gill and an increase over time was also observed (Fernandes et al. 2007a, b, c). However, the liver as the main homeostatic organ generally exhibits high copper concentrations (Grosell et al. 2003). In fact, the mean gill to liver-Cu ratio in *L. saliens* was 1:28, and the range of hepatic Cu content found (53–464 mg kg^{-1}) suggests an above-normal accumulation. According to Paris-Palacios et al. (2000) values of normal hepatic Cu content in fish liver were about 12 mg kg^{-1}. Furthermore, the increased Cu levels with fish length, noted in this study, indicates some loss of liver homeostatic capacity, as previously observed (Fernandes et al. 2007a).

Besides metal accumulation and associated gill histopathological changes, the stress caused by chronic metal exposure can also affect the plasma K^+ levels in *L. saliens.* In this system, Cu-liver was a better environmental indicator of Cu stress, rather than Cu-gill (Fernandes et al. 2007a). Additionally, plasma K^+ levels in fish from the lagoon were higher than this electrolyte measured in fish from the sea exposed to low concentrations of contaminants. Thus, the positive relationship between Cu-liver and K^+ concentrations can indirectly indicate that chronic exposure affects this plasma electrolyte. Plasma K^+ levels may increase in fish from the lagoon due to osmotic adjustment, when compensating for a decline of other serum components, or resulting from disruption of K^+ regulatory ability (Marcaldo-Allen et al. 2004). Plasma K^+ alterations noted in this study may also have been caused by the disruption of cell membrane integrity due to gill lesions. In fact, a positive relationship was established between epithelial lifting (severity and extent) and K^+ levels. A comparison of plasma K^+ levels between fish exhibiting epithelial lifting (6.40 ± 0.36 mEq/L) and fish without this gill change (4.44 ± 1.31 mEq/L) further reinforces this concept. Other investigations have also described an increase of K^+ levels with Cu acute toxicity (Mazon et al. 2002).

The mechanisms of acute Cu toxicity also include osmoregulatory disturbance of Na^+ uptake by the gill (Grosell et al. 2003; Taylor et al. 2004), related to inhibition of branchial Na^+/K^+-ATPase (Mazon et al. 2002). In contrast, the present results showed that plasma Na^+ levels in fish from the lagoon were not affected. No relationship between Na^+ levels and gill changes or metals were established, nor differences between Na^+ levels from the two populations indicate any affects on the Na^+/K^+-ATPase function. This may result from metal sequestration in metalloproteins and/or in lysosomes, decreasing in this way metal toxicity (Weis et al. 1986).

Plasma Ca^{2+} and Cl^- concentrations are parameters used to characterize the general osmoregulatory condition of the fish. Both of these plasma electrolytes generally decrease in cyprinids following exposure to different stressors (Jeney et al. 2002; Mazon et al. 2002). No trends in Ca^{2+} or Cl^- electrolytes in fish from the lagoon were found. The same electrolytes had similar levels in the two populations, which suggest that *L. saliens* did not show a stress response, because it may be adapted or the osmoregulatory dysfunction was attenuated by low salinities. Tsuzuki et al. (2001) reported that moderate salinities (3–5 ppt) can reduce the stress response and ionic unbalances, because less osmotic work is needed to maintain stable ion levels. Indeed, in this lagoon the annual salinity range was 0.17–2.6 ppt, which may have contributed to a mild ionic stress response.

The higher phosphorus concentration found in lagoon *L. saliens*, when compared with fish from the sea, could be a compensatory mechanism to maintain the number of osmotically active particles in the plasma (Mercaldo-Allen et al. 2004) and/or, as an important buffer involved in acid–base balance. In addition, the relationship between phosphorus levels and severity of hyperplasia may be explained by decreased cell integrity resulting in phosphorus release from the cell.

Our results show potential evidence of anthropogenic effects on blood chemistry parameters of *L. saliens* collected from Esmoriz-Paramos lagoon. The chronic heavy metal toxicity may involve a series of adjustments or adaptations that contribute to the leaping grey mullets long-term survival. The effect of chronic Cu and Zn exposure in adult *L. saliens* in this lagoon included high gill and liver metal content, histological damage to specific target organs (e.g the gills) and indirectly to ionoregulatory disturbances. The increase of plasma K^+ and phosphorus could be a consequence of changes in gill permeability and cell integrity, associated with the gill histopathological alterations. In conclusion, this study provides information about the nature of chronic adverse and adaptive effects on aquatic biota undergoing exposure to metals in Esmoriz-Paramos lagoon.

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