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Metallothionein-like proteins in the blue crab *Callinectes sapidus*: Effect of water salinity and ions

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ABSTRACT

The effect of water salinity and ions on metallothionein-like proteins (MTLP) concentration was evaluated in the blue crab *Callinectes sapidus*. MTLP concentration was measured in tissues (hepatopancreas and gills) of crabs acclimated to salinity 30 ppt and abruptly subjected to a hypo-osmotic shock (salinity 2 ppt). It was also measured in isolated gills (anterior and posterior) of crabs acclimated to salinity 30 ppt. Gills were perfused with and incubated in an isosmotic saline solution (ISS) or perfused with ISS and incubated in a hypo-osmotic saline solution (HSS). The effect of each single water ion on gill MTLP concentration was also analyzed in isolated and perfused gills through experiments of ion substitution in the incubation medium. *In vivo*, MTLP concentration was higher in hepatopancreas than in gills, being not affected by the hypo-osmotic shock. However, MTLP concentration in posterior and anterior gills significantly increased after 2 and 24 h of hypo-osmotic shock, respectively. *In vitro*, it was also increased when anterior and posterior gills were perfused with ISS and incubated in HSS. In isolated and perfused posterior gills, MTLP concentration was inversely correlated with the calcium concentration in the ISS used to incubate gills. Together, these findings indicate that an increased gill MTLP concentration in low salinity is an adaptive response of the blue crab *C. sapidus* to the hypo-osmotic stress. This response is mediated, at least in part, by the calcium concentration in the gill bath medium. The data also suggest that the trigger for this increase is purely branchial and not systemic.

1. Introduction

Salinity is a natural factor influencing several biochemical and physiological processes in euryhaline crustaceans (Bianchini et al., 2008). Changes in environmental salinity typically cause metabolic changes in these animals. Their response to lowering salinities is characterized by an increased gill oxygen consumption rate (Péqueux and Gilles, 1988; Lucu and Pavieié, 1995; Piller et al., 1995; Brown and Terwillinger, 1999). This indicates higher energy expenditure with the ionic and osmotic regulation processes, as well as a possible occurrence of amino acid oxidation (Gilles, 1983; Mantel and Farmer, 1983). In turn, increased oxygen consumption rates are closely related to increased generation of reactive oxygen species (ROS). These species are produced in all aerobic organisms due to an incomplete reduction of the oxygen to water during the oxidative phosphorylation at the mitochondrial level (Storey, 1996).

MT is a group of low molecular weight, cysteine-rich proteins, capable of binding both essential and non-essential metals. The MT behavior is dominated by the chemistry of the thiol (–SH) group. The metal-thiolate clusters within the MT molecules allow rapid exchanges of metallic ions between clusters and with other MT molecules. These characteristics of binding and transference of metals appear to be unique to MT and fundamental to its biological role. Proposed functions to MT include homeostasis of essential metals, detoxification of non-essential metals, and scavenging of ROS (Palmiter, 1998; Miles et al., 2000; Viarengo et al., 2000; Coyle et al., 2002).

MT protection against cellular oxidative stress has been shown in several aquatic invertebrates (Suzuki et al., 1996; Brouwer and Hoexum-Brouwer, 1998; Viarengo et al., 2000). Their involvement with the cellular antioxidant defense system has been reported by several authors (Anderson et al., 1999; Andrews, 2000; Cavaletto et al., 2002). Therefore, it is expected that an increased oxygen consumption rate induced by hypo-osmotic shock would lead to increased MT concentration in tissues of euryhaline crustaceans, as an adaptive response to environmental salinity changes. In fact, salinity-induced changes in MT expression in gills and hepatopancreas/digestive gland have been reported in aquatic invertebrates, including crustaceans (Legras et al., 2000; Mouneyrac et al., 2001; Leung et al., 2002).

MT is part of a core suite of biomarkers recognized and examined in the framework of biological effects quality assurance in monitoring programs (Mathiessen, 2000; Monserrat et al., 2007). Therefore,

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analysis of the salinity influence on MT concentration in tissues of aquatic animals, especially invertebrates, is essential for the adequate application of MT as a biomarker of metal exposure in biomonitoring programs in estuarine and coastal waters (Leung et al., 2002).

In light of the above, we analyzed in the present study the *in vivo* and *in vitro* influences of water salinity and ions on metallothionein-like proteins (MTLP) concentration in key tissues (gills and hepatopancreas) of the blue crab *Callinectes sapidus*. This crab was used as a model since it is the most abundant and widely distributed species of the genus *Callinectes* in America (Rathbun, 1930). This euryhaline crab occurs from Canada (Rathbun, 1930) to Argentina (Boschi, 1964), inhabiting estuarine areas. In the Patos Lagoon estuary (Southern Brazil), it is the most frequent crab species (Capitoli et al., 1978; Topin and Souza, 1982).

2. Materials and methods

2.1. Crab collection and acclimation

Adult male crabs *C. sapidus* (n=64; carapace length: 10.96±0.16 cm; wet mass: 104.2±4.6 g) in stage C or early D of the intermoult cycle (Drach and Tchernigovtzeff, 1967) were collected from clean (i.e. not metal contaminated) sites at the "Marinheiros Island" (32°02′S–52°12′W) in the Patos Lagoon estuary (Southern Brazil) (Baumgarten and Niencheski, 1998; Marcuzzo et al., 1998). Crabs were transferred to the laboratory and kept in natural seawater at salinity 30 ppt, for at least two weeks before tests (Piller et al., 1995). Water was continuously filtered (chemical and biological filters) and aerated. Temperature and photoperiod were fixed at 20 °C and 12L:12D, respectively. Crabs were fed at satiation with chopped fish three times a week.

After the laboratory holding period, crabs were divided into two groups (n=32 in each group). One group was used for the *in vivo* experiments while the other was used for the *in vitro* experiments, as described below.

2.2. In vivo experiment

Eight crabs held in seawater at salinity 30 ppt were cryoanesthetized. The hemolymph (1 mL) was collected by puncture at the basis of the 3rd or 4th pair of pereiopods. Samples were immediately frozen (−20 °C) until ionic and osmotic concentration analyses, as described below. Hepatopancreas and gills (anterior and posterior) were dissected, dried on filter paper, and stored at −80 °C until MTLP analysis, as described below.

Twenty four crabs held in seawater at salinity 30 ppt were abruptly transferred to saltwater at salinity 2 ppt. Water at salinity 2 ppt was prepared by dilution of natural seawater with distilled water. After 2, 6 and 24 h of hypo-osmotic shock, eight crabs at each experimental time were cryoanesthetized and had their tissues excised and stored as described above.

Samples (5 mL) from the different experimental media were collected and frozen (−20 °C) for further ionic and osmotic concentration analyses as described below.

2.3. In vitro experiments

In vitro experiments were performed on isolated and perfused gills of thirty two crabs held in seawater at salinity 30 ppt. Gills were perfused and incubated for 2 h following procedures described by Péqueux and Gilles (1978).

Crabs were cryoanesthetized and had their carapaces removed. Anterior gills 4 and posterior gills 5 or 6 were excised from their bases and carefully rinsed with an iso-osmotic saline solution (ISS). ISS had the following composition (in mM): NaCl 370, $KHCO_3$ 12, $CaCl_2$ 20, $MgSO_4$ 10, H_3BO_3 5, sodium citrate 15, and glucose 2.8. The pH was adjusted to 7.6 with Tris-Base. This saline solution was prepared considering the hemolymph ionic composition of blue crabs held in seawater at salinity 30 ppt (see Results section), as well as other saline solutions already used to perfuse and incubate crustacean gills (Péqueux and Gilles, 1978; Luquet et al., 2002; Tresguerres et al., 2003).

The hemolymph remaining in the excised gills was quickly removed gently flushing the gill with ISS using a plastic catheter (Sondaplast, #10) adapted to a plastic disposable syringe (5 mL). This catheter was inserted into the gill afferent vessel. After gill rinsing, plastic catheters (Sondaplast, #10) were introduced in the afferent and efferent vessels and held in place using a plastic clipper. The gill preparation was immersed in a glass beaker containing 15 mL of incubation saline solution. Composition of this saline solution depended on the experiment to be performed. Under symmetric conditions (control), gills (anterior and posterior) were perfused with and incubated in ISS for 2 h. To determine the hypo-osmotic shock effect on MTLP concentration, gills were perfused with ISS and incubated in a hypo-osmotic saline solution (HSS). The ionic composition of this saline solution was similar to that of saltwater at salinity 2 ppt (see Results section). HSS had the following composition (in mM): NaCl 35, $KHCO_3$ 0.5, $CaCl_2$ 0.5, $MgSO_4$ 5, H_3BO_3 5, and glucose 2.8. The pH was adjusted to 7.6 with Tris-Base.

The catheter inserted into the afferent vessel was connected to a glass balloon, serving as a pressure reservoir, which contained ISS. The pressure of perfusion corresponded to 15–20 cm of water column, a value similar to the normal hemolymph pressure in the infrabranchial sinus of crabs (Wilkens and Young, 1992; Burnett et al., 2006). The catheter inserted into the efferent vessel was ended into a glass beaker to collect the perfusate. The level of the incubation medium was continuously monitored over the perfusion time to check for possible leakages. Leaking preparations were discarded. Oxygen was continuously bubbled into the incubation solution.

The effect of isolated ions on MTLP concentration was analyzed through experiments of ion substitution in the incubation saline solution. ISS was used to perfuse and incubate gills. However, each ion tested (Cl^-, Na^+, K^+, Ca^{2+}, and Mg^{2+}) was completely replaced in the incubation saline solution by an equivalent ion at the same concentration to keep the saline solution osmolality unchanged. Cl^- was replaced by gluconate, Na^+ and Ca^{2+} by choline, and K^+ and Mg^{2+} by Na^+. The influence of Ca^{2+} concentration on MTLP concentration was also analyzed in gills perfused with ISS and incubated in ISS containing different calcium concentrations (0, 10, and 20 mM).

In all cases, gills were removed from the clip after 2 h of perfusion, dried on filter paper, weighed, and stored at −80 °C until MTLP concentration measurement as described below. Two anterior and two posterior gills from each crab were used. Four crabs were used to test the effect of changes in the incubation saline solution: control (20 mM Ca^{2+}-ISS), hypo-osmotic shock (HSS), Na^+-free ISS, Cl^--free ISS, K^+-free ISS, Mg^{2+}-free ISS, Ca^{2+}-free ISS, and 10 mM Ca^{2+}-ISS.

2.4. Ion concentration and osmolality measurements

Chloride concentration in crab hemolymph and experimental media samples was measured by titration (chloridometer PLM3, Jenway Ltd., England) while Na^+, K^+, and Ca^{2+} concentrations were determined by flame photometry (MicroNal, SP, Brazil). Mg^{2+} concentration was measured using a colorimetric reagent kit (Doles, GO, Brazil). Absorbance readings (λ=490 nm) were performed using a microplate reader (Bio-Tek EL_X-800, Vermont, USA). Hemolymph and experimental media osmolality was determined using a semi-micro osmometer (Knauer, Germany) based on the freezing depression point.

2.5. MTLP concentration measurement

MTLP concentration was measured in tissue samples according to the methodology described by Viarengo et al. (1997). Application of this methodology yields a partially purified metalloprotein fraction by acidic ethanol/chloroform fractionation of the tissue homogenate. During

Table 1
Osmolality (mOsmol/kg water) and ion concentration (mEq/L) in the experimental media (salinity 2 ppt and 30) and in the hemolymph of the blue crab acclimated to saltwater at salinity 30 ppt

Parameter	Salinity 2 ppt	Salinity 30 ppt	Hemolymph
Osmolality	83±2	855±5	853±8
Sodium	34±1	444±7	414±17
Chloride	36±3	451±3	406±8
Potassium	0.4±0.1	7.8±0.3	11.5±0.3
Magnesium	4.8±0.2	35.8±5.3	10.2±0.3
Calcium	0.5±0.1	13.9±0.2	20.8±0.9

Data are expressed as mean±standard error (n=8).

extract preparation, MT is denatured by low pH and high ionic strength. MT concentration in extracts is measured by spectrophotometry employing the Ellman's –SH reagent (5,5-dithio-bis 2-nitrobenzoic acid; DTNB). According to Viarengo et al. (1997), procedures performed during sample preparation allow obtaining a complete MT precipitation and to avoid oxidation of –SH groups, contamination by soluble low molecular weight thiols, and enzymatic protein degradation.

In the present study, tissue samples (hepatopancreas and gills) were homogenized in a buffer solution (sucrose: 500 mM, Tris–HCl: 20 mM, PMFS: 0.5 mM and β-mercaptoethanol 0.01%, pH 8.60). MTLP concentration in homogenates was determined by spectrophotometry (405 nm) using DTNB (Sigma-Aldrich, USA). Results were expressed in terms of GSH (μmol GSH/g of tissue wet mass), employing GSH standard solutions at different concentrations (15, 30, 60, 90 and 120 μmol GSH/L) to build a calibration curve.

2.6. Data analysis

Data were expressed as mean±standard error (n=8). Mean values from *in vivo* experiments were subjected to two-way (time and tissue as factors) analysis of variance (ANOVA). Mean values from *in vitro* experiments were subjected to one-way ANOVA (experimental condition as factor). Both analyses were followed by the Duncan's multiple comparison test and the significance level adopted was 95% (α=0.05). ANOVA assumptions (data normality and homogeneity of variances) were previously checked (Zar, 1984).

3. Results

Ionic composition and osmolality of the experimental media and hemolymph of crabs held in seawater at salinity 30 ppt are shown in Table 1. Osmolality and ionic composition of the hemolymph are similar to those measured in seawater at salinity 30 ppt, but much higher than those measured in saltwater at salinity 2 ppt.

Results from the *in vivo* experiment showed that MTLP were detected in all tissues analyzed. Prior to the hypo-osmotic shock, MTLP concentration was significantly higher in hepatopancreas than in gills (anterior or posterior). No significant difference was observed between anterior and posterior gills (Fig. 1).

Hepatopancreas MTLP concentration was not influenced by the *in vivo* hypo-osmotic shock. However, MTLP concentration in posterior gills was significantly increased 2 h after the hypo-osmotic shock, remaining elevated until 24 h of test. In anterior gills, a trend to an increased MTLP concentration was observed 2 and 6 h after the *in vivo* hypo-osmotic shock. A significant increased concentration was observed 24 h after the hypo-osmotic shock. After 24 h of hypo-osmotic shock, all tissues showed a similar MTLP concentration (Fig. 1).

The *in vitro* hypo-osmotic shock induced a significant increase in MTLP concentration in both anterior and posterior gills (Fig. 2). In anterior gills tested under symmetric conditions, i.e. perfused with and incubated in ISS, there was no significant effect of ions replacement in

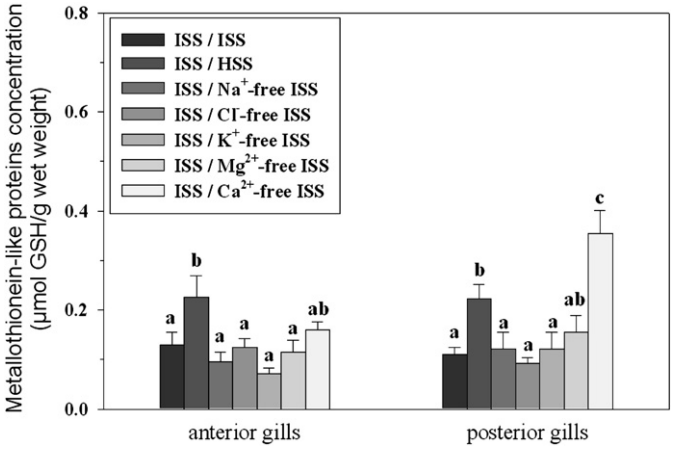

Fig. 2. Metallothionein-like proteins (MTLP) concentration in anterior (a) and posterior (b) gills of the blue crab *Callinectes sapidus* perfused with isosmotic saline solution (ISS) and incubated in either ISS or hypo-osmotic saline solution (HSS). In the ion replacement experiments, gills were incubated in Na^+-, Cl^--, K^+-, Mg^{2+}- or Ca^{2+}-free ISS. In the plot legends, solutions employed are represented as follows: perfusion saline solution/incubation saline solution. Different letters indicate significant different means (p<0.05) for anterior or posterior gills. Data are expressed as mean±standard error (n=8).

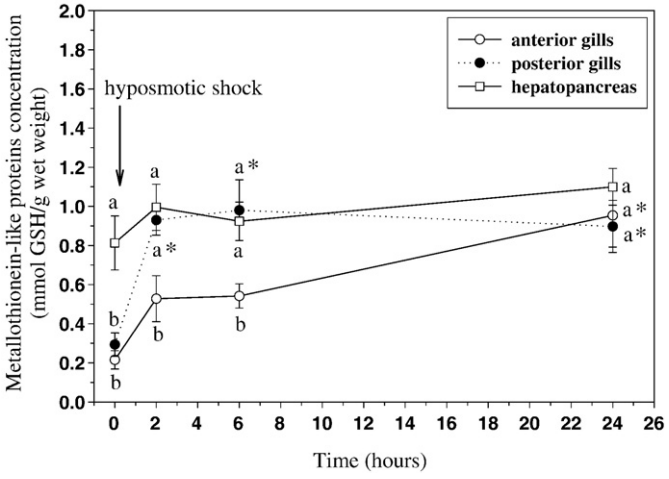

Fig. 1. Effect of *in vivo* hypo-osmotic shock on metallothionein-like proteins (MTLP) concentration in tissues of the blue crab *Callinectes sapidus*. Different letters indicate significant different means (p<0.05) between tissues for the same experimental time. * indicates significant different means from the beginning of the experiment (time zero) for each tissue. Data are expressed as mean±standard error (n=8).

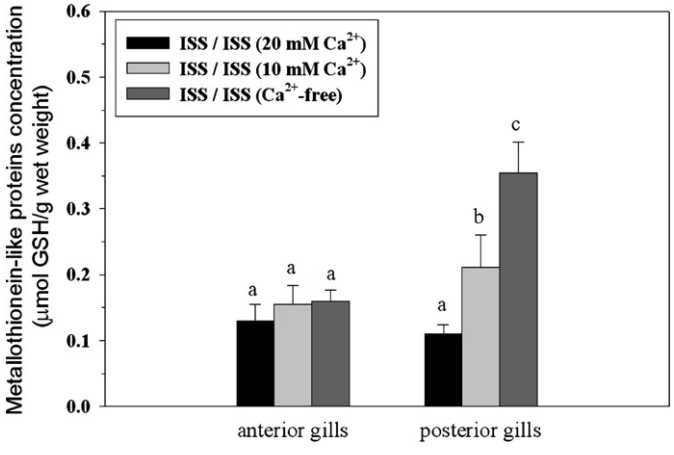

Fig. 3. Metallothionein-like proteins (MTLP) concentration in gills of the blue crab *Callinectes sapidus* perfused with isosmotic saline solution (ISS) and incubated in ISS containing different calcium concentrations (0, 10 and 20 mM). In the plot legends, solutions employed are represented as follows: perfusion saline solution/incubation saline solution. Different letters indicate significant different means (p<0.05) for anterior or posterior gills. Data are expressed as mean±standard error (n=8).

the incubation saline solution on MTLP concentration. On the other hand, a significantly higher MTLP concentration was observed when posterior gills were perfused with ISS and incubated in Ca^{2+}-free ISS (Fig. 2). Furthermore, an inverse relationship between MTLP concentration in posterior gills and Ca^{2+} concentration in the incubation saline solution was observed (Fig. 3).

4. Discussion

The increased levels of MTLP observed in anterior and posterior gills of the blue crab after either *in vivo* or *in vitro* hypo-osmotic shock could be a result of an increased production of reactive oxygen species (ROS) in low salinities. This hypothesis is based on the fact that MT is involved in the cellular antioxidant defense system in aquatic invertebrates, reacting with ROS (Anderson et al., 1999; Andrews, 2000; Cavaletto et al., 2002). At this point, it is important to note that MT can protect cells against the oxidative stress not only by acting as an oxyradical scavenger, but also through the metal binding/release dynamics (Suzuki et al., 1996; Brouwer and Hoexum-Brouwer, 1998; Viarengo et al., 2000).

Changes in salinity, especially the abrupt ones as applied in the present study, typically cause metabolic changes in crustaceans. In euryhaline crabs, including the blue crab *C. sapidus*, the response to lowering salinities is characterized by an increased gill oxygen consumption rate (Péqueux and Gilles, 1988; Lucu and Pavieié, 1995; Piller et al., 1995; Brown and Terwillinger, 1999). This indicates higher energy expenditure with the ionic and osmotic regulation processes, as well as the possible occurrence of amino acid oxidation (Gilles, 1983; Mantel and Farmer, 1983). Lucu and Pavieié (1995) observed that glucose and amino acids oxidation rates were 2-fold higher in gills of crabs *Carcinus mediterraneus* acclimated to diluted seawater than in those from crabs acclimated to full seawater. In turn, an increased oxygen consumption rate induces an elevated generation of ROS, which include $O_2^{\cdot-}$, H_2O_2 and $HO^{\cdot}$ (Storey, 1996).

Gills are generally responsible for the ion regulatory capacity in decapod crustaceans, including the blue crab *C. sapidus* (Cameron, 1978; Robinson, 1994; Piller et al., 1995; Towle et al., 1996). This crab species is able to maintain high hemolymph osmolarity in low salinities by actively pumping ions through the gills (Mantel and Farmer, 1983; Lucu 1990; Piller et al., 1995; Towle et al., 1996). In most euryhaline crabs, including the blue crab *C. sapidus*, posterior gills are the major sites for ion transport. They show higher activities of enzymes implicated in ion transport mechanisms and have more mitochondria-rich cells than anterior gills. Alternately, anterior gills are considered as mainly respiratory, showing passive Na^+ movements (Henry and Cameron, 1982; Lucu, 1990; Péqueux, 1995; Lucu and Towle, 2003; Henry, 2005; Burnett et al., 2006). Therefore, the differential time-course response of MTLP concentration observed between anterior and posterior gills after the hypo-osmotic shock could be related to the different physiological roles played by these different types of gills in decapod crustaceans.

In the present study, both *in vivo* and *in vitro* experiments were performed to verify the effect of water salinity on MTLP concentration in gills of the blue crab *C. sapidus*. In both cases, gills (anterior and posterior) were able to approximately double their MTLP concentration after the hypo-osmotic shock. This finding indicates that the trigger for the increase in MTLP concentration after the hypo-osmotic shock is purely branchial and not systemic. It is clear from the data reported in the present study that Ca^{2+} concentration in the environmental medium is an important factor triggering the gill MTLP synthesis, at least in posterior gills. In fact, an inverse relationship between Ca^{2+} concentration in the incubation medium and gill MTLP concentration was observed in the absence of an osmotic gradient through the isolated and perfused gill.

The higher levels of MTLP observed in gills incubated in a Ca^{2+}-free saline solution could also represent a protective response against a higher metal uptake rate in the absence of Ca^{2+} in the environmental medium. This statement is based on the fact that Ca^{2+} competes with other divalent metals for binding at physiologically active sites at gills, thus decreasing their transport and accumulation (Campbell, 1995; Wood et al., 1997; Paquin et al., 2000; Gensemer et al., 2002). For example, copper and cadmium could enter the gills and reach the hemolymph at least through one of the following mechanisms of calcium transport: Ca^{2+} channels, Na^+/Ca^{2+} exchanges or Ca^{2+} ATPase (Silvestre et al., 2004). Furthermore, a negative relationship between ambient Ca^{2+} concentration and gill Cd accumulation was also observed in mollusks (Roesijadi and Unger, 1993; Perceval et al., 2002). Taken together, these findings strongly suggest that the calcium concentration in the experimental medium is an important environmental signal to regulate MTLP synthesis, aiming to protect invertebrate gill cells against both oxidative damage and higher metal accumulation in low salinity conditions.

It has been reported that tissues directly involved in metal uptake, storage and excretion have a high capacity of MT synthesis (Amiard et al., 2006). In aquatic organisms, these proteins have been studied in the digestive gland (hepatopancreas) and gills of crustaceans (Canli et al., 1997; Pedersen et al., 1997; Legras et al., 2000; Mouneyrac et al., 2001; Brown et al., 2004; Silvestre et al., 2005). Generally, inter-organ differences in MT concentration are found. Results from the present study in the blue crab *C. sapidus* are in complete agreement with those previously reported for the crabs *Carcinus maenas* (Pedersen et al., 1997; Legras et al., 2000) and *Pachygrapsus marmoratus* (Legras et al., 2000; Mouneyrac et al., 2001). In all cases, a markedly higher MTLP concentration was observed in hepatopancreas than in gills. In fact, hepatopancreas has been described as the main organ involved in metal storage, accumulating more trace metals than gills (Pedersen et al., 1997; Legras et al., 2000).

As previously mentioned, MT is part of a core suite of biomarkers recognized and examined in the framework of biological effect quality assurance in monitoring programs (Mathiessen, 2000). However, if the MT measurement is to be incorporated into biomonitoring programs in estuarine environments, the salinity influence on MT concentration should be considered (Leung et al., 2002). According to results from the *in vivo* experiments performed in the present study, hepatopancreas MTLP are a more suitable biomarker for metal monitoring in estuarine and coastal areas than gill MTLP. This statement is based on the fact that hepatopancreas MTLP were refractive to salinity change and therefore will not confound metal–MTLP relationships useful for biomarking metal exposures. In fact, hepatopancreas MT was already suggested as a sensitive tool to detect metal contamination in crustaceans, such as copper in the shore crab *C. maenas* (Brown et al., 2004) and cadmium in the Norway lobster *Nephrops norvegicus* (Canli et al., 1997). However, previous information on possible influence of biotic factors and other abiotic factors than salinity on hepatopancreas MT synthesis is necessary for an adequate use this endpoint as a suitable biomarker in metal biomonitoring programs. For example, it was demonstrated that natural variability in hepatopancreas MT concentration associated with molt and reproductive cycles, season and sex can, in some cases, conceal the relationships with accumulated metal concentrations (Engel and Brouwer, 1993; Canli et al., 1997; Mouneyrac et al., 2001).

Another aspect to be considered is the possible incorporation of hepatopancreas MTLP in the context of current physiological/toxicity models being phased into use with water quality guidelines, such as the Biotic Ligand Model — BLM. Actually, the BLM reliability to establish water quality criteria for freshwaters (WQC) was recognized by the United States Environmental Protection Agency (USEPA, 2003). This model has incorporated the strong influence of abiotic factors on metal toxicity in freshwater species and has been calibrated considering the specific characteristics of the biotic ligand involved (Paquin et al., 2000; Paquin et al., 2002; Niyogi and Wood, 2004). Currently, many efforts are being made towards the extension of the freshwater BLM to saltwater conditions (Arnold et al., 2005, Grosell et al., 2007). It is important to

note that BLM is based on the premise that there is a strong correlation between metal concentration on or into the biotic ligand and its subsequent acute toxicity (Paquin et al., 2000; Paquin et al., 2002). Therefore, identification and characterization of major biotic ligands involved in metal accumulation and toxicity in marine animals is necessary to build a robust and reliable BLM version for marine conditions. In this context, it is important to consider that *C. sapidus* hepatopancreactic cells are able to take up copper and combine it with newly synthesized MT after either short-term exposure to dissolved copper or long-term exposure by means of copper-rich diets (Schlenk and Brouwer, 1993; Brouwer and Hoexum-Brouwer, 1998). Therefore, hepatopancreas MT would be a valuable parameter to be incorporated into the framework of a future BLM version for saltwater conditions.

In summary, results from the present study suggest that decreasing salinity induces increased levels of gill MTLP in the blue crab *C. sapidus*, likely as part of an adaptive response to hypo-osmotic stress. Furthermore, they suggest that the trigger for this response is purely branchial, being mediated by the environmental calcium concentration.

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