SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 9999, No. 9999, pp. 1–11, 2016

Printed in the USA

Environmental Toxicology

PHYSIOLOGICAL DIFFERENCES IN THE CRAB *UCIDES CORDATUS* FROM TWO POPULATIONS INHABITING MANGROVES WITH DIFFERENT LEVELS OF CADMIUM CONTAMINATION

Priscila Ortega,† Hector A. Vitorino,† Renata G. Moreira,† Marcelo A.A. Pinheiro,‡ Alaor A. Almeida,§ Márcio R. Custódio,† and Flávia P. Zanotto*†

†University of São Paulo, São Paulo, Brazil
‡Paulista State University, São Vicente, São Paulo, Brazil
§Paulista State University, Botucatu, São Paulo, Brazil

(Submitted 18 May 2016; Returned for Revision 14 June 2016; Accepted 20 June 2016)

Abstract: Crustaceans found in metal-contaminated regions are able to survive, and the authors investigated the physiological mechanisms involved by comparing populations from contaminated and noncontaminated areas. The objective of the present study was to measure the cellular transport of a nonessential metal (cadmium [Cd]) in gills and hepatopancreas of *Ucides cordatus*, together with cell membrane fluidity, metallothionein levels, and lipid peroxidation. The 2 populations compared were from a polluted and a nonpolluted mangrove area of São Paulo State, Brazil. The authors found, for the first time, larger Cd transport in gills and hepatopancreatic cells from crabs living in polluted mangrove areas. The cells also had lower plasma membrane fluidity, increased lipid peroxidation and less metallothionein compared to those from nonpolluted regions. The authors also found larger amounts of Cd in intracellular organelles of gills, but not in the hepatopancreas, from crabs in polluted regions. Therefore, in polluted areas, these animals showed higher Cd transport and lower plasma membrane fluidity and storage of Cd intracellularly in gill cells, whereas hepatopancreatic cells used metallothionein as their main line of defense. The findings suggest that crabs from polluted areas can accumulate Cd more easily than crabs from nonpolluted areas, probably because of an impairment of the regulatory mechanisms of cell membrane transport. *Environ Toxicol Chem* 2016;9999:1–11. © 2016 SETAC

Keywords: Cadmium Gill Hepatopancreas Membrane fluidity Cadmium transport

INTRODUCTION

Animals from estuarine and coastal regions are directly affected by local pollution through the disposal of chemicals and toxic metals, mainly from industries [1–3]. Cadmium (Cd), a major pollutant in mangrove areas, is a nonessential metal and is known to cause damage and to accumulate in animal tissues. In contact with crustaceans, it may cause physiological and morphological damage in gills and the hepatopancreas, organs that are the first entry sites in these organisms. Moreover, toxic metals are some of the strongest naturally occurring carbonic anhydrase inhibitors [4]. Metals such as silver ion (Ag^{+}), Cd (II) ion (Cd^{2+}), copper (II) ion (Cu^{2+}), and zinc ion (Zn^{2+}) are known to be inhibitory to carbonic anhydrase activity in vitro and to carbonic anhydrase–related physiological processes in vivo. For example, Ag^{+} is known to inhibit both gill carbonic anhydrase and ion transport in freshwater fish [4,5] as well as intestinal ion transport in marine fish [6]. Gill carbonic anhydrase from an estuarine crab (*Chasmagnathus granulata*) is highly sensitive to Cd^{2+}, with an inhibitory concentration of 2.15 μM [7]. Other studies showed that *Ucides cordatus* and *Callinectes danae* found in contaminated sites with toxic metals showed adaptive physiological changes, such as increased accumulation of toxic metals, reduced ionic hyporegulation, and increased levels of gill sodium–potassium adenosine triphosphatase (Na^{+}/K^{+}-ATPase) [1]. Other studies with crabs showed that Cd exposure similarly affected intracellular calcium (Ca) concentration [3,8]. Therefore, it is possible that toxic metal exposure in estuarine organisms can inhibit multiple ion transport processes both individually and through the inhibition of gill carbonic anhydrase. Furthermore, by compromising the osmoregulatory process, toxic metals could also exclude euryhaline species from the less saline regions of their natural habitat.

In addition, estuarine animals present strategies to signal the degree of pollution in a given location, which assist in their survival. One strategy is the presence of a metal binding protein, metallothionein [8–10]. These low–molecular weight proteins are extremely important to aquatic organisms, leading to increased tolerance to some toxic metals during exposure [11]. In general, metallothioneins play an important role in homeostasis and control of essential metals, mainly Cu and Zn [12,13]. Studies with mollusks in Cd-contaminated regions reveal an increase of metallothionein in the cytosol of gill cells, acting as an important stress marker [14]. Another stress marker of environmental pollution is lipid peroxidation, also associated with environmental excess of metals [15–18]. Several studies with vertebrates and invertebrates have shown that with rising pollution levels (particularly with respect to Cd), there is an increase in the antioxidant systems, including lipid peroxidation [19].

Environmental changes caused by human activities also interfere with biological membranes, transferring external signals to the internal milieu of cells [20]. In experiments with crabs (*Carcinus maenas*), mobilization of fatty acids from the hepatopancreas was a response to toxic metal contamination [21,22]. Long chain polyunsaturated fatty acids play a role in osmoregulation, nutrient assimilation, and ion transport.

* Address correspondence to fzanotto@usp.br
Published online 22 June 2016 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/etc.3537

Furthermore, changes in the proportion of monounsaturated fatty acids modulate protein activity. Reductions in some proteins and ionic channels are correlated with a reduction in polyunsaturated fatty acids and an increase in monounsaturated fatty acids, and both can influence the permeability of the cell membrane and Na^+/K^+-ATPase activity [21]. In another crab study, the breakdown of fatty acids was used as energy for detoxification and excretion of toxic metals [22].

Estuarine crab gills are usually divided into the anterior gill, which is a typical gas exchange epithelium made up of thin cells, and the posterior gill, which is essential for the transport of ions [23]. The hepatopancreas is characterized by 4 different cell types: embryonic cells, which originate the other cell types; resorptive and fibrillar cells, both important for absorption of nutrients; and blister cells, responsible for digestion and storage of nutrients [23,24]. In addition, these organs exchange ions and metals with the environment through Ca channels, exchangers, and Zn channels [23,25,26].

To further understand the physiological mechanisms that enable the survival of these organisms in highly stressful environments, it is essential to study populations from areas with different levels of pollution. Thus, the aim of the present study was to compare the physiological responses of a mangrove crab, *U. cordatus*, living in polluted and unpolluted mangroves with respect to Cd. For this, Cd cellular transport together with Cd localization in gills and hepatopancreas, composition of membrane phospholipids, and levels of metallothionein and lipid peroxidation were measured.

MATERIALS AND METHODS

Study area

The Itanhaém district (São Paulo state) is part of the Itanhaém estuary (24°10′14.76″S–46°48′2.96″W), which has a high degree of metal contamination. Landfills and dumps, especially, are considered high-risk sources that contribute to the contamination of estuarine systems [27]. Studies verified the presence of Cd in this estuarine system, mainly in the river Piaçaguera, which receives effluents from fertilizer industries in Cubatão. Also, they found high levels of Cd in sediments of that river, indicative of a possible source of this metal linked to local industries [27].

The Juréia-Itatins Ecological Station is located on the south coast of São Paulo (24°26′7.43″S, 47°4′46.12″W). It has an area of approximately 80 000 ha, covering land belonging to the districts of Itariri, Miracatu, Pedro de Toledo, Iguape, and Peruíbe. This is an important biodiversity [28] reserve of the Atlantic Forest still preserved and considered a conservation unit. Conservation units have been the best way to protect biodiversity because, with increasing population growth and reduction of natural habitats, they are one of a few natural places for most biota intolerant of human presence [28]. The conservation unit Juréia-Itatins Ecological Station was created in 2006. At the same time, the Juréia-Itatins Ecological Station was transformed into a protected area mosaic and the Barra do Una Sustainable Development Reserve [28].

Animals

The mangrove crab *U. cordatus* Linnaeus (Decapoda, Ucididae) was captured on March 2013 and 2014 at polluted and unpolluted areas. Water and sediment ($n = 3$ each) were collected from 3 subareas inside of *U. cordatus* burrows. Water was collected using a plastic hose and stored in polypropylene flasks properly labeled. In each subarea sediment samples of the superficial 10 cm were collected by hand with nitrile gloves and placed into labeled flasks. Animals were brought to the Laboratory of Physiology at the University of São Paulo, where they were acclimatized for 7 d in the vivarium. Crabs were kept in tanks filled with artificial brackish water (salinity 20 ppm), gravel, filtered water, and pieces of brick to provide an emersion area. The photoperiod (12:12-h light:dark) was constant, as was the temperature (22 ± 3 °C). Only intermolt males were used to avoid different metabolic influences. For each set of experiments, 4 animals were used from each mangrove area.

Field animals were collected in the same mangrove areas listed in the *Study area* section and brought to the University of São Paulo, and the tissues were collected immediately, without acclimatization. These were called "crabs from field." In a different group, animals were collected and brought to the university, where they remained in the vivarium for 7 d before the tissues and cells were collected. These animals were called "crabs lab held." After the acclimatization period, the animals from each mangrove area were cryo-anesthetized, and samples of the gills (anterior and posterior) and hepatopancreas were collected, placed into Eppendorf tubes, and frozen until analysis (–80 °C).

Cellular dissociation

After acclimatization for 7 d, gills (anterior and posterior) were submitted to cell dissociation [3] using an enzymatic method, where 10 mL of the extraction solution (sodium chloride [NaCl] 395 mM, potassium chloride 10 mM, sodium bicarbonate 2.5 mM, monosodium phosphate 2.5 mM, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane- sulfonic acid 3.75 mM, glucose 1 mM, and ethylenediamine tetraacetic acid [EDTA] 0.9 mM; pH buffered to 7.8) were mixed with 200 μL of trypsin [29] (0.05%). The gills were immersed in this solution and kept on ice (15 min), minced with scissors (15 min), filtered in thin mesh at 30-μm nylon mesh, put in Falcon tubes (15 mL), and centrifuged (10 min at 115 *g* at 5 °C). After centrifugation, the pellet was resuspended in fresh extraction solution and kept on ice.

The protocols for hepatopancreatic dissociation and other procedures [29] were as follows. The hepatopancreas was added to a beaker with 15 mL of extraction solution and stirred for 30 min, using a proportion of 2:1 (volume:tissue mass). Then, the solution was filtered in a mesh of 30 μm, centrifuged (5 min, 115 *g*), and stored for separation in the sucrose gradient. The final volume of cell suspension was 2 mL. The different cell types were collected using a sucrose gradient [29]. Sucrose concentrations of 10%, 20%, 30%, and 40% were dissolved in saline solution prepared before. Next, 2 mL of each sucrose concentration was layered carefully in 15-mL tubes. After this, the hepatopancreatic cell suspension was added to the top and centrifuged (115 *g* for 5 min). Next, the layers containing cells and sucrose were collected with a Pasteur pipette and kept on ice.

Cellular transport

Gill and hepatopancreatic cells previously dissociated were labeled with 1 μL of FluoZin-3 AM (Molecular Probes) for 1 h under shaking at 200 rpm and room temperature. This probe crosses the cellular membrane and becomes fluorescent (by esterification) in the presence of Cd. After, the cells were centrifuged (405 *g* for 5 min) and then washed in the extraction solution, without EDTA. The cell suspension was

distributed in enzyme-linked immunosorbent assay (ELISA) plates (180 μL/well, corresponding to 23×10^4 cells) in series with Cd chloride ($CdCl_2$) concentrations of 0.15 mM, 0.25 mM, 0.5 mM, 0.75 mM, and 1.0 mM. Cellular transport was analyzed in a fluorimeter (BioTek), with emission of 525 nm and excitation of 495 nm with measurements each 90 s in real time. A calibration curve was prepared to transform arbitrary fluorescence units into intracellular Cd^{2+} concentrations [30]. At the end of the experiment, 180 μL of cells not exposed to Cd were added to ELISA plates with 50 μL of Triton X-100 20% for 15 min. To obtain the minimum fluorescence, we added 50 μL of *N*-hydroxyethyl-ethylenediamine-triacetic acid (1 mM). For maximum fluorescence, we added 50 μL of Triton X-100 20% for 15 min and 50 μL of $CdCl_2$ (5 mM) in different batches of nonexposed cells. Transport of Cd was interpreted through the fluorescence increase that originated a hyperbolic curve that follows the Michaelis-Menten equation. Intracellular Cd for every experiment was calculated according to the equation

$$\left[Cd^{2+}\right]_i = K_d \times \left(\frac{F - F_{min}}{F_{max} - F}\right)$$

where $[Cd^{2+}]_i$ is the intracellular free Cd concentration (nanomoles), K_d is the dissociation constant of FluoZin (1.88), F is the actual fluorescence measured, F_{min} is the minimum fluorescence, and F_{max} is the maximum fluorescence [3].

Lipid and fatty acid analysis

Lipid and fatty acid analyses were performed in quadruplicate [31]. Extraction of the total lipid content from the gills and hepatopancreas was performed using a mixture of chloroform: methanol:water (2:1:0.5) [32,33]. Lipid extracts were separated into polar (phospholipids) and neutral (triglycerides) lipids via an activated silica column (Z. Yang, 1995, Master's thesis, University of Newfoundland, St. John's, Newfoundland, Canada). Methylation of the polar fraction was performed using acetyl chloride (5% hydrogen chloride [HCl] in methanol) [34], and fatty acid composition was determined from the methyl ester content via a Varian model 3900 gas chromatograph, coupled to a flame ionization detector. Fatty acids were identified by comparing retention times using known methyl ester standards (Supelco, 37 components [Sigma-Aldrich], and Mixture Me93, Qualmix polyunsaturated fatty acid fish M, and menhaden oil [Larodan]). Fatty acid methyl esters were analyzed using a capillary column (CP Wax 52 CB, 0.25 μm thickness, inner diameter 0.25 mm, and 30 m length). Hydrogen was used as the carrier gas at a linear velocity of 22 cm s^{-1}. The programmed temperature was as follows: 170 °C for 1 min, a 2.5 °C min^{-1} ramp to 240 °C, and then a final hold time of 5 min. The injector and flame ionization detector temperatures were 250 °C and 260 °C, respectively. At the end of the experiment, we have percentages of saturated fatty acids, which decrease the membrane fluidity; monounsaturated fatty acids, which increase the membrane fluidity; and polyunsaturated fatty acids, which increase even more the membrane fluidity.

Measurement of lipid peroxide with ferrous oxidation–xylenol

The standard assay for measuring lipid peroxidation in tissue extracts was performed as follows [15,35,36]. Frozen tissue samples were rapidly weighed and homogenized in 100% cold (5 °C) high-performance liquid chromatography–grade methanol (1:9 w:v). Homogenates were centrifuged (1000 *g*), and then supernatants were removed. For the standard assay, the following reagents were added sequentially in an ELISA plate: 0.25 mM iron(II) sulfate, 25 mM sulfuric acid, 0.1 mM xylenol orange, and water for a final volume of 0.9 mL. A sample of tissue extract (2–100 μL) was then added, and the final volume was adjusted to 1 mL with deionized water. Blanks were prepared by replacing tissue extracts with deionized water. Samples were incubated at room temperature until the reaction was complete (1 h), and absorbance was read at 580 nm. With tissue samples, a further addition was then made of 5 μL of 1 mM cumene hydroperoxide, and absorbance at 580 nm was read again. Levels of lipid hydroperoxide were expressed as cumene hydroperoxide equivalents.

Metallothionein assay

Metallothionein was measured as described [37] with modification. The gills and hepatopancreas of the crabs were rapidly removed and homogenized using a proportion 3:1 (volume:tissue mass) of 0.5 M sucrose, 20 mM tris-(hydroxymethyl) aminomethane (Tris)-HCl buffer (pH 8.6), containing 0.5 mM phenylmethylsulfonyl fluoride and 0.01% β-mercaptoethanol. Aliquots of the homogenate (20 μL) were stored at −80 °C. The homogenate was centrifuged (30 000 *g* for 20 min), followed by addition of 1.05 mL cold (−20 °C) absolute ethanol and 80 μL of chloroform per 1 mL of the resulting supernatant. Subsequently, the samples were centrifuged (6000 *g* for 10 min, at 4 °C). Supernatants were added to cold ethanol using the proportion 3:1 (volume:supernatants), stored at −20 °C for 1 h, and centrifuged in a swinging rotor (6000 *g* for 10 min). Pellets were washed with ethanol: chloroform:homogenization buffer (87:1:12), centrifuged again (6000 *g* for 10 min), and suspended in 300 μL of 5 mM Tris-HCl and 1 mM EDTA (pH 7). The concentration of metallothionein was estimated by the concentration of reduced glutathione (GSH) [37] and quantified in a spectrophotometer using Ellman's reagent (2 M NaCl, 5,5′-dithio-bis-[2-nitrobenzoic acid] in 0.43 M and 0.2 M phosphate buffer, pH 8.0) at 412 nm.

Cd location in gills and hepatopancreatic cells

Glass microscope slides were previously immersed in an aquarium for 24 h to allow the formation of a biofilm and improve cell adhesion. Subsequently, slides were washed in running water and placed in an oven to dry for 24 h. Cells from gills and hepatopancreas were separated and labeled with FluoZin-3 AM as described in *Cellular transport*, placed on slides with the aid of a Pasteur pipette, and covered with glass coverslips. When the probe crossed the cellular membrane, it became fluorescent (by esterification) in the presence of Cd [38]. The preparation was incubated with 1 mM $CdCl_2$ solution and observed with a fluorescence microscope (Zeiss) with excitation at 495 nm and emission at 525 nm. Photographs were taken immediately before and after incubation with $CdCl_2$, by checking the fluorescence change in each organ studied, over 5 min. Variation of the fluorescence was calculated through analysis of each photograph by the program Image J, by comparing the difference between the diffuse fluorescence that occurred in the cytoplasm and the punctual fluorescence that was observed in organelles. The photographs with clear images are shown in Figures 1 and 2.

Statistical analysis

For the tests performed, we used parametric analysis through analysis of variance (ANOVA), testing for normality and equality of variance. For the metallothionein and lipid

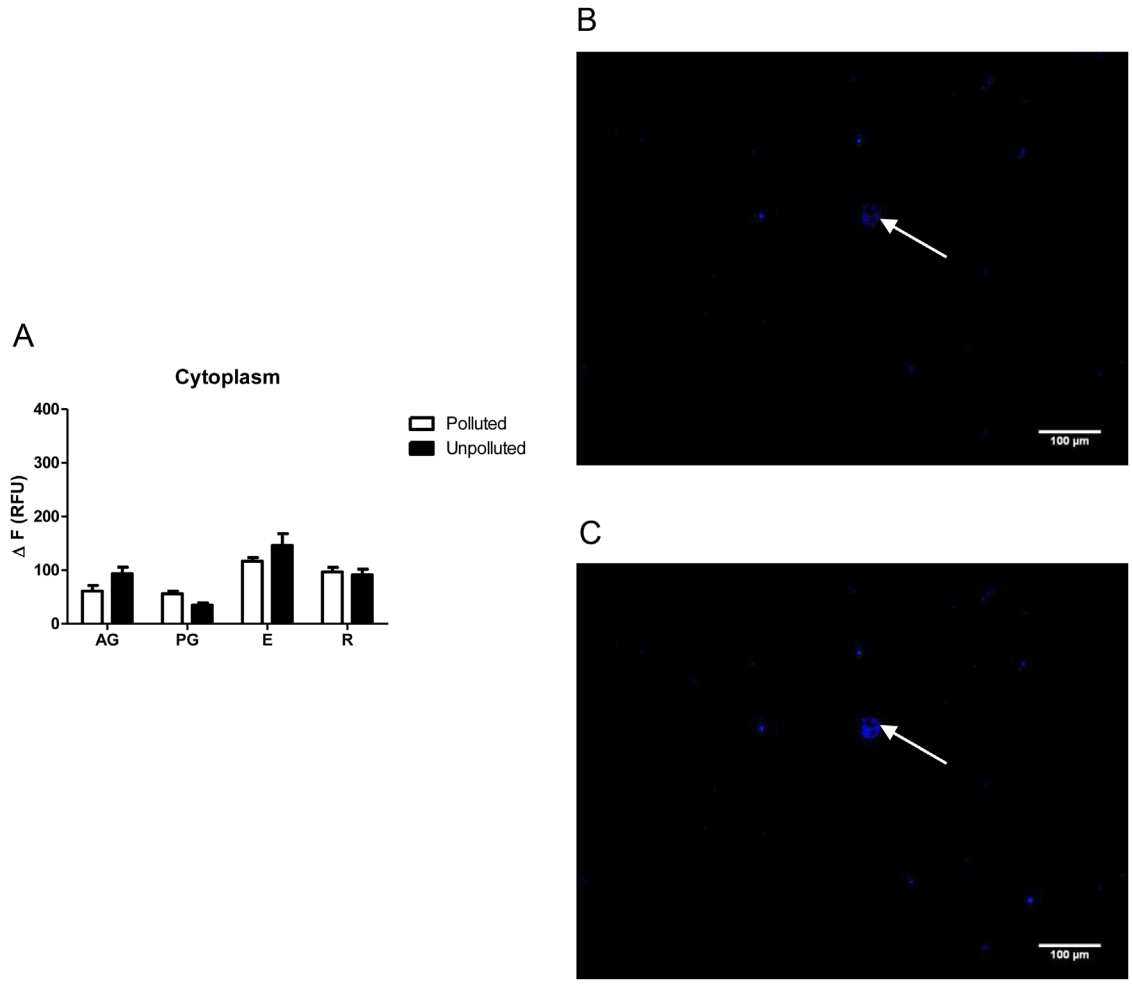


Figure 1. Fluorescence variation in the cytoplasm in anterior gills, posterior gills, and hepatopancreatic embryonic and resorptive cells of animals from polluted (Itanhaém) and unpolluted (Juréia) areas (**A**). Images show embryonic cells labeled with FluoZin 3 AM before (**B**) and after (**C**) addition of cadmium (1 mM). Arrows show the fluorescence variation in the cytoplasm of embryonic cells before (**B**) and after (**C**) addition of cadmium (1 mM). Mean ± standard error ($n = 4$). *Significant differences between areas ($p = 0.003$). AG = anterior gills; E = embryonic cells; F = fluorescence; PG = posterior gills; R = resorptive cells; RFU = relative fluorescence unit.

peroxidation analyses, we used the Tukey post hoc test. In the experiment with phospholipids, we separated crabs from field and crabs lab held to compare some physiological differences that could appear during the acclimatization processes, using ANOVA. Analyses were performed using Sigma Stat® for Windows® Ver 3:10, with a significance level of 0.05.

RESULTS

The concentration of Cd was measured in environmental matrices (water and sediment) from polluted and unpolluted mangrove areas, as described in *Materials and Methods*. In sediment samples, we observed the presence of Cd in contaminated areas. The sediment had 0.50 ± 0.02 μg/g of Cd, whereas for all the other samples, including water samples, the concentrations were below the detection limit.

A significant fluorescence signal associated with higher Cd transport (Figure 3) was observed only in the posterior gills of animals from polluted areas compared to those from unpolluted areas (ANOVA, $p = 0.019$ for maximum velocity and $p = 0.035$ for Michaelis constant). For hepatopancreatic cells (Figure 4), resorptive, fibrillar, and embryonic cells of animals from polluted areas showed a significant fluorescence increase and higher transport of Cd (ANOVA $p = 0.002$, $p = 0.008$, and $p = 0.0001$, respectively) compared to those from unpolluted areas. Blister cells, however, showed the same Cd transport between polluted and unpolluted areas.

In gill cells, only monounsaturated fatty acids of anterior gills from crabs from field showed higher values compared to animals from unpolluted areas (Figure 5; ANOVA, $p = 0.009$). The posterior gills of crabs from field from polluted areas showed a decrease of omega-6 compared to crabs from field from unpolluted areas (Figure 6; ANOVA $p = 0.018$). And anterior and posterior gills of crabs from field of polluted areas showed a decrease in the sum of C20-22 n6 (long chains of fatty acids; ANOVA $p = 0.023$ and $p = 0.018$). In crabs lab held, the anterior gills of animals of polluted areas showed an increase in the sum of C18 n6 fatty acids (long chains) compared with animals from unpolluted mangrove areas (Figure 7; ANOVA $p = 0.03$).

In the hepatopancreas, the percentages of saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids in phospholipids from crabs from field and crabs lab held belonging to polluted and unpolluted mangrove areas are presented in Figure 8. Crabs from field of polluted areas showed an increase in monounsaturated fatty acid (ANOVA $p = 0.004$)

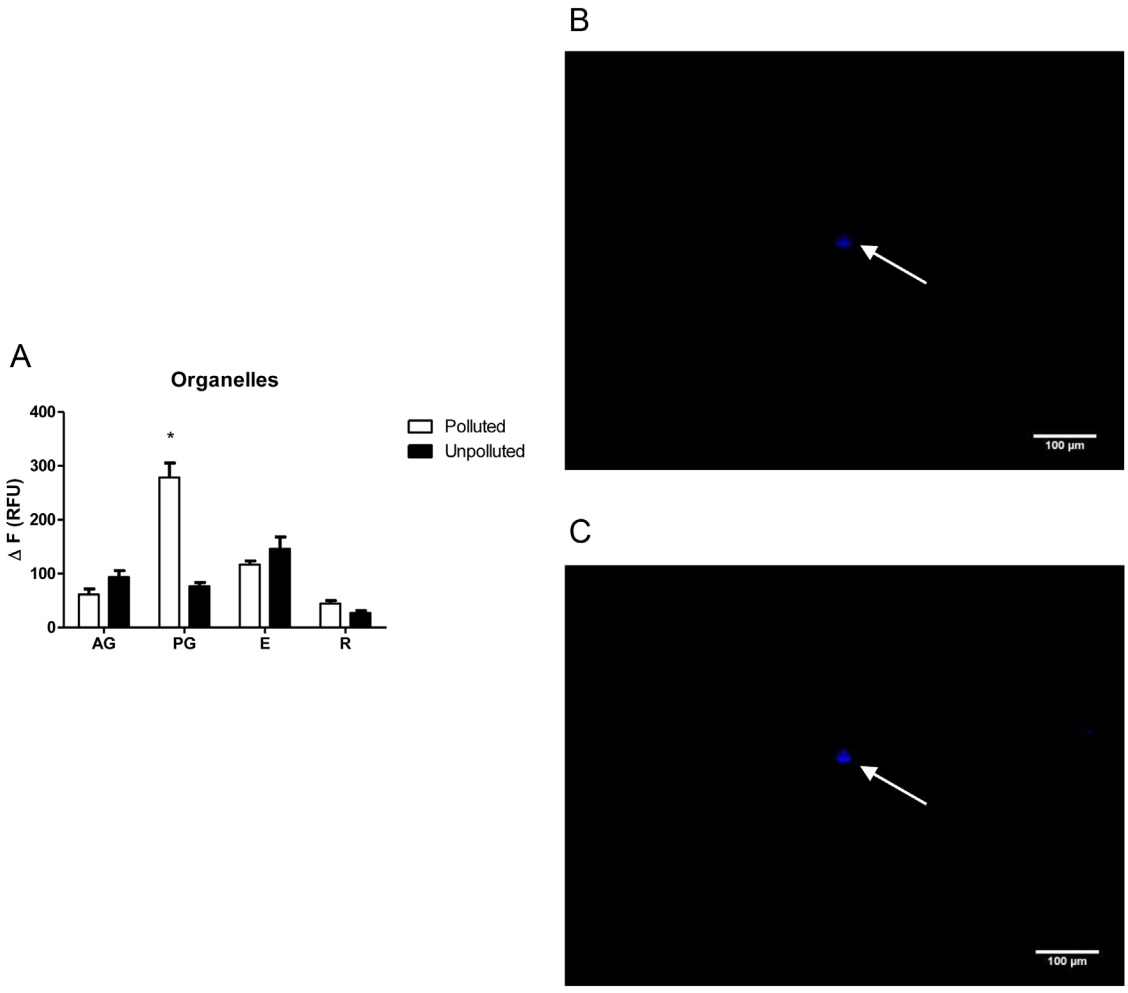
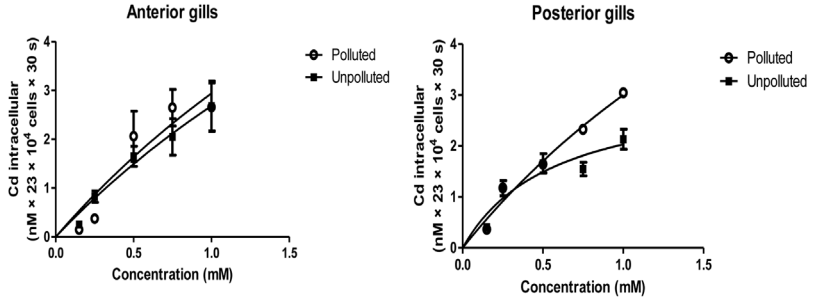


Figure 2. Fluorescence variation in the organelles in anterior gills, posterior gills, and hepatopancreatic embryonic and resorptive cells of animals from polluted (Itanhaém) and unpolluted (Juréia) areas (**A**). Images show posterior gill cells labeled with FluoZin 3 AM before (**B**) and after (**C**) addition of cadmium (1 mM). Arrows show the fluorescence variation in the organelles of posterior gill cells before (**B**) and after (**C**) addition of cadmium (1 mM). Mean ± standard error ($n = 4$). *Significant differences between areas ($p = 0.003$). AG = anterior gills; E = embryonic cells; F = fluorescence; PG = posterior gills; R = resorptive cells; RFU = relative fluorescence unit.

and a decrease in polyunsaturated fatty acid (C18 n3, short chain of fatty acids; ANOVA $p = 0.022$) and n3 (omega 3; ANOVA $p = 0.001$) compared to animals from unpolluted areas. In crabs lab held, crabs from polluted areas presented a decrease in C18 n3 (short chain) in the hepatopancreas compared with animals from unpolluted regions (ANOVA $p = 0.014$).

Higher lipid peroxidation levels were detected in anterior gills from polluted areas compared with unpolluted ones



Region	Km	Vmax
Polluted (Itanhaém)	3.907	14.42
Unpolluted (Juréia)	3.910	13.16

Region	Km	Vmax
Polluted (Itanhaém)	3.177 *	12.53 *
Unpolluted (Juréia)	0.5684	3.185

$p < 0.05$

Figure 3. Cadmium transport from anterior and posterior gills from polluted and unpolluted areas (Itanhaém and Juréia, respectively; $p = 0.019$ for maximum velocity and $p = 0.035$ for Michaelis constant). Mean ± standard error ($n = 3$) for the total experiment. Cd = cadmium; K_m = Michaelis constant; Vmax = maximum velocity.

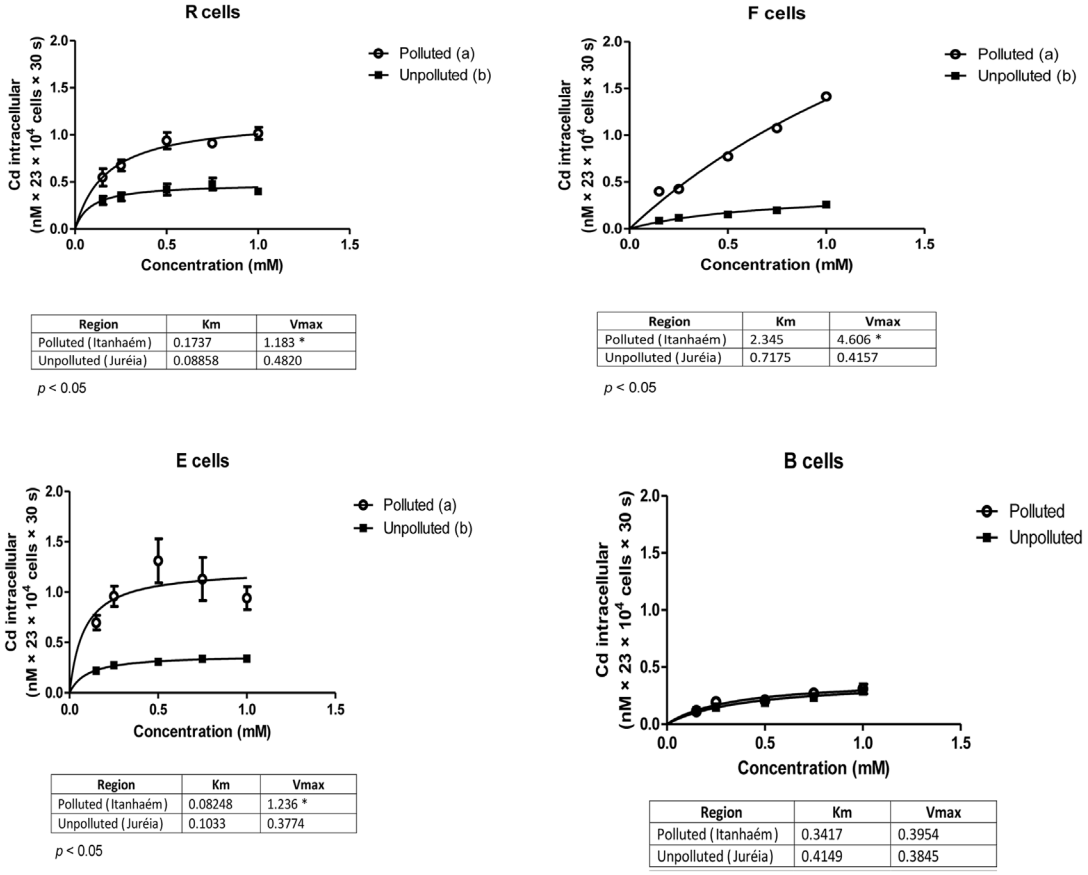


Figure 4. Cadmium transport from hepatopancreatic cells (resorptive, fibrillar, embryonic, and blister cells) from polluted and unpolluted areas (Itanhaém and Juréia, respectively). Mean ± standard error ($n = 3$). *Significant difference in Michaelis constant or maximum velocity among groups. Different letters mean significant differences among groups ($p = 0.002$, $p = 0.008$, and $p = 0.0001$, respectively). B = blister; Cd = cadmium; E = embryonic; F = fibrillar; K_m = Michaelis constant; R = resorptive; Vmax = maximum velocity.

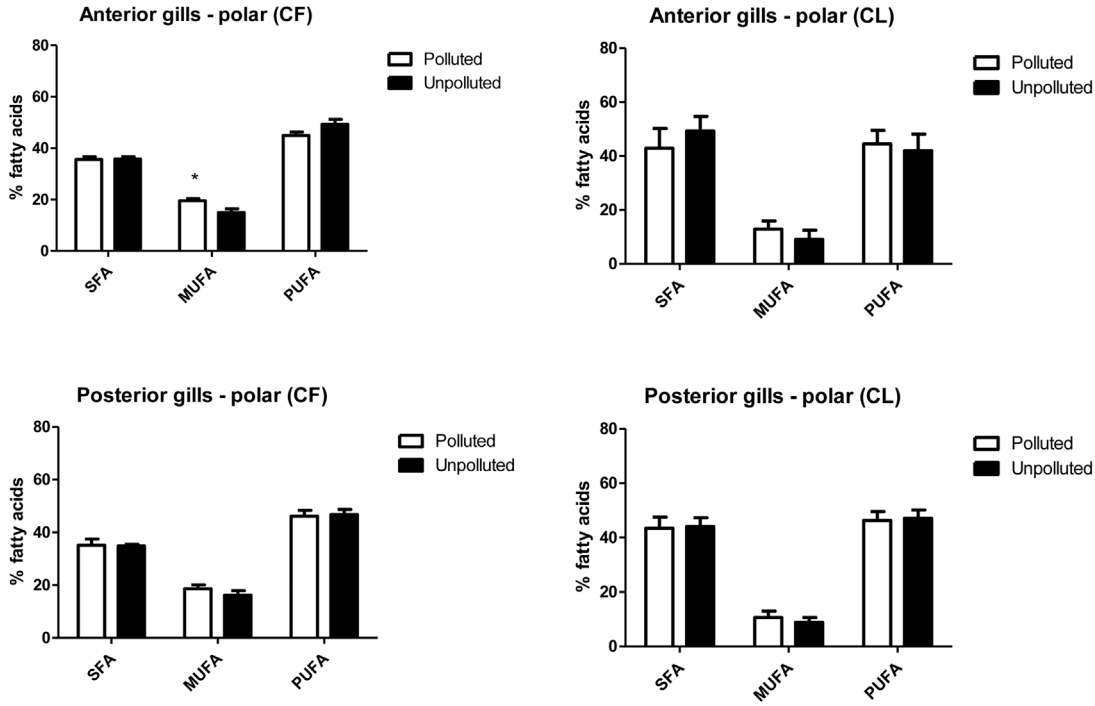


Figure 5. Percentage of fatty acids from phospholipids: saturated, monounsaturated, and polyunsaturated in anterior and posterior gills of crabs from field and crabs lab held from polluted and unpolluted mangrove areas (Itanhaém and Juréia, respectively). Mean ± standard error ($n = 4$). *Significant difference between areas ($p = 0.009$). CF = crabs from field; CL = crabs lab held; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; SFA = saturated fatty acid.

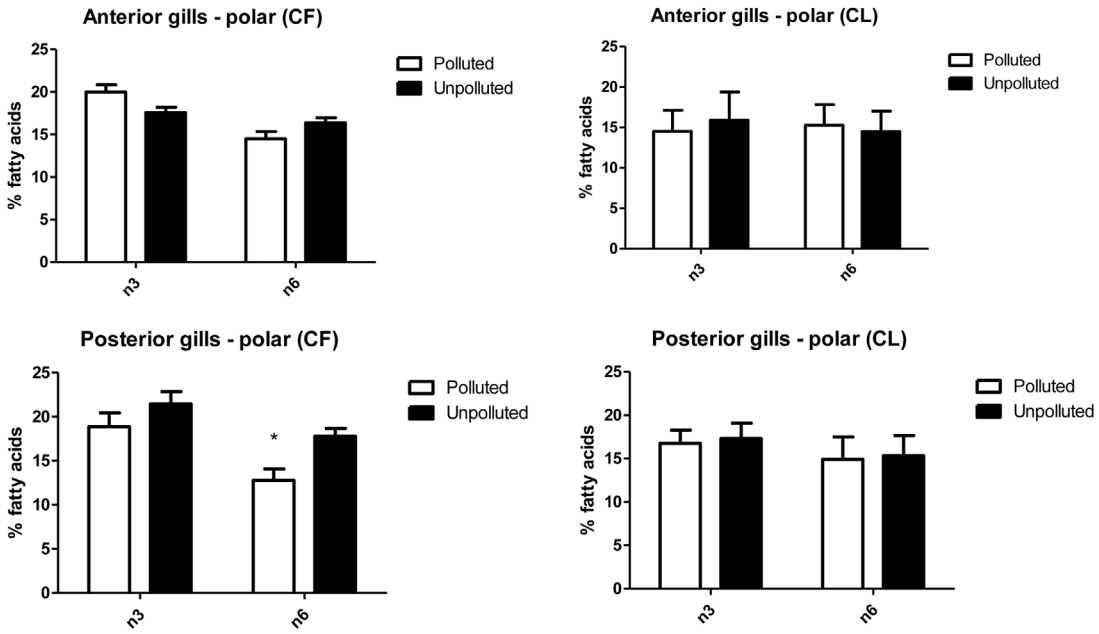


Figure 6. Percentage of omega 3 (n3) and omega 6 (n6) fatty acids from phospholipids in anterior and posterior gills of crabs from field and crab lab held from polluted and unpolluted mangrove areas (Itanhaém and Juréia, respectively). Mean ± standard error ($n = 4$). *Significant difference between areas ($p = 0.018$). CF = crabs from field; CL = crabs lab held.

(Figure 9). In addition, posterior gills showed the highest lipid peroxidation in polluted compared with unpolluted areas. The same was found for the hepatopancreas (ANOVA $p = 0.0001$ for anterior and posterior gills and hepatopancreas).

The highest concentration of metallothioneins in relation to GSH (Figure 10) was detected in the hepatopancreas compared with gills (0.24 ± 0.05 μmol GSH μg protein^{-1} against 0.13 ± 0.01 μmol GSH μg protein^{-1}; ANOVA $p = 0.0001$). There was also a significant difference between crabs from unpolluted and polluted mangrove areas for both tissues ($p = 0.007$ for anterior gills, $p = 0.022$ for posterior gills, and $p = 0.007$ for hepatopancreas). When we compared the

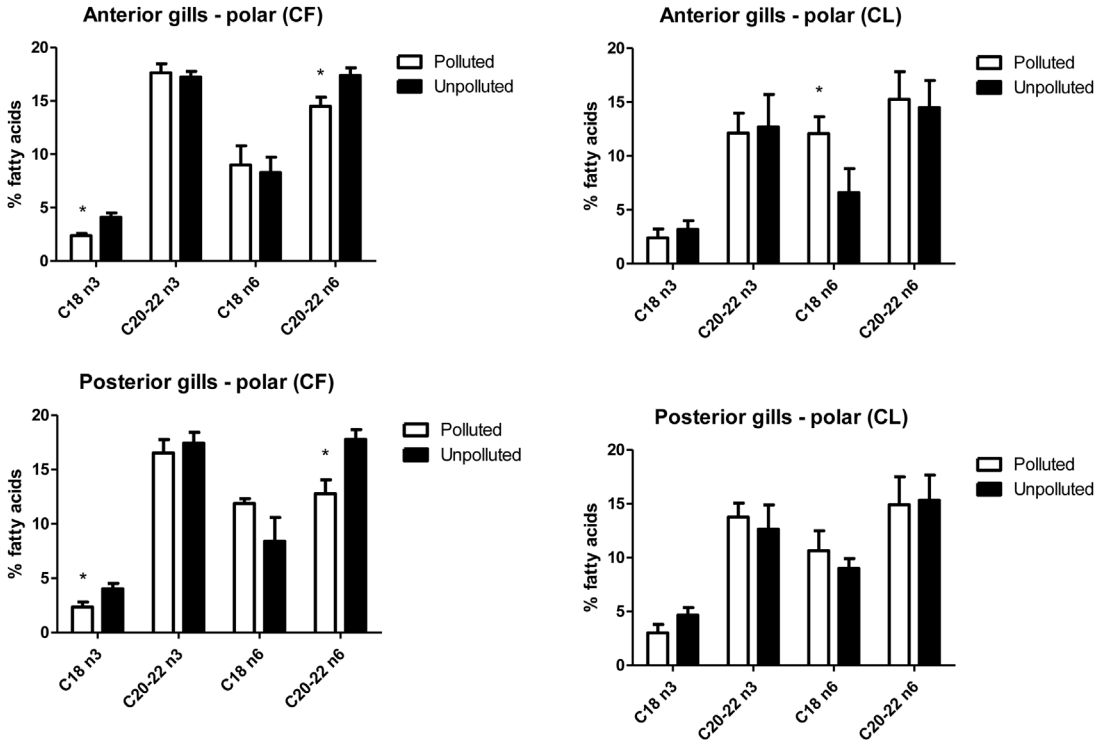


Figure 7. Percentage of fatty acids of phospholipids in anterior and posterior gills of crabs from field and crabs lab held from polluted and unpolluted mangrove areas (Itanhaém and Juréia, respectively). Mean ± standard error ($n = 4$). *Significant difference between areas ($p = 0.023$, $p = 0.018$, and $p = 0.03$, respectively). C18n3 = sum of C18 n3 fatty acids; C20-22n3 = sum of C20 and C22 n3 fatty acids; C18n6 = sum of C18 n6 fatty acids; C20-22n6 = sum of C20 and C22 n6 fatty acids; CF = crabs from field; CL = crabs lab held.

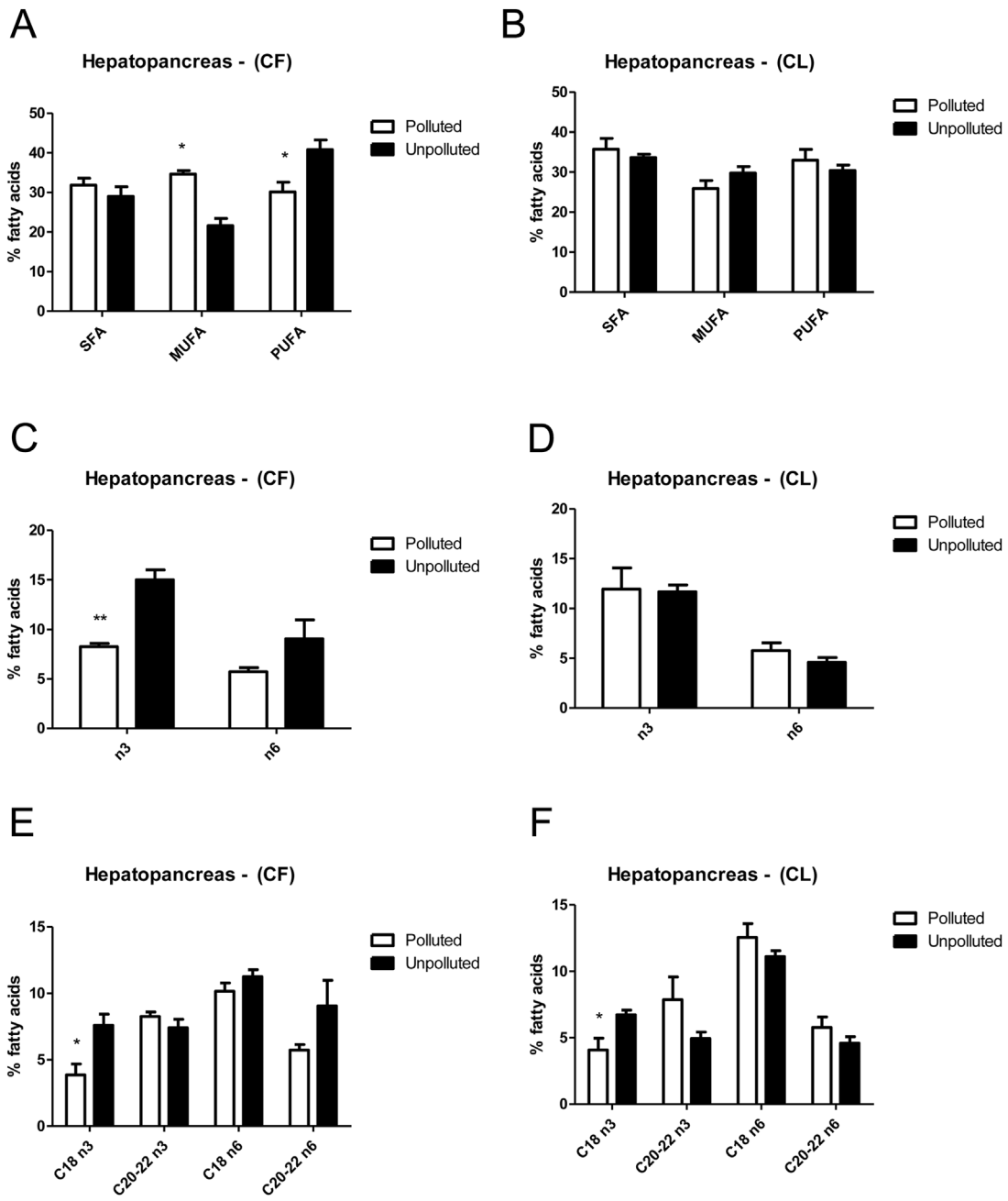


Figure 8. Percentage of fatty acids from polar fraction of hepatopancreatic phospholipids: (**A**,**B**) saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids; (**C**,**D**) omega 3 (n3) and omega 6 (n6) polyunsaturated fatty acids; (**E**,**F**) C18n3, C20-22n3, C18n6, and C20-22n6. Crabs from field and crab lab held from polluted and unpolluted mangrove areas (Itanhaém and Juréia, respectively). Mean ± standard error ($n = 4$). *Significant difference between areas ($p = 0.004$, $p = 0.022$, $p = 0.001$, and $p = 0.014$, respectively). C18n3 = sum of C18 n3 fatty acids; C20-22n3 = sum of C20 and C22 n3 fatty acids; C18n6 = sum of C18 n6 fatty acids; C20-22n6 = sum of C20 and C22 n6 fatty acids; CF = crabs from field; CL = crabs lab held; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; SFA = saturated fatty acid.

variation of fluorescence in the cytoplasm and organelles before and after incubation with 1 mM of $CdCl_2$, we detected higher fluorescence in organelles of the posterior gills of animals from polluted mangrove areas than those from unpolluted ones (Figures 1 and 2; ANOVA $p = 0.003$).

DISCUSSION

Organisms are known to display physiological and biochemical mechanisms of tolerance to environmental chronic contamination [1,39,40]. Therefore, our first approach to study populations of *U. cordatus* from polluted and unpolluted environments was the measurement of Cd transport in gills and the hepatopancreas. Surprisingly, in animals from polluted regions, the cells of the posterior gills transported and accumulated more intracellular Cd than the same cells found in crabs from unpolluted areas. Similarly, the same occurs in the embryonic, resorptive, and fibrillar cells from the hepatopancreas. The gill epithelia of crustaceans is particularly rich in Na^+/K^+-ATPase, which is a key enzyme that provides energy for ionic transport processes. In studies with *Callinectes danae*, increased activity of this enzyme was

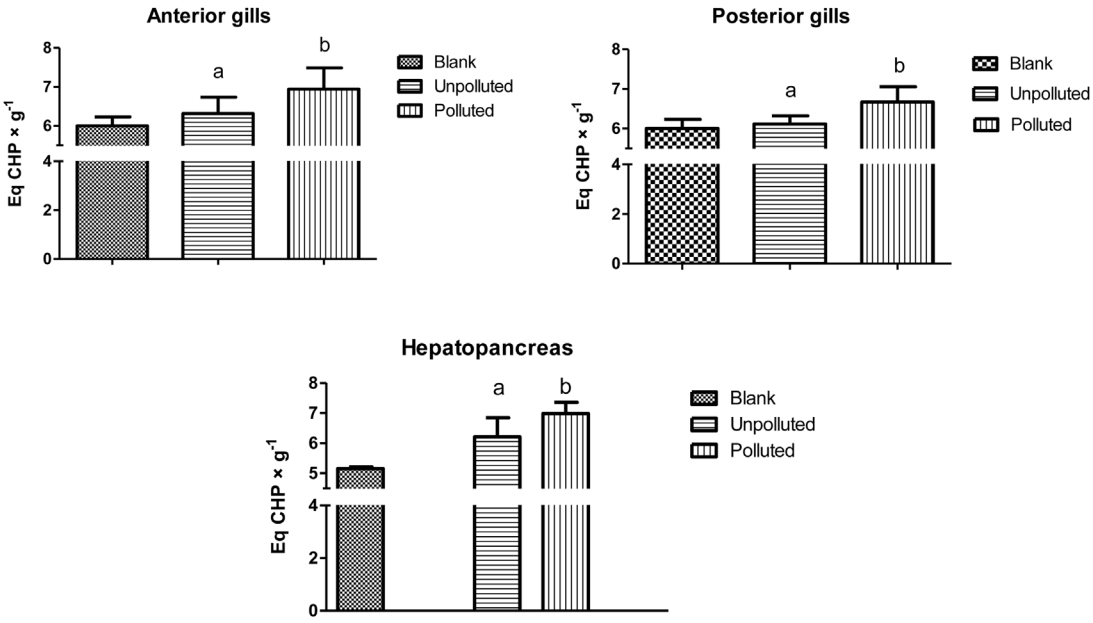


Figure 9. Lipid peroxidation in gills (anterior and posterior) and hepatopancreas of crabs from polluted and unpolluted mangrove areas (Itanhaém and Juréia, respectively). Mean ± standard error ($n = 4$). Different letters mean significant differences among groups ($p = 0.0001$ for anterior and posterior gills and for hepatopancreas). CHP = cumene hydroperoxide.

found in gills of animals from polluted areas compared with nonpolluted areas [1]. In studies with *Eriocheir sinensis* submitted to acute and chronic Cd exposure, there was a decrease of the Na^+/K^+-ATPase activity during acute exposure and no effect in chronic exposure [41,42]. In fish, an increase of Na^+/K^+-ATPase was confirmed when some species were exposed chronically to Cu (e.g., trout, *Oncorhynchus mykiss*) [43] or in chronically contaminated environments (e.g., black fin, *Goodea atripinnis*) [44].

Our results suggest that a greater transport of Cd in posterior gills could be linked to an increased activity of Na^+/K^+-ATPase in animals from polluted areas, favoring a higher Cd entry. Other studies [45] reveal an altered Cd^{2+} influx by changing the membrane potential across the gills, caused by the addition of Na^+-free solution in perfused gills of *C. maenas*. In a study with *U. cordatus* [3], ouabain, an Na^+/K^+-ATPase inhibitor, affected Cd transport in the anterior and posterior gills. According to these authors, a change in Na^+ equilibrium could alter the exchanger Cd^{2+}/Ca^{2+} (Na^+) and increase the influx of Cd^{2+}. Therefore, in regions highly polluted with Cd, an increase of Cd^{2+} influx could be linked to a change in cell membrane potential and the activity of Na^+/K^+-ATPase.

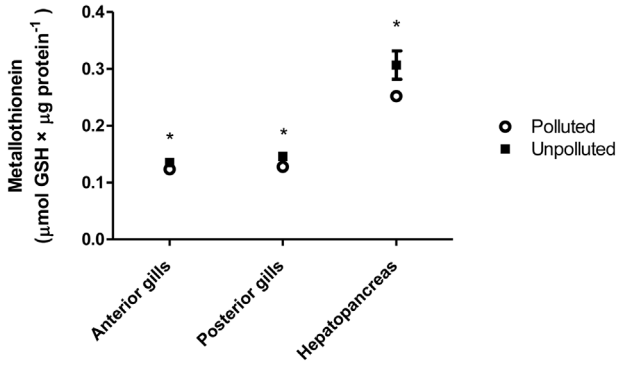


Figure 10. Concentration levels of metallothionein for hepatopancreas and gill tissues from animals collected from polluted and unpolluted regions. *Significant differences between polluted and unpolluted regions in each tissue. GSH = reduced glutathione.

After this, we investigated how Cd accumulates in gill and hepatopancreatic cells intracellularly. When the Cd probe crosses the cellular membrane, the cell becomes fluorescent (by esterification) in the presence of Cd [38]. We verified the diffuse fluorescence variation (cytoplasm) and punctual fluorescence variation (organelles or vacuoles) in each photograph, comparing control (without Cd) and experimental (Cd-incubated), using Image J. Cadmium accumulation occurred mainly in organelles, vacuoles, and granules of posterior gill cells. In general, metal detoxification in cells is confirmed by metallothionein presence in cytoplasm. Alternatively, in studies using X-ray microprobe analysis, toxic metals can also be stored in vacuoles of different types: A (phosphate and Zn), B (sulfur groups, binding Cd, Cu, mercury, and Ag), C (iron through ferritin groups), and D, which is extracellular and filled with Ca concretions [46]. In addition, these authors noted that metals could accumulate in lysosomes, endoplasmic reticulum, and/or mitochondria, before elimination through the feces. Thus, one may suggest that the organelles and vacuoles present in posterior gills have a significant role in the detoxification of toxic metals such as Cd, suggesting that the metal could have accumulated in these structures. Alternatively, vacuoles and/or granules apparently did not accumulate Cd in hepatopancreatic cells, as observed in the present study. This fact suggests the importance of metallothionein in the hepatopancreas cytoplasm as the main site for detoxification of toxic metals, unlike gill cells [13,47]. Interestingly, the metal handling strategy for *Anodonta woodiana*, a bivalve found in contaminated sites, was different for each organ: Cd was found mainly in the cytoplasm, bound to metallothioneins in the digestive gland, whereas for gills the metal was found in granules [13], similar to our results.

Environmental contamination may also influence biological membranes, especially in organs having direct contact with the

environment [20]. With regard to gills, a higher percentage of monounsaturated fatty acid was found in anterior gills of crabs from polluted areas compared with nonpolluted ones. According to the literature, monounsaturated fatty acids can be used as an energy source for physiological repair processes [20,48]. In vertebrates, studies show the action of an enzyme, stearoyl-coenzyme A Δ9 desaturase, which adds an unsaturation between carbons 9 and 10, converting them into monounsaturated forms [49]. A hypothesis for the higher presence of monounsaturated fatty acids in the anterior gills of *U. cordatus* from contaminated regions would be the presence of a similar desaturase enzyme to provide more energy for physiological processes such as respiration, which is carried out mainly in the anterior gills of this species [29,50]. For crabs from field from polluted areas, our results showed a decrease in the long chain fatty acids C18 n3 and C20-22 n6 in anterior and posterior gills. This fact contributes to an increased membrane fluidity [51–53], important for osmoregulatory mechanisms. In addition, there was lower polyunsaturated fatty acid in the plasma membrane of hepatopancreatic cells of crabs from polluted areas, which decrease membrane fluidity, a possible physiological mechanism to decrease the impact caused by environmental pollution.

An increase in lipid peroxidation was observed in hepatopancreas and gills, especially in animals from polluted regions. In gills, there is a specific region for cellular respiration, usually with higher oxygen concentration, that can produce free radicals and therefore increase the lipid peroxidation process [17,54]. The hepatopancreas, in turn, is an organ for detoxification. This process occurs to decrease the concentration of toxic metals. With the accumulation of metals, there is a stimulus for the formation of hydroxyl radicals, which could cause oxidative stress by their reaction with macromolecules, ultimately leading to cell damage, such as apoptosis and lipid peroxidation [54]. Therefore, an increase in lipid peroxidation in gills and hepatopancreas leads to a decrease in polyunsaturated fatty acid (C18 n3 and C20-22 n6) and an increase in monounsaturated fatty acid.

We found larger amounts of metallothionein in hepatopancreas and gills of animals from unpolluted regions. Comparison between crabs from field and crabs lab held did not show a statistically significant difference. It is possible that the acclimatization process did not affect the metallothionein levels. Studies with invertebrates showed that metallothionein has an important role in detoxification as a fast process when animals are exposed to metals and is also critical in cellular homeostasis [13,47]. The induction of metallothionein depends not only on high levels of metals but also on which metal is present in the environment, changes in salinity, the species involved, and their physiological processes. Thus, animals from polluted environments may exhibit low levels of metallothionein, as well as an inverse pattern [13,47]. For example, in oysters translocated from an unpolluted region to a polluted one [55], the researchers verified lower levels of metallothionein than in specimens from more contaminated areas [13]. One hypothesis would be that animals from polluted regions could present physiological modifications that permit their survival in a contaminated environment and that an increase in the production of metallothionein would be unnecessary. Also, there are tissue-related variations in metallothionein concentrations that reflect their physiological role in metal handling strategies. In addition, the influence of contamination factors limits the possibility of using metallothionein concentration as a biomarker of metal exposure [13].

CONCLUSIONS

It was observed for the first time that crabs living in polluted environments had higher gill and hepatopancreatic Cd transport. Also, they displayed increased monounsaturated fatty acid, decreased polyunsaturated fatty acid, and higher lipid peroxidation in both gills and hepatopancreas. Animals from nonpolluted environments, on the other hand, had a higher percentage of polyunsaturated fatty acid, together with greater amounts of metallothionein. These data corroborate the hypothesis that crabs from polluted environments have different physiological mechanisms to deal with chronic metal exposure: accumulating more metal intracellularly and increasing lipid oxidative stress. The detoxification mechanisms for Cd differed between organs: the gills accumulated Cd in intracellular organelles, whereas for the hepatopancreas, Cd binds to metallothionein present in the cell cytoplasm.

Acknowledgment—We thank the laboratory technician V. Alberto from the University of São Paulo for lipid methodology support and M. Hermes-Lima from the University of Brasilia for the lipid peroxidation methodology. The present study was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (to P. Ortega) and a FAPESP grant (2009/15546-3, to F.P. Zanotto).

Data availability—Data, associated metadata, and calculation tools are available from P. Ortega (priortega218@gmail.com).

REFERENCES

1. Harris RR, Santos MCF. 2000. Heavy metal contamination and physiological variability in the Brazilian mangrove crabs *Ucides cordatus* and *Callinectes danae* (Crustacea: Decapoda). *Mar Biol* 137: 691–703.
2. Rotter S, Sans-Piché F, Streck G, Altenburger R, Schmitt-Jansen M. 2011. Active bio-monitoring of contamination in aquatic systems: An in situ translocation experiment applying the PICT concept. *Aquat Toxicol* 101:228–236.
3. Ortega P, Custódio MR, Zanotto FP. 2014. Characterization of cadmium plasma membrane transport in gills of a mangrove crab *Ucides cordatus*. *Aquat Toxicol* 157:21–29.
4. Christensen GM, Tucker JH. 1976. Effects of sellected water toxicants on the in vitro activity of fish carbonic anhydrase. *Chem Biol Interact* 13:181–192.
5. Morgan IJ, Henry RP, Wood CM. 1997. The mechanism of acute silver nitrate toxicity in freshwater rainbow trout (*Oncorhynchus mykiss*) is inhibition of gill Na^+ and Cl^- transport. *Aquat Toxicol* 38:145–163.
6. Grosell M, De Boeck G, Johannsson O, Wood CM. 1999. The effects of silver on intestinal ion and acid-base regulation in the marine teleost fish, *Parophrys vetulus*. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* 124:259–270.
7. Vitale AM, Monserrat JM, Castilho P, Rodriguez EM. 1999. Inhibitory effects of cadmium on carbonic anhydrase activity and ionic regulation of the estuarine crab *Chasmagnathus granulata* (Decapoda, Grapsidae). *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* 122:121–129.
8. Bjerregaard P, Bjørn L, Nørum U, Pedersen KL. 2005. Cadmium in the shore crab *Carcinus maenas*: Seasonal variation in cadmium content and uptake and elimination of cadmium after administration via food. *Aquat Toxicol* 72:5–15.
9. Mouneyrac C, Amiard-Triquet C, Amiard JC, Rainbow PS. 2001. Comparison of metallothionein concentrations and tissue distribution of trace metals in crabs (*Pachygrapsus marmoratus*) from a metal-rich estuary, in and out of the reproductive season. *Comp Biochem Physiol C Toxicol Pharmacol* 129:193–209.
10. Gao A, Wang L, Yuan H. 2012. Expression of metallothionein cDNA in a freshwater crab, *Sinopotamon yangtsekiense*, exposed to cadmium. *Exp Toxicol Pathol* 64:253–258.
11. Ma W, Wang L, He Y, Yan Y. 2009. Effect of 1-butyl-3-methylimidazolium tetrafluoroborate on the wheat (*Triticum aestivum* L.) seedlings. *Environ Toxicol* 24:296–303.
12. Silvestre F, Duchêne C, Trausch G, Devos P. 2005. Tissue-specific cadmium accumulation and metallothionein-like protein levels during acclimation process in the Chinese crab *Eriocheir sinensis*. *Comp Biochem Physiol C Toxicol Pharmacol* 140:39–45.

13. Amiard JC, Amiard-Triquet C, Barka S, Pellerin J, Rainbow PS. 2006. Metallothioneins in aquatic invertebrates: Their role in metal detoxification and their use as biomarkers. *Aquat Toxicol* 76:160–202.
14. Giguère A, Couillard Y, Campbell PGC, Perceval O, Hare L, Pinel-Alloul B, Pellerin J. 2003. Steady-state distribution of metals among metallothionein and other cytosolic ligands and links to cytotoxicity in bivalves living along a polymetallic gradient. *Aquat Toxicol* 64:185–200.
15. Hermes-Lima M, Willmore WG, Storey KB. 1995. Quantification of lipid peroxidation in tissue extracts based on Fe(III) xylenol orange complex formation. *Free Radic Biol Med* 19:271–280.
16. Monserrat JM, Geracitano LA, Pinho GLL, Vinagre TM, Faleiros M, Alciati JC, Bianchini A. 2003. Determination of lipid peroxides in invertebrates tissues using the Fe(III) xylenol orange complex formation. *Arch Environ Contam Toxicol* 45:177–183.
17. Wang J, Zhang P, Shen Q, Wang Q, Liu D, Li J, Wang L. 2013. The effects of cadmium exposure on the oxidative state and cell death in the gill of freshwater crab *Sinopotamon henanense*. *PLoS One* 8:1–9.
18. Vitorino HA, Mantovanelli L, Zanotto FP. 2015. Iron metallodrugs: Stability, redox activity and toxicity against *Artemia salina*. *PLoS One* 10:1–11.
19. Xuan R, Wang L, Sun M, Ren G, Jiang M. 2011. Effects of cadmium on carbohydrate and protein metabolisms in the freshwater crab *Sinopotamon yangtsekiense*. *Comp Biochem Physiol C Toxicol Pharmacol* 154:268–274.
20. Vigh L, Los DA, Horváth I, Murata N. 1993. The primary signal in the biological perception of temperature: Pd-catalyzed hydrogenation of membrane lipids stimulated the expression of the desA gene in *Synechocystis* PCC6803. *Proc Natl Acad Sci USA* 90:9090–9094.
21. Ribeiro CS. 2012. A influência térmica na dinâmica das membranas celulares: Uma contribuição na conservação de *Steindachneridion parahybae* (Siluriformes: Pimelodidae), uma espécie de peixe ameaçada de extinção. PhD thesis. Universidade de São Paulo, São Paulo, São Paulo, Brazil (in Portuguese).
22. Styrishave B, Faldborg Petersen M, Andersen O. 2000. Influence of cadmium accumulation and dietary status on fatty acid composition in two colour forms of shore crabs, *Carcinus maenas*. *Mar Biol* 137:423–433.
23. Ortega P, Santos RA, Lacouth P, Rozas EE, Custódio MR, Zanotto FP. 2014. Cytochemical characterization of gill and hepatopancreatic cells of the crab *Ucides cordatus* (Crustacea, Brachyura) validated by cell metal transport. *Iheringia Série Zoologia* 104:347–354.
24. Chavez-Crooker P, Garrido N, Ahearn GA. 2001. Copper transport by lobster hepatopancreatic epithelial cells separated by centrifugal elutriation: Measurements with the fluorescent dye Phen green. *J Exp Biol* 204:1433–1444.
25. Ahearn GA, Mandal PK, Mandal A. 2001. Biology of the $2Na^+/1H^+$ antiporter in invertebrates. *J Exp Zool* 289:232–244.
26. Fujishiro H, Yano Y, Takada Y, Tanihara M, Himeno S. 2012. Roles of ZIP8, ZIP14, and DMT1 in transport of cadmium and manganese in mouse kidney proximal tubule cells. *Metallomics* 4:700–708.
27. Silva JM, Martins MBG, Cavalheiro AJ. 2010. Caracterização anatômica e perfil químico da lâmina foliar de *Laguncularia racemosa* (L.) Gaertn, de manguezais impactados e não impactados do litoral de São Paulo. *Iheringia Série Botânica* 65:123–132 (in Portuguese).
28. Ferreira CP. 2005. Percepção ambiental na estação ecológica de Juréia-Itatins. PhD thesis. Universidade de São Paulo, São Paulo, São Paulo, Brazil (in Portuguese).
29. Ortega P, e Sá MG, Custódio MR, Zanotto FP. 2011. Separation and viability of gill and hepatopancreatic cells of a mangrove crab *Ucides cordatus*. *In Vitro Cell Dev Biol Anim* 47:346–349.
30. Zanotto FP, Baptista BB. 2011. ATP pulse and calcium homeostasis in cells from hepatopancreas of *Dilocarcinus pagei*, a freshwater crab. *Comp Biochem Physiol A Mol Integr Physiol* 158:432–437.
31. Mello PH, Araújo BC, Venturieri RLL, Moreira RG. 2012. Fatty acids as a tool to compare cachara (*Pseudoplatystoma reticulatum*) (Siluriformes: Pimelodidae) and hybrid (*Pseudoplatystoma corruscans* × *Pseudoplatystoma reticulatum*) larvae during early development. *Aquaculture International* 20:1139–1160.
32. Folch J, Less M, Stanley GH. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* 226: 497–503.
33. Parrish CC. 1988. Dissolved and particulate marine lipid classes: A review. *Mar Chem* 23:17–40.
34. Cristie WW, Han X. 2010. *Lipid Analysis: Isolation, Separation, Identification and Lipidomic Analysis*, 4th ed. Oily Press, Bridgwater, UK.
35. Jiang ZY, Hunt JV, Wolff SP. 1992. Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low density lipoprotein. *Anal Biochem* 202:384–389.
36. Jiang ZY, Woollard ACS, Wolff SP. 1991. Lipid hydroperoxide measurement by oxidation of Fe^{2+} in the presence of xylenol orange. Comparison with the TBA assay and an iodometric method. *Lipids* 26: 853–856.
37. Viarengo A, Ponzano E, Dondero F, Fabbri R. 1997. A simple spectrophotometric method for metallothionein evaluation in marine organisms: An application to Mediterranean and Antarctic molluscs. *Mar Environ Res* 44:69–84.
38. Kawanai T, Fujinaga M, Koizumi K, Kurotani I, Hashimoto E, Satoh M, Imai S, Miyoshi N, Oyama Y. 2011. Some characteristics of membrane Cd^{2+} transport in rat thymocytes: An analysis using Fluo-3. *BioMetals* 24:903–914.
39. Hebert PD, Luiker MM. 1996. Genetic effects of contaminant exposure: Towards an assessment of impacts on animal populations. *Sci Total Environ* 191:23–58.
40. Rotter S, Sans-Piché F, Streck G, Altenburger R, Schmitt-Jansen M. 2011. Active bio-monitoring of contamination in aquatic systems: An in situ translocation experiment applying the PICT concept. *Aquat Toxicol* 101:228–236.
41. Silvestre F, Traush G, Péqueux A, Devos P. 2004. Uptake of cadmium through isolated perfused gills of the Chinese mitten crab, *Eriocheir sinensis*. *Comp Biochem Physiol A Mol Integr Physiol* 137:189–196.
42. Kang X, Mu S, Li W, Zhao N. 2010. Toxic effects of cadmium on crabs and shrimps. In Acree W, ed, *Toxicity and Drug Testing*. InTech, Rijeka, Croatia. [cited 2016 February 15]. Available from: http://www.intechopen.com/books/toxicity-and-drug-testing/toxic-effects-of-cadmium-on-crabs-and-shrimps
43. Mcgeer JC, Szebedinszky C, Mcdonald DG, Wood CM. 2000. Effects of chronic sublethal exposure to waterborn Cu, Cd or Zn in rainbow trout 1: Iono-regulatory disturbance and metabolic costs. *Aquat Toxicol* 50:231–243.
44. López-López E, Sedeño-Díaz JE, Soto C, Favari L. 2011. Responses of antioxidant enzymes, lipid peroxidation, and Na^+/K^+-ATPase in liver of the fish *Goodea atripinnis* exposed to Lake Yuriria water. *Fish Physiol Biochem* 37:511–522.
45. Pedersen TV, Bjerregaard P. 2000. Cadmium influx and eflux across perfused gills of the shore crab, *Cracinus maenas*. *Aquat Toxicol* 48: 223–231.
46. Ahearn GA, Mandal PK, Mandal A. 2004. Mechanisms of heavy-metal sequestration and detoxification in crustaceans: A review. *J Comp Physiol B Biochem Syst Environ Physiol* 174:439–452.
47. Legras S, Mouneyrac C, Amiard JC, Amiard-Triquet C, Rainbow PS. 2000. Changes in metallothionein concentrations in response to variation in natural factors (salinity, sex, weight) and metal contamination in crabs from a metal-rich estuary. *J Exp Mar Biol Ecol* 246:259–279.
48. Figueiredo J, Penha-Lopes G, Anto J, Narciso L, Lin J. 2008. Potential fertility and egg development (volume, water, lipid, and fatty acid content) through embryogenesis of *Uca rapax* (Decapoda:Brachyura: Ocypodidae). *J Crust Biol* 28:528–533.
49. Paton CM, Ntambi JM. 2009. Biochemical and physiological function of stearoyl-CoA desaturase. *Am J Physiol Endocrinol Metab* 297:28–37.
50. Freire CA, Onken H, McNamara JC. 2008. A structure-function analysis of ion transport in crustacean gills and excretory organs. *Comp Biochem Physiol A Mol Integr Physiol* 151:272–304.
51. Chapelle S, Dandrifosse G, Zwingelstein G. 1976. Metabolism of phospholipids of anterior or posterior gills of the crab *Eriocheir sinensis* M. EDW, during the adaptation of this animal to media of various salinities. *Int J Biochem* 7:343–351.
52. Chapelle S. 1977. Lipid composition of tissues of marine crustaceans. *Biochem Syst Ecol* 5:241–248.
53. Chapelle S, Zwingelstein G. 1984. Phospholipid composition and metabolism of crustacean gills as related to changes in environmental salinities: Relationship between Na^+/K^+-ATPase activity and phospholipids. *Comp Biochem Physiol B* 78:363–372.
54. Yang J, Liu D, Jing W, Dahms HU, Wang L. 2013. Effects of cadmium on lipid storage and metabolism in the freshwater crab *Sinopotamon henanense*. *PLoS One* 8:1–9.
55. Geffard A, Amiard-Triquet C, Amiard JC, Mouneyrac C. 2001. Temporal variations of metallothionein and metal concentrations in the digestive gland of oysters (*Crassostrea gigas*) from a clean and a metal-rich site. *Biomarkers* 6:91–107.