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Orientadora

À minha família, que sempre me apoiou com carinho; ao meu amor, que sempre esteve ao meu lado. Em memória de minha avó Noemia, que deve estar orgulhosa no plano superior.

“Em algum lugar, algo incrível espera para ser conhecido”

Carl Sagan

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INTRODUÇÃO GERAL

Introdução Geral

Ao longo da evolução dos crustáceos, surgiram adaptações especializadas das células epiteliais, principalmente aquelas encontradas nas brânquias e no hepatopâncreas, que possibilitaram uma melhoria no seu desempenho, como a respiração e absorção de nutrientes^{1,2}.

As brânquias são um importante órgão relacionado com a troca de íons com a água, transporte de gases, processos enzimáticos relacionados à respiração, osmorregulação, absorção e excreção de amônia. Além disso, apresentam canais que transportam cálcio, sódio, cloreto, entre outros íons³⁻⁵. Ademais, as brânquias possuem células específicas: células estriadas ligadas ao transporte e osmorregulação, células principais referentes à respiração e presentes nas brânquias anteriores, hemócitos associados à coagulação e circulação, e células pilares relacionadas à sustentação das brânquias (Fig. 1)^{4,6,7}.

Fig. 1: Representação esquemática da morfologia e da camada epitelial da brânquia posterior de caranguejo. 1) cutícula; 2) células estriadas; 3) células pilares; 4) espaço hemolinfático; 5) septo intralamelar. Na brânquia anterior, a cutícula poderia estar alinhada com as células estriadas e pilares. As células principais não estão presentes no esquema. Adaptado de⁴.

O hepatopâncreas, por sua vez, possibilita a absorção e estocagem de nutrientes, síntese de enzimas digestivas, detoxificação de xenobióticos e balanço de cálcio⁸. Assim, há quatro tipos celulares associados a esses processos: células embrionárias (E) que originam os outros tipos celulares; as de reabsorção (R) que absorvem os nutrientes; as fibrilares (F) e as armazenadoras ou “blister” (B), responsáveis pela digestão, degradação de partículas e estocagem de nutrientes (Fig. 2)^{8,9}.

Fig. 2: A) Vitorino, H. A., 2016 (Photographer, IQ-USP). Caranguejo *Ucides cordatus* com a carapaça exposta mostrando o hepatopâncreas no seu interior. B) Vitorino, H. A., 2016 (Photographer, IQ-USP). Tubo Falcon contendo os tipos celulares hepatopancreáticos E (embrionárias), R (absorptivas), F (fibrilares) e B (blister), após o processo de Separação por Gradiente de Sacarose.

Estudos demonstraram que metais potencialmente tóxicos podem contaminar os animais através das brânquias e hepatopâncreas^{10–12}, dependendo da concentração do metal no sedimento, água ou fontes de alimentação¹³. Trabalhos realizados com as brânquias de *Penaeus japonicus* demonstraram que a exposição do órgão a 2mg/L de cádmio dissolvido em água durante 15 dias resulta em um aumento de nefrócitos e uma diminuição na camada epitelial do filamento, tornando-se mais fina. Já a exposição durante 4 dias à mesma concentração de cádmio resultou em importantes alterações nos filamentos branquiais como necrose e obstrução do vaso hemolinfático.

Além disso, os filamentos tornaram-se desorganizados, com células epiteliais separadas da cutícula e perda de organelas citoplasmáticas¹⁴. Portanto, pode-se verificar que a contaminação dos animais por metais tóxicos leva a alterações estruturais e fisiológicas, validando a importância de se conhecer as adaptações e suas consequências em diferentes níveis de organização biológica.

Área de estudo

Município de Itanhaém, pertencente à Baixada Santista, faz parte do estuário de Itanhaém, apresentando alto grau de contaminação por metais. Em estudos realizados com sedimentos dessa região, verificou-se a presença de altos índices de arsênio, apresentando toxicidade no sedimento e bioacumulação em tecidos de caranguejos coletados no local¹⁵. Os aterros sanitários e, principalmente, os lixões, são considerados fontes de alto risco, que contribuem para a contaminação deste sistema estuarino¹⁶. Estudos verificaram a presença de cádmio neste sistema, principalmente no Rio Piaçaguera, corpo receptor de efluentes das indústrias de fertilizantes, em Cubatão. Também foram encontrados altos níveis de cádmio nos sedimentos deste mesmo rio, sendo indicativo de uma possível fonte do metal associada às indústrias locais¹⁶.

Estudos realizados no Estado de São Paulo com *Ucides cordatus* provenientes do Rio Casqueiro (região poluída) e Guaratuba, São Paulo (região não poluída)¹³, resultaram em altos índices de cádmio presente no hepatopâncreas e brânquias dos animais da região poluída em comparação à região não poluída. Deste modo, observa-se que o cádmio encontra-se amplamente difundido neste ecossistema, concentrando-se próximo às fontes do poluente. A contaminação do sedimento e da coluna d'água indica níveis de cádmio que aparentemente provocam efeitos nocivos à biota (Fig. 3)¹⁶.

Já a Estação Ecológica Juréia-Itatins situa-se no Litoral Sul de São Paulo, sob as coordenadas 24°26'7.43" S e 47° 4'46.12" W. Possui uma área de aproximadamente 80000ha abrangendo terras pertencentes aos municípios

de Itariri, Miracatu, Pedro de Toledo, Iguape e Peruíbe. Esta é uma importante reserva de biodiversidade da Mata Atlântica ainda preservada, sendo considerada uma Unidade de Conservação (UC). As UCs têm sido a melhor forma de proteção da biodiversidade, pois, com a elevação do crescimento populacional e redução dos habitats naturais, essas unidades têm se tornado os únicos redutos da maior parte da biota intolerante à ação humana^{17,18}. A UC da Estação Ecológica de Juréia-Itatins foi criada pelo Decreto Estadual nº 24.646/1986 e regulamentada pela Lei nº 5.649/1987. Em 2006, por força da legislação estadual (nº 12.406/2006), a Estação Ecológica de Juréia-Itatins foi transformada em Mosaico de Unidades de Conservação, sendo a Barra do Una a Reserva de Desenvolvimento Sustentável¹⁹.

Em estudos realizados em Juréia¹⁸, foram constatados a presença de cromo no sedimento, nas folhas de *R. mangle* e no hepatopâncreas de *U. cordatus*, apresentando, porém, um baixo impacto genotóxico nesses animais. Já em regiões como Cubatão e Bertioga, ocorreu em *U. cordatus* um impacto genotóxico cinco vezes acima da normalidade estabelecida, chegando até sete vezes mais alta para a região de São Vicente. Estes dados apontam um maior impacto ambiental, com maior interferência antropogênica nas regiões de São Vicente, Cubatão e Bertioga, e menor ação humana na região de Juréia, confirmando essa Estação Ecológica como uma Unidade de Conservação, que mantém a região livre de poluição²⁰ (Fig. 3).

Fig. 3: Ilustração representativa das regiões de Juréia (área não poluída, seta vermelha) e Itanhaém (área poluída, seta preta). Adaptado de: www.diariodeiguape.com

O Metal Cádmio

O cádmio é um metal de transição, tóxico, contido em pilhas e baterias descartadas, sem aparente função fisiológica para os animais, sendo um metal não essencial. Pode se acumular em tecidos de acordo com a concentração externa do metal^{13,21}, porém em baixas concentrações também pode causar toxicidade.

Vários estudos mostram que altas concentrações de cádmio podem ser letais para os crustáceos, causando efeitos fisiológicos adversos, especialmente na respiração e osmorregulação¹³. As brânquias, como são cruciais para a respiração, ao entrar em contato com altas concentrações do metal, podem sofrer também danos morfológicos resultando em deficiência respiratória e osmorregulatória¹³.

Estudos têm focado na retirada do Cd do meio aquático e sua acumulação, mas pouca informação existe sobre seu transporte celular. Em um trabalho desenvolvido com transporte de cádmio em brânquias de *U. cordatus*²², observou-se que o influxo de cádmio poderia ocorrer através de

canais de Ca^{2+} , trocadores $\text{Na}^+/\text{Ca}^{2+}(2\text{H}^+)$ ou ainda com a ação da Na^+/K^+ ATPase, evidenciando o papel do cádmio como metal oportunista. Em crustáceos, a retirada de metais ocorre também através das brânquias, onde o cádmio poderia atravessar o epitélio branquial pelas junções paracelulares, ou por via transcelular^{10,22}. Já em trabalhos desenvolvidos com caranguejos²³, observou-se que o cálcio (Ca) compete com o Cd em *Eriocheir sinensis* e o influxo deste último aumenta com a retirada de Ca do meio externo, aumentando assim a toxicidade do cádmio. Esses resultados parecem sugerir que o Cd pode entrar por trocadores de Ca^{2+} como o $\text{Na}^+/\text{Ca}^{2+}$ (NCX), alterando o equilíbrio do processo de transporte, sendo observado resultados semelhantes²². Este trabalho, através da ação de inibidores, demonstrou o transporte de cádmio em brânquias de *U. cordatus*, e a importância dos canais de Ca^{2+} , trocadores $\text{Na}^+/\text{Ca}^{2+}(2\text{H}^+)$ e da Na^+/K^+ ATPase como rotas para a entrada de cádmio nas células. Também, verificou-se para *Carcinus maenas* que o cádmio influencia a quantidade de ácidos graxos monoinsaturados presentes nas brânquias e hepatopâncreas²⁴. Estes notaram que com o aumento da concentração de cádmio, ocorre uma diminuição na porcentagem de ácidos graxos monoinsaturados na membrana. O hepatopâncreas apresenta grande capacidade de acumular metais para posterior detoxificação, necessitando de energia para tal função. Esta energia seria obtida através da oxidação de ácidos graxos monoinsaturados, ocorrendo assim a sua diminuição no órgão. Esta diminuição é mais pronunciada para ácidos graxos saturados e monoinsaturados, quando comparados aos polinsaturados, indicando um metabolismo seletivo^{24,25}.

Contaminação ambiental

Animais provenientes de regiões estuarinas e costeiras são afetados diretamente pela poluição local, através do descarte de produtos químicos e metais tóxicos das indústrias^{13,22,26}. O cádmio, um dos principais poluentes presentes em mangues, acarreta danos celulares, podendo ainda, se acumular nos tecidos. Quando em contato com o animal, pode causar prejuízos fisiológicos e morfológicos em brânquias e hepatopâncreas, órgãos que são os

primeiros sítios de entrada de substâncias e de troca com o ambiente. Essa entrada pode ocorrer via canais de cálcio, trocadores Na^+/H^+ e, possivelmente, por canais de zinco^{22,27,28}. Em carpas, altos níveis de poluição ambiental podem ocasionar lesões oxidativas, genotoxicidade, atividade apoptótica e distúrbios endócrinos²⁹. Em estudos realizados com *Carcinus maenas*, a contaminação por cádmio pode afetar a concentração de cálcio intracelular, o tamanho do caranguejo, o ciclo de muda, o tamanho dos ovários e o estágio reprodutivo³⁰.

Contudo, animais estuarinos podem apresentar mecanismos que indicam o grau de poluição de um determinado local, assim como processos para auxiliar na sua sobrevivência, como a presença de proteínas ligantes a metais (p. ex., metalotioneínas)^{30–32}. Essas proteínas de baixo peso molecular são extremamente importantes para os organismos aquáticos, apresentando diversas funções, como um aumento da tolerância ao metal durante sua acumulação³³. De maneira geral, as metalotioneínas são proteínas que exercem um importante papel na homeostase e no controle de metais essenciais (p. ex, Cu e Zn). Em um segundo momento, como estão ligadas a outros metais, podem também exercer um papel na detoxificação^{34,35}. Em estudos realizados com moluscos, notou-se que em ambientes contaminados com cádmio, houve um aumento de metalotioneínas presentes no citosol de células branquiais, variando com o gradiente de contaminação local e atuando como um importante sinalizador de estresse em relação ao ambiente³⁶. Um outro processo fisiológico que pode atuar como um sinalizador de estresse em relação a poluição ambiental é a peroxidação lipídica (LPO), onde, com o aumento da toxicidade e da presença de metais, aumenta-se os níveis de LPO^{37–40}. Vários estudos realizados com vertebrados e invertebrados demonstraram que, com o aumento dos níveis de poluição, principalmente com relação ao metal cádmio, aumentou-se o estresse, com impacto ao sistema antioxidativo⁴¹.

As mudanças ambientais causadas por ação antrópica também podem interferir nas membranas biológicas, através de um sistema de percepção por proteínas da membrana, transferindo os sinais do ambiente para o meio interno celular⁴². Em experimentos realizados com caranguejos *Carcinus maenas*,

notou-se que houve uma regulação fisiológica com relação às mudanças ambientais sofridas, principalmente pela mobilização dos ácidos graxos presentes no hepatopâncreas. Neste estudo, os pesquisadores observaram uma relação entre a quebra de ácidos graxos deste órgão com o aumento da liberação de energia, ocasionando em acúmulo do cádmio, detoxificação e sua excreção do mesmo²⁴. Outros estudos apontaram uma maior tolerância dos animais quando expostos a longos períodos em meios contaminados. Em estudos realizados com plantas foi constatado que, em altas concentrações de poluentes por longos períodos de tempo, os vegetais apresentaram tolerância e a geração de formas resistentes aos poluentes⁴³. Em componentes do fitoplâncton notou-se que, em comunidades cultivadas em locais poluídos, houve uma maior tolerância ao contaminante. Assim, mudanças na sensibilidade ao poluente foram consistentes com as mudanças fisiológicas, bioquímicas e nos ciclos reprodutivos das espécies, indicando claramente que o tempo de exposição ao contaminante interferiu na sobrevivência do fitoplâncton²⁶. Em caranguejos, por sua vez, percebeu-se algo muito semelhante. Estudos realizados com *Ucides cordatus* e *Callinectes danae* provenientes de locais contaminados com metais tóxicos, indicam mudanças fisiológicas, como maior acúmulo de metais tóxicos, redução da habilidade hiporregulatória, com posterior aumento da regulação e dos níveis de Na^+ / K^+ ATPase, devido ao longo tempo de exposição ao contaminante¹³.

Dessa maneira, ressalta-se a importância de estudos com o transporte celular em brânquias e hepatopâncreas, como realizado nos capítulos 1 e 2 desse trabalho, pois são os órgãos onde ocorrem o primeiro contato com o contaminante através da água e da alimentação, respectivamente. Com essa pesquisa, pode-se identificar os principais transportadores e canais associados com o influxo e efluxo de cádmio. Além disso, comparar populações de caranguejos provenientes de locais com diferentes níveis de poluição, como desenvolvido no capítulo 3, torna-se essencial para entender, em nível celular, quais os mecanismos e as modificações fisiológicas e morfológicas que poderiam permitir a sobrevivência desses animais em ambientes altamente estressantes.

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Capítulo 1

Characterization of cadmium plasma
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Characterization of cadmium plasma membrane transport in gills of a mangrove crab *Ucides cordatus*

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ABSTRACT

Membrane pathway for intracellular cadmium accumulation is not fully elucidated in many organisms and has not been studied in crab gill cells. To characterize membrane Cd^{2+} transport of anterior and posterior gill cells of *Ucides cordatus*, a hypo-hyper-regulating crab, a change in intracellular Cd^{2+} concentration under various experimental conditions was examined by using FluoZin, a fluorescent probe. The membrane Cd^{2+} transport was estimated by the augmentation of FluoZin fluorescence induced by extracellular application of CdCl_2 and different inhibitors. Addition of extracellular calcium (Ca^{2+}) to the cells affected little the fluorescence of FluoZin, confirming that Cd^{2+} was the main ion increasing intracellular fluorescence. Ca^{2+} channels blockers (nimodipine and verapamil) decreased Cd^{2+} influx as well as vanadate, a Ca^{2+} -ATPase blocker. Chelating intracellular Ca^{2+} (BAPTA) decreased Cd^{2+} influx in gill cells, while increasing intracellular Ca^{2+} (caffeine) augmented Cd influx. Cd^{2+} and ATP added at different temporal conditions were not effective at increasing intracellular Cd^{2+} accumulation. Ouabain (Na/K-ATPase inhibitor) increased Cd^{2+} influx probably through a change in intracellular Na and/or a change in cell membrane potential. Routes of Cd^{2+} influx, a non-essential metal, through the gill cell plasma membrane of crabs are suggested.

1. INTRODUCTION

Estuarine and coastal areas are regions affected by discharge of heavy metals, mainly cadmium. Cadmium (Cd) plays no role as a trace element and is considered a toxic metal with unknown physiological function for animals. One exception, however, is shown by some diatoms, and in the absence of Zn^{2+} , use Cd^{2+} in the active site of their carbonic anhydrase (Lane and Morel, 2000; Thévenod, 2010). It is known that crabs exposed to Cd up-regulate several antioxidant enzymes, and chronic exposure affects more extensively the metabolic and protein expression profiles than acute exposure to Cd (Silvestre et al., 2006). Cd also causes detrimental effects in respiratory and osmoregulatory functions (see Silvestre et al., 2006).

Membrane pathway for intracellular cadmium transport is not fully elucidated in many organisms (Bridges and Zalups, 2005; Gagnon et al., 2007; Ojo and Wood, 2008; Thévenod, 2010; Verheyen et al., 2012; Kwong and Niyogi, 2012; Li et al., 2012a,b) and has not been studied in crab gill cells, apart from work on perfused gills (Pedersen and Bjerregaard, 1995; Lucu and Obersnel, 1996; Bondgaard and Bjerregaard, 2005). At the cellular level, it is assumed that Cd^{2+} is taken up by cells through transporters for other essential elements as a consequence of low specificity of most membrane transporters (Rainbow and Luoma, 2011). Work with mammals, adopting methodologies for Cd^{2+} transport using radiotracers, or sensitive dyes, has shown unequivocally that Cd^{2+} can enter cells through divalent metal transporters (DMT1), Zn related protein transporters (ZIP) and through mediated receptor endocytosis as a Cd-metallothionein complex, using cell energy (see review by Thévenod, 2010). Other modes of transport such as Ca^{2+} channels are still controversial in mammals (Thévenod, 2010).

For aquatic organisms, studies using whole animals with radioactive tracers show that Cd^{2+} entry in gill epithelium occurs probably via calcium transport routes in fish (Perry and Flik, 1988; Wicklund Glynn et al., 1994), mollusks (Roesijadi and Unger, 1993) and crustaceans (Borowitz and McLaughlin, 1992; Pedersen and Bjerregaard, 1995; Lucu and Obersnel, 1996). Nørum et al. (2005) observed a postmolt increase in cadmium influx in the crab *Carcinus maenas* due to increased transport of cadmium, occurring at least

partly, by accidental uptake via calcium transporting proteins. In fish, Cd^{2+} affect Ca^{2+} homeostasis, as both ions compete at the chloride cells of the gills (Lock et al., 1987; Matsuo et al., 2005), and apparently the competition occurs with basolateral Ca^{2+} -ATPase and apical Ca^{2+} channels (Lock et al., 1987; Matsuo et al., 2005). For the crab *C. maenas*, Cd^{2+} caused an irreversible inhibition of the osmoregulatory enzyme Na^+/K^+ ATPase (Postel et al., 1998) and a strong inhibition of the respiratory enzyme carbonic anhydrase, affecting the regulation of multiple ions indirectly (Vitale et al., 1999). Additionally, once Cd^{2+} enters a cell, different mechanisms of intracellular detoxification are found. For example, in the bacteria *Staphylococcus aureus* and the yeast *Saccharomyces cerevisiae*, Cd^{2+} is discarded from the cell through a P-type ATPase (Adle et al., 2009; Tommasini et al., 1996; Mielniczki-Pereira et al., 2011). In mammals, however, this mechanism is still debatable (Thévenod, 2010). The binding of Cd^{2+} to metallothionein is an established mechanism of intracellular detoxification in animals and crustaceans (Wallace and Luoma, 2003; Van Kerkhove et al., 2010). In plants, Cd^{2+} is extruded as free ion and as a complexed form, energized by ATP and by an electrochemical gradient (Migocka et al., 2011).

Crustacean's gills are a selective organ that acts between external and internal environment, serving for gas exchange, osmolyte transport, nitrogenous excretion and acid–base balance as well as volume regulation (Lucu and Towle, 2003). In hypo-hyperregulating crabs, such as *Ucides cordatus*, the posterior gills harbor ionocytes, whereas the anterior gills display a typical gas-exchange epithelium made up of thin cells (see Sá and Zanotto, 2013, for differences in Cu^{2+} transport between both gills). In addition, earlier work has shown that these crabs have functional differences in adjacent gills, enabling strong hypo-hyper-ion regulation (Martinez et al., 1999). Flux-ratio analysis showed active uptake of Na^+ in one gill filament and active extrusion in another, suggesting that hypo-hyper-regulation is performed in different gills (Martinez et al., 1999). It is recognized that gills are immediately exposed to the environment and are the first organs affected by pollutants when ambient water is contaminated. Cadmium exposure, for example, causes a decrease in the functional lamellar area of the gills, impairing energy production (Xuan et al., 2014). In addition, waterborne metals are greatly affected by the water chemistry: amounts of $[\text{Ca}]$

and [Mg] (i.e. hardness), pH, alkalinity, dissolved organic matter and other complexing anions (Ojo and Wood, 2008). There is nowadays large evidence of interactions between different metals and in vitro cell studies of Cd^{2+} transport, such as this, and the interaction with different metals or ions, can be later validated by in vivo experiments. In this case, manipulations of the animal's external environment can be a mechanism that controls the amount of toxic metals that may accumulate in the animals via cellular influx.

Therefore, the objective of this work was to characterize gill cadmium transport (Cd^{2+}) in the presence of calcium, ATP and specific inhibitors of different transporters, in isolated gill cells of the mangrove crab *U. cordatus*. Toxic metal transport and interaction with other ions in cells is an essential part for a better understanding of metal toxicity. In addition, *U. cordatus* is an important source of human food, and could be a possible vehicle of cadmium contamination and transference to human populations. By studying the process of Cd^{2+} transport at the cellular level, unstudied in crab gill cells, the present work answers some questions and open avenues for further research on metal transport interactions in isolated cells of crustaceans.

2. MATERIAL AND METHODS

2.1. Animals

The animals were collected in Itanhaém, São Paulo coast, at the Pescadores Beach, and then, brought to University of São Paulo, where they were acclimatized in the vivarium of the University. The crabs were kept in tanks filled with artificial sea water at 20ppm, gravel, water filter and pieces of brick for the emersion of the animals. Photoperiod (12:12) was constant as well as the temperature ($22 \pm 3^\circ\text{C}$). Only males in the intermolt period were used. For each set of experiment, $N = 4$ animals were used.

2.2 Cellular Dissociation

The gills were separated in anterior and posterior regions. Then, for the gill cells dissociation, the enzymatic method was used, where 10 mL of the

extraction solution (NaCl: 22.47 g; KCl: 0.59 g; NaHCO₃: 0.22 g; NaH₂PO₄: 0.03 g; Hepes: 0.95 g; glucose: 0.18 g; EDTA: 0.37 g) were mixed with 200 µL of Trypsin (0.05%; Ortega et al., 2011). The gills were immersed in solution and kept in ice, for 15 min. After that, the gills were minced with scissors for 15 min more. The minced gills were filtered in thin mash at 30 µm nylon mesh, put in Falcon tubes (15 mL) and centrifuged for 10 min at 115 × g at 5°C. After the centrifugation the pellet was resuspended in extraction solution and kept in ice.

2.3. Cellular Transport

The gill cells previously dissociated were labeled with FluoZin-3 am (1 µL) during 1 h under shaking at 200 rpm at room temperature. After, the cells were centrifuged at 405 × g for 5 min and then washed in the extraction solution, without EDTA. In the fluorimeter (Biotek), the emission used was 525 nm and the excitation 495 nm. The cellular transport was measured each 90 s in real time. In an Elisa plate, 180 µL of cells were added with CdCl₂ concentrations of 0.5, 1.0 and 1.5 µM and at 0.5, 1.0 and 1.5 mM concentrations. The variation in fluorescence was measured for each concentration.

A calibration curve was prepared to transform arbitrary fluorescence units into intracellular Cd²⁺ concentrations, based on Zanotto and Baptista (2011). At the end of the experiment, 180 µL of cells without the exposition of cadmium were added to an Elisa plate with 50 µL of Triton X-100 20% for 15 min. To obtain the minimum fluoresce (F_{min}), it was added 50 µL of HEDTA (1 mM). For the maximum fluorescence (F_{max}), we added 50 µL of Triton X-100 20% for 15 min and 50 µL of CdCl₂ (5 mM) in different batch of cells. Intracellular cadmium for every experiment was calculated according to the equation:

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

where $[Cd^{2+}]_i$ is the intracellular free cadmium concentration (nM); 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.

The cellular viability was assayed using the Trypan Blue method, where 20 μL of Trypan Blue were added to 200 μL of cells (Tennant, 1964) and put in a Neubauer chamber. The viability was quantified by the visualization of the stained cells (unviable) and translucent cells (viable) in four quadrants and the result was multiplied by 10^4 . For all experiments, were used 23×10^4 cells.

2.4. Experimental design

To investigate cadmium transport, we added the following inhibitors: (a) verapamil (1 μM) and nimodipine (11 μM), which blocks slow calcium channels (Ahearn and Franco, 1993) and L-type voltage-dependent calcium channels (VDCC; Gavazzo et al., 2005) respectively; (b) ATP (1 mM), which inhibits Ca^{2+} -ATPase and Cu^{2+} -ATPase (Wheatly et al., 1999); (c) ouabain (2 mM), a Na^+/K^+ -ATPase inhibitor (Zare and Greenaway, 1998); (d) vanadate (100 μM), a Na^+/K^+ and Ca^{2+} -ATPase inhibitor (Zare and Greenaway, 1998); (e) caffeine (100 μM), which facilitates the release of calcium from the endoplasmic reticulum (Goudeau and Goudeau, 1998); and (f) BAPTA (2 mM) that chelates intracellular Ca^{2+} .

Cells were also incubated with extracellular Ca^{2+} at 1 mM or 20 mM . After, CdCl_2 was added at different concentrations and the change in fluorescence was followed.

For experiments with ATP, the cells were exposed to the following treatments: (a) CdCl_2 alone at 1.0 mM ; (b) CdCl_2 + ATP at 1 mM added together; (c) CdCl_2 followed by ATP after 90 s, both at 1 mM ; (d) ATP followed by CdCl_2 after 90 s, at 1 mM . Then, the fluorescence change was analyzed as intracellular Cd^{2+} .

In the case of experiments with BAPTA, an intracellular Ca^{2+} chelator, cells were incubated with BAPTA at 2 mM together with FluoZin dye during 1 h. Cellular Cd^{2+} transport was performed using CdCl_2 in the same concentrations as above.

2.5. Data analysis

Data was collected on 4 animals. One-Way ANOVA was used to compare different inhibitors (treatments) against control. Cadmium transport was also compared between treatments using the kinetic variables, K_m and V_{max} , when the data was displayed as Cd transport against Cd concentration. Whenever the data was presented as Cd transport against time, all experimentals (different inhibitors or ATP) were compared against control. The statistical package used was Sigma Stat and the data was verified for normality and homogeneity of variance. Data are displayed as means $\pm$ SE.

3. RESULTS

Before the start of the experiments with Cd^{2+} transport, we tested FluoZin to see whether the fluorescence of the dye could suffer interference with Ca^{2+} ions. To test that, we added both Cd^{2+} and Ca^{2+} at 1 mM, together or separated, in the presence of isolated cells. The results show that the fluorescence change (arbitrary units) due to Ca^{2+} is at least 3x less than the fluorescence due to Cd^{2+} alone, and the change due to Cd^{2+} and Ca^{2+} together is similar to Cd^{2+} alone (Fig. 1, ANOVA, $P < 0.05$). These results show that the probe/dye used is sensitive to Cd^{2+} only, and reinforce that any change in intracellular Ca^{2+} would not affect our results.

Figure 1: Fluorescence change (ΔF) as arbitrary units against Cd^{2+} and Ca^{2+} added together or separated, both at 1 mM, in anterior and posterior gill cells.

Next, we tested different concentrations of Cd^{2+} and followed gill cell viability for 2 h (within the experimental transport time) after gill cell exposure to Cd^{2+} . Cd^{2+} added to the cells in μM amounts elicited a change in intracellular Cd^{2+} at levels much lower levels compared to Cd^{2+} added in mM levels (0.15 against $1.0 \text{ nM} \times 23 \times 10^4 \text{ cells} \times 90 \text{ s}^{-1}$; Figs. 2A, B and 3). However, the errors associated were much larger for Cd^{2+} in μM amounts. We then tested the cell viability using Trypan Blue at the end of cell exposure to Cd^{2+} (Table 1). There was no difference in cell viability among control and treatments after 2 h. Therefore, we used Cd^{2+} in mM amounts for next experiments due to a lower amount of variation in the data collected.

Figure 2: Variation of intracellular Cd^{2+} concentration (ΔCd) against extracellular Cd^{2+} , for anterior and posterior gill cells. A. Cd^{2+} added in μM amounts; B. Cd^{2+} added in mM amounts.

Figure 3: Relationship between intracellular Cd^{2+} ($\text{nM} \times 23.10^4 \text{ cells}^{-1}$) and time (s) for anterior and posterior gill cells exposed to Cd^{2+} and Ca^{2+} (1mM for both). Cd^{2+} was added alone or Cd^{2+} and Ca^{2+} ($\text{Cd}^{2+} + \text{Ca}^{2+}$) were added together.

Table 1: Cell viability (%) at the end of Cd^{2+} exposure (2 hrs) for anterior and posterior gill cells exposed to Cd^{2+} at 1.5 μM and 1 mM. There was no difference among treatments (ANOVA; $P > 0.05$).

Cell viability (after 2 hrs) (%)	Control (0 Cd)	Anterior gills (Cd^{2+} 1mM)	Anterior gills (Cd^{2+} 1.5 μM)	Posterior gills (Cd^{2+} 1mM)	Posterior gills (Cd^{2+} 1.5 μM)
	69.2 $\pm$ 5.0%	57.4 $\pm$ 7,4%	68.6 $\pm$ 4,7%	50.1 $\pm$ 5,9%	49.9 $\pm$ 5,9%

Next, Cd^{2+} influx at different concentrations was compared between anterior and posterior gill cells (Fig. 4). There was no statistical difference between K_m and V_{max} for both. Anterior and posterior gill cells transport Cd^{2+} in similar amounts, although there was a slightly lower K_m for posterior gill cells, indicating a higher affinity.

Treatment	Km	Vmax
Anterior gill	0.5237	2.241
Posterior gill	0.1809	1.772

Figure 4: Relationship between intracellular Cd^{2+} ($\text{nM} \times 23.10^4 \text{ cells}^{-1} \times 90 \text{ sec}^{-1}$) and Cd^{2+} concentration (mM) for both anterior and posterior gill cells. K_m and V_{max} values are shown and are similar for both gills statistically.

Fig. 5A and B shows the effect Cd transport for cells exposed to the Ca^{2+} channel inhibitor nimodipine. Both K_m and V_{max} for Cd transport were reduced for cells incubated with nimodipine (ANOVA, $P < 0.001$). V_{max} was around 3x lower and K_m 2x smaller compared to control (Fig. 5A). For posterior gill cells the inhibition occurred only for V_{max} (ANOVA, $P < 0.001$, Fig. 5B). Gill cells exposed to verapamil (Fig. 5C and D), caused a decrease in V_{max} and K_m for anterior gill cells, while for posterior gill cells, V_{max} decreased while K_m increased 2x (ANOVA, $P < 0.001$, Fig. 5C and D). Vanadate exposure, an inhibitor of Ca-ATPase, caused a decrease in V_{max} only for anterior gill cells, while for posterior gill cells there was an increase in K_m (a decrease in transport affinity) and decrease in V_{max} (ANOVA, $P < 0.001$, Fig. 5E and F).

Figure 5: Relationship between intracellular Cd^{2+} ($\text{nM} \times 23.10^4 \text{ cells}^{-1} \times 90 \text{ sec}^{-1}$) and Cd^{2+} concentration (mM) for both anterior and posterior gill cells and different inhibitors. A and B. cells were incubated with nimodipine for 3 min at $11\mu\text{M}$; C and D. cells were incubated with verapamil for 3 min at $1\mu\text{M}$; E and F. Cells were incubated with vanadate for 3 min at $100\mu\text{M}$. Statistical differences between Km and Vmax are shown as asterisks for each Figure (One-Way ANOVA).

Anterior and posterior gill cells were incubated with extracellular Ca^{2+} at 1 and 20 mM or caffeine. After 30 s Cd at 1 mM was added alone or to the cells exposed to Ca^{2+} and caffeine (Fig. 6A and B). Results showed an increase for Cd influx for anterior gill cells exposed to caffeine (ANOVA, $P < 0.05$, Fig. 6A) but not for the other treatments. The same was seen for posterior gill cells, although the increase in Cd influx for cells treated with caffeine was not different from cells exposed to Cd and Ca^{2+} at 1 mM (ANOVA, $P < 0.05$, Fig. 6B). The effect of caffeine was, however, higher than cells exposed to the control (Cd alone).

Figure 6: Relationship between intracellular Cd^{2+} ($\text{nM} \times 23.10^4 \text{ cells}^{-1}$) and time (s) for anterior and posterior gill cells exposed to Cd^{2+} at 1 mM and different inhibitors or ions. Control at Cd 1mM; caffeine (100 μM); Cd and Ca (1mM) and Cd at 1mM and Ca at 20 mM. A. anterior gill cells; B. posterior gill cells. The vertical line represent the addition of Cd^{2+} (1mM) to the gill cells incubated with each different compound for 3 min. Different letters represent significant statistical difference (ANOVA, $P < 0.05$).

Anterior and posterior gill cells were incubated with vanadate, ouabain and vanadate + ouabain together. After 30 s, Cd at 1 mM was added alone or to the cells exposed to each inhibitor. Fig. 7A shows the effect of Cd^{2+} uptake for anterior gill cells. Intracellular Cd^{2+} was higher in the presence of ouabain compared to Cd alone, but lower when both ouabain and vanadate were added together (ANOVA, $P < 0.05$). The same trend was seen for posterior gill cells, an increase of Cd influx but at higher levels compared to anterior gill cells (Fig. 7B, ANOVA, $P < 0.05$). In addition, Cd transport in the presence of vanadate

was statistically lower than control, together with vanadate and ouabain added together (Fig. 7B, ANOVA, $P < 0.05$).

Figure 7: Relationship between intracellular Cd^{2+} (nM $\times 23.10^4$ cells $^{-1}$) and time (s) for anterior and posterior gill cells exposed to Cd^{2+} at 1 mM and different inhibitors. Vanadate (100 μM); Ouabaine (2mM) and Vanadate + Ouabaine together. The vertical line represent the addition of Cd^{2+} (1mM) to the gill cells incubated with each inhibitor for 3 min. Different letters represent significant statistical difference (ANOVA, $P < 0.05$).

Anterior and posterior gill cells incubated with BAPTA, an intracellular Ca^{2+} chelating agent, resulted in strong inhibition of intracellular Cd^{2+} influx for both K_m and V_{max} (Fig. 8), suggesting a dependence of Cd^{2+} transport and intracellular Ca^{2+} stores.

Figure 8: Relationship between intracellular Cd^{2+} ($\text{nM} \times 23.10^4 \text{ cells}^{-1} \times 90 \text{ sec}^{-1}$) and Cd^{2+} concentration (mM) for both anterior and posterior gill cells incubated with BAPTA, an intracellular Ca^{2+} quelator, for 1 h. Km and Vmax values are shown, and there was a statistically significant difference for Km and Vmax for both gill cells.

To understand the mechanism of Cd^{2+} influx and the effect of ATP, anterior and posterior gill cells were exposed to Cd^{2+} alone, Cd^{2+} followed by ATP after 20 s, ATP followed by Cd^{2+} after 20 s and Cd^{2+} + ATP added to the cells at the same time (Fig. 9A and B). The results showed that Cd^{2+} and ATP added in different order did not affect the intracellular levels of the metal (Kruskal–Wallis One Way Analysis of Variance on Ranks, $P > 0.05$). The experiment was performed for both anterior and posterior gill cells, with larger increases in intracellular Cd^{2+} for posterior gill cells compared to anterior gill cells (Fig. 9B).

Fig. 10 shows an integrative view of the suggested mechanisms of Cu transport in crab gill cells according to the results reported here. Overall Cd enter gill cells through: (a) Ca^{2+} channel – nimodipine and verapamil inhibited; (b) $\text{Cd}^{2+}/\text{Ca}^{2+}(2\text{Na}^+)$ exchanger where intracellular Na^+ increase result in augmented Cd^{2+} influx, increased (caffeine) or decreased intracellular Ca^{2+} (BAPTA), increase or decrease Cd^{2+} influx, respectively. However there was no effect of increased extracellular Ca^{2+} , (c) Na^+/K^+ -ATPase inhibited by ouabain, increased Cd^{2+} influx probably through intracellular Na^+ alteration; (d) Ca^{2+} -ATPase, vanadate sensitive, decreased Cd^{2+} influx, an effect not explained here by an elevation of intracellular Ca^{2+} but through a probable effect in cell membrane potential, resulting in altered Cd^{2+} influx.

Figure 9: Relationship between intracellular Cd²⁺ (nM x 23.10⁴ cells⁻¹) and time (s) for anterior and posterior gill cells exposed to Cd²⁺ and ATP (1mM for both) added at different time points according to the hatched line. Cd²⁺ alone; Cd²⁺ and ATP; ATP and Cd; and Cd²⁺ + ATP added simultaneously. (+) and (-) represent the addition or not of the different compounds below the hatched line. There was no statistical difference among the treatments.

Fig. 10. Working model for Cd²⁺ transport in *Ucides cordatus* gill cells. Cd²⁺ enters gill cells through: (a) Ca²⁺ channel, nimodipine and verapamil inhibited; (b) Cd²⁺/Ca²⁺ (2Na⁺) exchanger where intracellular Na⁺ increase result in augmented Cd²⁺ influx, increased (caffeine) or decreased intracellular Ca²⁺ (BAPTA), increase or decrease Cd²⁺ influx, respectively. However there was no effect of increased extracellular Ca²⁺; (c) Na⁺/K⁺-ATPase inhibited by ouabain,

increased Cd^{2+} influx probably through intracellular Na^+ alteration; (d) Ca^{2+} -ATPase, vanadate sensitive, decreased Cd^{2+} influx, an effect not explained here by an elevation of intracellular Ca^{2+} but through a probable effect in cell membrane potential, resulting in altered Cd^{2+} influx.

4. DISCUSSION

The use of FluoZin as a fluorescent probe for monitoring changes in intracellular concentration of cadmium was shown here to be effective to monitor this metal influx. The results strongly suggest that Cd^{2+} transport from gill cells of *U. cordatus*, a hypo-hyper-regulating crab occurs through Ca^{2+} transporting proteins due to the transporter sensitivity to Ca^{2+} channel blockers. Additionally the transporter is vanadate sensitive, an ATPase blocker. In the presence of ATP, however, Cd^{2+} influx did not change. Also, the transporter is highly dependent on intracellular Ca^{2+} stores and Na^+ and/or membrane potential, both affecting Cd^{2+} transport. Extracellular Ca^{2+} , on the other hand, did not affect Cd uptake and there were no differences between anterior and posterior gill cells for uptake of Cd^{2+} .

For crustaceans, it has been shown that Cd^{2+} influx is affected by Ca^{2+} concentration using, in these cases, perfused gills cells (Lucu and Obersnel, 1996; Silvestre et al., 2004; Bondgaard and Bjerregaard, 2005). In addition, Cd^{2+} affects Na^+/K^+ -ATPase activity when administered in the basolateral side of perfused gills of the crab *C. maenas* (Postel et al., 1998). Another work with perfused gills of *Eriocheir*, an euryhaline crab like the one studied here (Silvestre et al., 2004), the authors did not see Cd^{2+} influx through posterior gills, only for anterior ones. However, they saw Cd^{2+} accumulation in both gills. Influx rates for *Eriocheir* in that work were around $0.17 \text{ nmol Cd}^{2+} \text{ g}^{-1} \text{ gill w w h}^{-1}$ (Silvestre et al., 2004). Although it was measured here influx through isolated cells from posterior and anterior gills, intracellular Cd^{2+} accumulation was in the nanomolar range as well. Usually there are 4 different types of cells in crustaceans gills in general (reviewed by Freire et al., 2008), and we expect higher amount of ionocytes from posterior gills compared to anterior gills (see Ortega et al., in press). Interestingly, Pedersen and Bjerregaard (2000) measured influx and efflux across perfused gill cells of *Carcinus* and influx rates were also in the nanomolar range but, surprisingly, efflux rates were very similar

to influx rates in their work. In addition, a change in membrane potential difference from negative to positive across perfused gills in that work apparently altered Cd^{2+} influx, caused by the addition of a Na^+ -free solution to the perfused gills (Pedersen and Bjerregaard, 2000). It was seen here a Na^+ ion effect, through the inhibition of Na^+/K^+ -ATPase and as a result, an augmentation in Cd^{2+} influx. It seems that a change in the membrane potential alters Cd^{2+} cell influx rates. More details on the types of cells found in gills of crustaceans are revised elsewhere (Freire et al., 2008) and were characterized in Ortega et al. (in press).

4.1. Cd^{2+} and Ca^{2+} channels

The addition of Ca^{2+} and Cd^{2+} together to the extracellular medium did not affect Cd^{2+} influx, although Ca^{2+} channel blockers inhibited Cd^{2+} influx. Apparently, the rapid passage of both ions simultaneously through diffusion using channels is not limiting for each ion, however blocking the channel affects Cd^{2+} uptake in gill cells. Epithelial Ca^{2+} channels, in contrast with excitable cells, have been characterized in intestine and kidney of mammals (Nilius et al., 2001) and have a constitutive presence of Ca^{2+} permeability at physiological membrane potentials (Den Dekker et al., 2003) and, in addition, allow passage of other divalent cations. Interestingly, intracellular ATP is necessary to maintain epithelial Ca^{2+} channel activity steady in mammals, although the mechanism of ATP-induced activation is not known yet (Den Dekker et al., 2003). This could be an explanation for results found here on Cd^{2+} influx through epithelial channels in crabs and the fact that Cd^{2+} gill cell transport was affected by vanadate. Epithelial Ca^{2+} channel has not been elucidated for crabs, though. Energy dependence for cell Cd^{2+} uptake has also been reported for oyster gills (Roesijadi and Unger, 1993), and Cd^{2+} transport was also inhibited by calcium channel blockers (see review by Wright, 1995). Moreover, Li et al. (2012a) saw that a freshwater crab exposed to Cd^{2+} had decreased levels of Na^+/K^+ -ATPase and Ca^{2+} -ATPase in gills, followed by a lower metallothionein mRNA levels, which is known to chelate intracellular Cd^{2+} in general.

Pancreatic cells possess L-type voltage-dependent calcium channels (VDCC) and represent a pathway of Cd^{2+} influx in these cells (Gavazzo et al.,

2005). Nimodipine is a specific inhibitor of these VDCC and was shown to modulate Cd^{2+} influx for insulinoma (pancreatic) cells (Gavazzo et al., 2005). It was seen here a similar effect of the same blocker on Cd^{2+} influx, although the presence of an L-type Ca^{2+} channel has not been characterized in crustacean gill cells. Verapamil, also an L-type Ca^{2+} channel inhibitor, was also effective at inhibiting Cd^{2+} influx seen in our results.

4.2. Cd^{2+} and intracellular Ca^{2+}

A study with rat enterocytes, cells of epithelial origin, showed that extracellular as well as intracellular Ca^{2+} binding sites for Ca^{2+} transporters have affinities for Cd^{2+} two orders of magnitude higher than for Ca^{2+} (Verbost et al., 1987), also observed for fish gills (Verbost et al., 1988). They concluded that Cd^{2+} affect intracellular Ca^{2+} homeostasis due to the extreme sensitivity of the Ca-pumping ATPase in basolateral plasma membrane and other internal ATP-driven Ca^{2+} transport systems. Here, chelating intracellular Ca^{2+} was affecting Cd^{2+} transport negatively, suggesting a dependence of Cd^{2+} for intracellular Ca^{2+} stores. Similarly, the use of caffeine, responsible for releasing Ca^{2+} from sarcoplasmic reticulum (Mandal et al., 2005), affected Cd influx positively. Therefore, lowering of intracellular Ca^{2+} and an increase of the same ion inside gill cells were affecting intracellular Cd^{2+} influx, strongly suggesting that a $\text{Ca}^{2+}/\text{Na}^{+}$ exchanger in the gill cell plasma membrane could also exchange Cd^{2+} .

4.3. Cd, $\text{Na}^{+}/\text{K}^{+}$ -ATPase and effects of ATP In addition, the use of extracellular Ca^{2+} was not affecting intracellular Cd^{2+} accumulation as seen for intracellular Ca^{2+} .

Here, ouabain affected positively Cd^{2+} influx and again, an increase in intracellular Na^{+} , through inhibition of the $\text{Na}^{+}/\text{K}^{+}$ -ATPase, could possibly be another factor to reinforce the presence of a $\text{Cd}^{2+}/\text{Ca}^{2+}(\text{Na}^{+})$ exchanger in gill cells. Interestingly, the positive effect of ouabain in these cells was lost when vanadate and ouabain were added together, now through a larger decrease in intracellular Cd^{2+} influx. The reason for such effect is not clear at the moment.

Probably inhibitors affecting at the same time Na^+/K^+ -ATPase plus Ca^{2+} -ATPase generate a large change in major intracellular ions and in the cell electrochemical gradient that affects negatively Cd^{2+} influx and overcomes the effect of ouabain alone.

The presence of a Cu^{2+} -ATPase in crab gill cells was suggested by Sá and Zanotto (2013), as seen by the presence of ATP causing increased influx of intracellular Cu^{2+} . Cd^{2+} , studied here, was not affected by ATP. The essentiality of the metal Cu^{2+} for crustaceans, in contrast with Cd^{2+} , suggest basic differences between transport of both ions. Here some inhibitors had a positive effect on Cd^{2+} influx, not seen for Cu^{2+} (Sá and Zanotto, 2013) and extracellular Ca^{2+} had no effect on Cd^{2+} transport, different from effects seen with intracellular Ca^{2+} . In addition, Zn^{2+} transport in lobster gill cells (Sá et al., 2009), another essential metal, shares many similarities with Cu^{2+} transport in crabs (Sá and Zanotto, 2013), unlike the non-essential metal Cd^{2+} studied here.

5. Conclusions

In conclusion, the present work elucidates the mechanisms of Cd^{2+} influx, a non-essential metal, in crab gill cells. Although the Cd concentrations used here were not similar to environmental Cd^{2+} , it still represents, in a larger scale, how Cd penetrate epithelial cells and how it interacts with other ions. Cd influx appears to occur through Ca^{2+} channels, and further routes of Cd^{2+} influx are evident, that is, through the gill cell plasma membrane $\text{Cd}^{2+}/\text{Ca}^{2+}$ (Na^+) exchanger due to a dependence of Cd^{2+} on intracellular Ca^{2+} and Na^+ (see Fig. 10 for details). Additionally, alterations in the transporter Na^+/K^+ -ATPase through ouabain, augmented Cd influx. The metal influx or efflux is not affected by ATP, and the presence of a Cd^{2+} -ATPase to get rid of excess Cd, as seen in bacterial cells (Thévenod, 2010; Mielniczki-Pereira et al., 2011), appears very unlikely here.

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Capítulo 2

Characterization of cadmium transport in hepatopancreatic cells from a mangrove crab *Ucides cordatus*: the role of calcium.

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Characterization of cadmium transport in hepatopancreatic cells of a mangrove crab *Ucides cordatus*: the role of calcium.

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Abstract

Cadmium is a toxic metal, present in batteries and discarded in estuaries and mangrove habitats. Apart from that, it is a non-essential metal that causes toxic effects in many organisms. Cadmium accumulates in gills and hepatopancreas and it is possible that its influx occurs by calcium routes. The objective of this study was to characterize the transport of cadmium and the role of calcium in different cell types from hepatopancreas of the mangrove crab *Ucides cordatus*. For this, the hepatopancreas was dissociated by magnetic stirring and after that separated by a sucrose gradient. Then, the cells were labeled with FluoZin 3 and different CdCl_2 concentrations were added together with a variety of inhibitors. The results showed that Cd^{2+} transport occurs differently in each cell type and is partially explained by the function the cells perform in the hepatopancreas. Embryonic (E) and Resorptive (R) cells transported more Cd compared to Fibrillar (F) and Blister (B) cells. R cells responded to Ca^{2+} channel inhibitors and intracellular Ca^{2+} manipulations positively, as the other cell types, but in a stronger way. B cells were the least responsive to Ca^{2+} channel inhibitors and, unlike the other cells, responded negatively to intracellular Ca^{2+} manipulations. The results indicate that Ca^{2+} affects the transport of Cd^{2+} in hepatopancreatic cells of *Ucides cordatus* and uses Ca^{2+} channels to enter these cells. In addition, information about Ca concentration in the environment could be used as a mitigating factor for Cd accumulation.

Keywords: *Ucides cordatus*, hepatopancreas, cells, CdCl_2 transport, inhibitors.

1. Introduction

Cadmium (Cd^{2+}) is a toxic metal (Kang *et al.*, 2012) and is released into the air, water and soil by industry and through human activities. Cd^{2+} is not an essential metal for animals and, in high concentrations leads to a range of adverse effects. In aquatic ecosystems, Cd^{2+} has a high solubility in water, and accumulates easily in aquatic species, such as mollusks and crustaceans (Liu *et al.*, 2013). Cadmium accumulation occurs mainly in gills and hepatopancreas, because these organs are the first in contact with the environment (Silvestre *et al.*, 2005; Sokolova and Lanning, 2008).

The hepatopancreas is composed of four major cell types: E cells (embryonic cells originating the other cell types), R cells (absorptive cells), F cells (secretion of digestive enzymes) and B cells (intracellular digestion and excretion of enzymes and xenobiotics) (Chavez-Crooker *et al.*, 2001; Kang *et al.*, 2012). The hepatopancreas are considered as detoxification organs in crustaceans and reduce the adverse effects of toxic metals in the circulation (Kang *et al.*, 2012). Therefore, we expect that Cd^{2+} can be transported by hepatopancreatic cells through membrane transport processes. At the cellular level, it is known that Cd^{2+} is taken up by gill cells through transporters for other essential ions (Ortega, *et al.*, 2014). Ahearn *et al.* (1999) studied the transcellular transport of hepatopancreatic epithelial cells using membrane vesicles from lobster *Homarus americanus* and observed that the metal Cd^{2+} can enter the cells by the $2\text{Na}^+/\text{H}^+$ antiporter and, inside the cells, the metal can be stored in vesicles and detoxified. One other possibility is the transport through hepatopancreatic cells via Ca^{2+} channels or through competition between Ca^{2+} and Cd^{2+} . For aquatic organisms, studies using radioactive tracers show that Cd^{2+} can enter the gill epithelium by calcium transport routes in fish, mollusks and crustaceans (Perry and Flik, 1988; Wicklund Glynn *et al.*, 1994; Roesijadi and Unger, 1993; Ortega *et al.*, 2014). This possibility occurs by the accidental uptake via calcium transport proteins or through competition by the apical calcium channel or basolateral Ca^{2+} -ATPase, as demonstrated by

Matsuo *et al* (2005) in gill chloride cells of fish and Ahearn *et al* (1999), in lobster hepatopancreas.

To date, the only study in isolated hepatopancreatic cells and metal transport in crustaceans looked at Cu^{2+} transport (Chavez-Crooker *et al.*; 2001). Copper uptake by epithelial cells of lobster hepatopancreas occurred in the presence of calcium. Ca^{2+} appeared to affect the kinetics constants for Cu transport, suggesting that both metal binding and its rate of transport across the membrane were influenced by calcium. Zinc was a competitive inhibitor of Cu transport in E and R cells, because both copper and zinc use the same calcium-dependent, carrier-mediated, transport system for uptake into epithelial cells. Moreover, in Ahearn *et al.*, (1994) using a brush-border membrane vesicles from hepatopancreas, both zinc and cadmium inhibited the uptake of calcium by sensitive or insensitive amiloride carrier processes. Other studies by Ahearn *et al.*, (1999) suggested that, once inside hepatopancreatic epithelial cells, cadmium serves as an apparent intracellular regulator for iron uptake. In studies by Vitorino *et al* (submitted, 2016), the authors observed an increase in iron transport, mainly in E cells, reflecting their demand for nutrients to allow cell replication and differentiation. In the same work, the researchers noted that iron internalization occurred by divalent metal transporters, in the case of free iron in solution, or by endocytosis or passive diffusion, in case of iron nanoparticles.

Cadmium transport in isolated hepatopancreatic cells has not been studied in a mangrove crab, and the membrane pathway for intracellular cadmium transport is not fully elucidated in many organisms (Ortega *et al.*, 2014). Therefore, it is not known how Cd^{2+} enters hepatopancreatic cells in these organisms. Thereby, the objective of this work was to characterize Cd transport in isolated cells of the hepatopancreas from a mangrove crab, *Ucides cordatus*, using different inhibitors for Ca^{2+} .

2. Materials and methods

2.1 Animals

Male crabs, in the intermoult period, were collected at Praia dos Pescadores beach, Itanhaém, São Paulo, and brought to the vivarium of the University of São Paulo for 1 week acclimation. The crabs were kept in tanks with seawater at 20 ppt, gravel, water filter and pieces of brick for the emersion of the animals. Photoperiod was constant (12:12) and temperature ($22 \pm 3^{\circ}\text{C}$). For each set of experiments, N=4 animals were used.

2.2 Cellular dissociation

For hepatopancreatic cells dissociation, a magnetic stirring method was used, where the organ was immersed in 10 mL of the extraction solution (composed of mmol l^{-1} of 395 NaCl, 10 KCl, 2.5 NaHCO_3 , 2.5 NaH_2PO_4 , 3.75 Hepes, 1 glucose and 0.9 EDTA, pH buffered to 7,8), agitated for 15 min, filtered using a 30 μm nylon mesh and centrifuged for 10 min at 115 x g at 5°C . The pellet was suspended in extraction solution and cells were separated using the Sucrose gradient method (Ortega *et al.*, 2011), where concentrations of sucrose (10, 20, 30 and 40%) were prepared and added to a Falcon tube (15 mL), forming a discontinuous gradient, and centrifuged for 5 min at 115 x g at 5°C . After this, the pellet from each gradient was separated.

2.3 Cellular transport

The hepatopancreatic cells previously dissociated were labeled with FluoZin 3 (1 μL) during 1 hour under shaking at 200 rpm at room temperature. After, the cells were centrifuged at 405 x g for 5 min and then washed in extraction solution, without EDTA. In the fluorimeter (Biotek), the emission used was 525 nm and the excitation 495 nm. The cellular transport was then measured each 90 s in real time. In an Elisa plate, 180 μL of cells were added with CdCl_2 concentrations of 0.15; 0.5; 0.75, 1.0 and 1.25 mM, respectively. The variation in the fluorescence was measured for each concentration.

The calibration curve was prepared based on Zanotto and Baptista (2011). At the end of the experiment, 180 μL of each cell type without cadmium were added to an Elisa plate with 50 μL of Triton X-100 20% for 15 min. To obtain the minimum fluorescence (F_{min}), 50 μL of HEDTA (1 mM) was added.

For the maximum fluorescence ($F_{\max}$), 50 μL of Triton X-100 20% was added during 15 min together with 50 μL of CdCl_2 (5 mM) in a different batch of cells. Intracellular cadmium was calculated using the following equation:

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

where $[\text{Cd}^{2+}]_i$ is the intracellular free cadmium concentration (nM); K_d is the dissociation constant of FluoZin 3 am (1.88); F is the actual fluorescence measured; $F_{\min}$ is the minimum fluorescence observed and $F_{\max}$ is the maximum fluorescence verified.

2.4 Experimental design

To investigate cadmium transport, the following inhibitors were added: (a) verapamil (1 μM) and nimodipine (111 mM), inhibitors that block slow calcium channels and L-type voltage-dependent calcium channels, respectively (Ahearn and Franco, 1993; Gavazzo *et al.*, 2005): (b) ouabain (2 mM), a Na^+/K^+ -ATPase inhibitor (Zare and Greenaway, 1998): (c) vanadate (100 μM), a Na^+/K^+ and Ca^{2+} -ATPase inhibitor (Zare and Greenaway, 1998): (d) BAPTA (2 mM) a calcium intracellular chelator and (e) caffeine (20mM) which release intracellular calcium from endoplasmic reticulum.

For experiments using BAPTA, ouabain and caffeine, specifically, each inhibitor was incubated with hepatopancreatic cells and FluoZin 3 during 1 h. After, cellular cadmium transport was measured through time (90 s), using 1mM of CdCl_2 .

2.5 Data analysis

One-Way ANOVA was used to compare different inhibitors (treatments) against control. Cadmium transport was also compared between treatments using the kinetic variables, K_m and $V_{\max}$, for data displayed as Cd transport

against Cd concentration and/or time, after verifying the kinetic parameters fit using SigmaPlot. The statistical package used was Sigma Stat and the data were verified for normality and homogeneity of variance. Data are displayed as means $\pm$ SE.

3. Results

The results demonstrated differences in cadmium influx, depending on the cell type. There was a cadmium influx at all Cd concentrations, but E cells and R cells showed a higher Cd transport kinetics, K_m and V_{max} , compared to F and B cells ($P < 0.05$; Figure 1). These last two cell types showed similar transport kinetics between them.

Cells	Vmax	Km
E	2.91 $\pm$ 1.04 (*)	1.84 $\pm$ 0.98
R	5.36 $\pm$ 3.75(*)	3.94 $\pm$ 3.43 (*)
F	0.57 $\pm$ 0.12	1.21 $\pm$ 0.44
B	0.89 $\pm$ 0.26	3.36 $\pm$ 1.25

Figure 1: Cadmium transport ($\text{nM} \times 23.10^4 \text{ cells}^{-1} \times 90 \text{ secs}^{-1}$) in hepatopancreatic cells of a mangrove crab *Ucides cordatus* at Cd concentrations of 0.15, 0.25, 0.5, 0.75, 1.0 and 1.25 mM added to the different cells. $N = 4$, $P < 0.05$.

For E cells (Figure 2), cadmium transport kinetics was statistically different between control and the inhibitors nimodipine and vanadate. There was a decrease in K_m and V_{max} in the presence of these inhibitors ($P < 0.05$). R cells, (Figure 3), showed differences for Cd transport compared to control: nimodipine change K_m and V_{max} compared to control (decrease); for vanadate, there was a decrease in K_m , and for verapamil, a decrease in K_m and V_{max} . These differences were significant ($P < 0.05$ and $P < 0.001$; Figure 3). F cells (Figure 4) showed a difference in transport ($P < 0.05$) using the inhibitor nimodipine, vanadate and verapamil, with a decrease in K_m and V_{max} . Finally, B cells (Figure 5) showed significant differences for cadmium transport in the presence of nimodipine, vanadate and verapamil, with a decrease in K_m and V_{max} ($P < 0.05$).

E cells	V_{max}	K_m
Control	2.91 ± 1.04	1.84 ± 0.98
Nimodipine	0.69 ± 0.041 (*)	0.21 ± 0.05 (*)

(*) $P < 0.05$

E cells	V_{max}	K_m
Control	2.91 ± 1.04	1.84 ± 0.98
Vanadate	0.41 ± 0.02 (*)	0.12 ± 0.02 (*)

(*) $P < 0.05$

E cells	V_{max}	K_m
Control	2.91 ± 1.04	1.84 ± 0.98
Verapamil	1.53 ± 0.48	1.33 ± 0.68

Figure 2: Cadmium transport ($\text{nM} \times 23.10^4 \text{ cells}^{-1} \times 90 \text{ secs}^{-1}$) in E cells from hepatopancreas of a mangrove crab *Ucides cordatus* at Cd concentrations of 0.15, 0.25, 0.5, 0.75, 1.0 and 1.25 mM in the presence of nimodipine (111 mM), vanadate (100 μM) and verapamil (1 mM), added to the cells. N = 4, $P < 0.05$.

Figure 3: Cadmium transport ($\text{nm} \times 23.10^4 \text{ cells}^{-1} \times 90 \text{ secs}^{-1}$) in R cells of a mangrove crab *Ucides cordatus* at Cd concentrations of 0.15, 0.25, 0.5, 0.75, 1.0 and 1.25 mM in the presence of nimodipine (111 mM), vanadate (100 μM) and verapamil (1 mM), added to the cells. N = 4, $P < 0.05$; $P < 0.001$.

Figure 4: Cadmium transport ($\text{nM} \times 23.10^4 \text{ cells}^{-1} \times 90 \text{ secs}^{-1}$) in F cells of a mangrove crab *Ucides cordatus* at Cd concentrations of 0.15, 0.25, 0.5, 0.75, 1.0 and 1.25 mM in the presence of nimodipine (111 mM), vanadate (100 μM) and verapamil (1 mM), added to the cells. N = 4, P<0.05.

Figure 5: Cadmium transport ($\text{nm} \times 23.10^4 \text{ cells}^{-1} \times 90 \text{ secs}^{-1}$) in B cells of a mangrove crab *Ucides cordatus* at Cd concentrations of 0.15, 0.25, 0.5, 0.75, 1.0 and 1.25 mM in the presence of nimodipine (111 mM), vanadate (100 uM) and verapamil (1 mM), added to the cells. N = 4, P<0.05.

It the presence of ouabain (inhibitor of Na^+/K^+ ATPase) and caffeine (responsible for Ca^{2+} efflux from sarcoplasmic reticulum) we observed that E, R and F cells showed a significantly higher transport kinetics compared to control cells. For B cells, on the other hand, results showed the opposite, a decrease of Cd transport kinetics in the presence of ouabain and caffeine. In contrast, the inhibitor BAPTA did not interfere with cadmium transport for E, F and B cells.

For R cells, K_m for Cd transport was decreased (affinity increased) in the presence of BAPTA (Figure 6A, B, C and D).

Figure 6: Cadmium transport ($\text{nM} \times 23.10^4 \text{ cells}^{-1} \times 90 \text{ secs}^{-1}$) in hepatopancreatic cells of a mangrove crab *Ucides cordatus* in relation to time, using Cd concentration at 1.0 mM, in the presence of BAPTA (2 mM), ouabain (2 mM) and caffeine (20 mM) added to the cells. $N = 4$. $P < 0.05$.

Table 1 shows an integrative review of the influx of cadmium for each cell type from hepatopancreas and in the presence of different inhibitors. Overall, Cd enter by calcium channel (inhibited by nimodipine and verapamil) in F and R cells, and the transport is also affected by intracellular calcium in all cell types.

Table 1: Summary of cadmium transport in different cell types from hepatopancreas, in the presence of different inhibitors. The negative effect in Cd transport was indicated by the negative symbol (-), the positive effect was indicated by the positive symbol (+) and when the Cd transport was not affected by the inhibitor, we use the term NE (no effect).

Cell Type	Inhibitors					
	Nimodipine	Vanadate	Verapamil	BAPTA	Ouabain	Caffeine
E	-	-	-	NE	+	+
R	-	-	-	-	+	+
F	-	NE	-	NE	+	+
B	-	-	-	NE	-	-

4. Discussion

Cadmium transport occurs differently for each hepatopancreatic cell because of different functional characteristics of each cell, that reflect the routes a toxic metal such as Cd^{2+} could enter these cells. E and R cells have higher Cd^{2+} transport, about 3 to 5 times higher (both K_m and V_{max}) when compared to F and B cells. In addition, R-cells are highly affected by Ca^{2+} inhibitors compared to the other cell types, and show increased affinity for Cd^{2+} when intracellular Ca^{2+} is manipulated. B cells, on the other hand, are affected in the opposite manner compared to the other cell types, decreasing Cd transport when intracellular Ca is manipulated.

Cadmium is a toxic metal, non- essential to animals and, in high concentrations, cause cell damage (Kang *et al.*, 2012). Work with a freshwater crab, *Sinoptamon henanense*, showed that Cd accumulates in the hepatopancreas, known as an important detoxification organ (Wu, *et al.*, 2013). Cd is believed to enter cells via $\text{Na}^+ / \text{Ca}^{2+}$ exchanger and through Ca^{2+}

channels, showing direct competition with calcium because of their similar ionic radii (Ahearn *et al.*, 1999; Matsuo *et al.*, 2005). Another study with cadmium transport in gill cells of *Ucides cordatus*, we observed that cells present in posterior gills had increased Cd^{2+} transport compared with cells located in anterior gills. Cells from posterior gills have a greater amount of mitochondria, and are responsible for ion transport and osmoregulation. In contrast, cells from the anterior gills are responsible for cellular respiration processes (Ortega *et al.*, 2014). The transport affinity for Cd^{2+} is higher in gills compared to hepatopancreas. Likewise, the hepatopancreatic R-cells are well known for ion transport and absorption in this organ, having a larger amount of mitochondria and ion exchangers (Chavez-Crooker *et al.*, 2001; Kang *et al.*, 2012; Liu *et al.*, 2013; Ortega *et al.*, 2014) compared to the other cell types. Thus, cadmium transport is higher in R cells due to their major transport role. For Embryonic (E cells), a high mitotic activity are present in these cells, which are constantly replicating. This could similarly explain the higher Cd^{2+} transport observed here (Vogt, 1994; Chavez-Crooker *et al.*, 2001; Kang *et al.*, 2012; Liu *et al.*, 2013; Ortega *et al.*, 2014).

F cells, in turn, are responsible for digestive enzyme synthesis, transcellular nutrient transport such as glucose and aminoacids, and may also act in metal detoxification (Verri *et al.*, 2001; Fiandra *et al.*, 2006). These cells showed a lower transport of Cd^{2+} when compared with R and E cells. As F cells have a role in detoxification of metals, cadmium could possibly enter this cell, but could be subsequently stored in supranuclear vacuoles or lysosomes, which are specific regions of detoxification in these cells (Vogt, 1994). As a consequence, the process of rapid detoxification could be responsible for a decrease in intracellular Cd^{2+} fluorescence, as observed in the results presented here. The same reasoning can be used for B cells. These cells have a primary function of metal detoxification, and have vacuoles which occupy most of the cell, where cadmium could be stored, causing a similar decrease in intracellular cell fluorescence (Chavez-Crooker *et al.*, 2001; Kang *et al.*, 2012; Liu *et al.*, 2013; Ortega *et al.*, 2014).

In the presence of inhibitors, each cell exhibited different behavior with respect to Cd^{2+} transport. R cells, in the presence of nimodipine, vanadate and

verapamil, showed a significant decrease in Cd transport. Thus, it can be suggested that cadmium transport in R cells occurs via L-type calcium channel and slow calcium channels, and in addition, a Ca^{2+} -ATPase inhibitor also affected Cd^{2+} transport (Ahearn *et al.*, 1999; Ahearn *et al.*, 2004). Moreover, R cells showed an increase Cd transport affinity in the presence of ouabain and caffeine, and a decrease in the presence of BAPTA. This fact suggests a relation between intracellular calcium and cadmium transport (Ahearn *et al.*, 1999; Ahearn *et al.*, 2004; Ortega *et al.*, 2014). R cells contain microvilli in the apical region (Vogt, 1994; Chavez-Crooker *et al.*, 2001; Ortega *et al.*, 2014). This specific cellular region is responsible for transport, and allow more influx of Cd^{2+} , that can substitute Ca^{2+} by competition. Some studies revealed that there is a $\text{Ca}^{2+} / \text{Na}^{+}$ exchanger in membrane border epithelium of crustacean's hepatopancreas. Toxic metals, such as cadmium, could act as a competitive inhibitors of Ca^{2+} uptake by the brush border membrane exchanger (Ahearn *et al.*, 1994, 2001; Zhuang *et al.*, 1995; Zhuang and Ahearn, 1996). So, our results strongly suggested that a $\text{Ca}^{2+} / \text{Na}^{+}$ exchanger in the cell plasma membrane could also exchange Cd^{2+} . A study in rat enterocytes showed that intracellular Ca^{2+} binding sites have high affinity for Cd^{2+} , which is two orders of magnitude higher than for Ca^{2+} (Verboost *et al.*, 1988; Ortega *et al.*, 2014). So, Cd^{2+} could affect intracellular Ca^{2+} homeostasis due to the extreme sensitivity of the Ca-ATPase and other internal Ca^{2+} transport systems. In our study, we verified that, in the presence of BAPTA, an intracellular Ca^{2+} chelating agent, Cd^{2+} transport was affected negatively, suggesting a dependence for intracellular Ca^{2+} stores. The use of caffeine, responsible for releasing Ca^{2+} from sarcoplasmic reticulum (Mandal *et al.*, 2005), affected the transport of Cd^{2+} positively, suggesting that is necessary the presence of intracellular Ca^{2+} for the Cd^{2+} transport.

E cells are constantly replicating, and could contribute to increased cellular transport (Vogt, 1994). For E cells, cadmium transport was affected by nimodipine (inhibitor responsible for L-type calcium channel blocking), vanadate (inhibitor of $\text{Na}^{+} / \text{K}^{+}$ ATPase and Ca^{2+} -ATPase) and verapamil (inhibitor of slow calcium channels). It can be suggested that cadmium transport in E cells occurs via calcium channels, and in addition, a Ca^{2+} -ATPase transporter was also

involved (Ahearn *et al.*, 1999; Ahearn *et al.*, 2004). E cells, however, showed elevated cadmium transport in the presence of ouabain and caffeine. This means that intracellular calcium affects the input or output of Cd^{2+} , as well as the presence of Na^+ or changes in membrane potential (Ahearn *et al.*, 1999; Ahearn *et al.*, 2004; Ortega *et al.*, 2014). In our studies, ouabain affected positively Cd^{2+} influx again and, an increase in intracellular Na^+ , through inhibition of the Na^+ / K^+ ATPase, could possibly be another factor to reinforce the presence of a $\text{Cd}^{2+} / \text{Ca}^{2+}(\text{Na}^+)$ exchanger in hepatopancreatic cells (Ortega *et al.*, 2014). In addition, a change in membrane potential caused by intracellular Na^+ could change Cd^{2+} influx rate.

F cells, in turn, showed a decrease in cadmium transport in the presence of nimodipine and verapamil. According to these results, we suggest that Cd^{2+} input occurs via an L-type calcium channel and slow calcium channels (Ahearn *et al.*, 1999; Ahearn *et al.*, 2004). F cell showed an increased transport in the presence of ouabain and caffeine. We also suggest that intracellular calcium in these cells directly affects Cd^{2+} transport (Ahearn *et al.*, 1999; Ahearn *et al.*, 2004; Ortega *et al.*, 2014).

Finally, Cd transport in B cells was affected by nimodipine, verapamil and vanadate. As these cells have the main function of storage, it is suggested that cadmium can enter these cells by calcium channel or $\text{Ca}^{2+} / 2\text{Na}^+$, and may have been stored inside the cell (Vogt, 1994; Ahearn *et al.*, 1999; Ahearn *et al.*, 2004; Ortega *et al.*, 2014). In this case, B cell was not affected by the presence of BAPTA, and contrary to what was seen in the other cell types, there was a decrease in Cd^{2+} transport in the presence of ouabain and caffeine. We suggest that there is a competition between cadmium and intracellular calcium in these cells (Ortega *et al.*, 2014). These cells also play a role in detoxification and accumulation, which is important for the bioindicator animal (Vogt, 1994; Ortega *et al.*, 2014), and Cd^{2+} could enter these cells and be sequestered by vacuoles or chelating proteins like metallothioneins. In a study by Ahearn *et al.*, (2004), the authors suggested that some granules with toxic metals can occur in membrane-bound vesicles or vacuoles that could be involved in Cd^{2+} transport to the lysosome for later excretion (Havelaar *et al.*, 1998; Ahearn *et al.*, 2004).

5. Conclusions

Cadmium transport occurs differently in each cell type and is partially explained by the function each cell perform in the hepatopancreas. E and R cells have higher Cd cell transport compared to F and B cells. E and R cells responded to Ca^{2+} channel inhibitors and intracellular Ca^{2+} manipulations more strongly than the other cell types. B cells responded to Ca^{2+} channel inhibitors and, unlike the other cells, responded negatively to intracellular Ca^{2+} manipulations. F cells showed an intermediary response to Ca^{2+} manipulations. Therefore, the diversity of response of each hepatopancreatic cell to Cd^{2+} transport and the effect of Ca^{2+} reflects the role of this versatile organ in crustaceans: secretion and production of digestive enzymes, together with an absorption and transport role.

Moreover, metal transport studies show how crabs living in mangrove areas can be used as bioindicators of toxic metal contamination and how non-essential toxic metals can enter their cells. In addition, knowledge and manipulations of environmental Ca^{2+} can help mitigate Cd accumulation.

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Capítulo 3

Physiological differences in the crab *Ucides cordatus* from two populations inhabiting mangroves with different levels of cadmium contamination

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Physiological differences in the crab *Ucides cordatus* from two populations inhabiting mangroves with different levels of cadmium contamination

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Abstract

Crustaceans found in metal contaminated regions are able to survive and we decided to investigate the physiological mechanisms involved by comparing populations from contaminated and non-contaminated regions. The objective of this study was to measure the cellular transport of a non-essential metal (Cd, cadmium), in gills (G) and hepatopancreas (H) of *Ucides cordatus*, together with cell membrane fluidity, metallothionein levels (MT) and lipid peroxidation (LPO). The two populations compared were from a polluted (P) and a non-polluted (NP) mangrove area of São Paulo State, Brazil. We found, for the first time, a larger Cd transport in G and in H cells from crabs living in P mangrove areas. The cells also had lower plasma membrane fluidity, increased LPO and less MT compared to NP regions. We also found larger amounts of Cd in intracellular organelles of G from crabs in P regions, but not in H. Therefore, in polluted areas, these animals showed higher Cd transport, lower plasma membrane fluidity and storage of Cd intracellularly in G cells, while H cells used metallothionein as their main line of defense.

Keywords: cadmium, gills, 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, a major pollutant in mangrove areas, is a non-essential metal and is known to cause damage and accumulation in animals' tissues. In contact with crustaceans, it may cause physiological and morphological damage in gills and hepatopancreas, organs that are the first entry sites in these organisms. Moreover, toxic metals are some of the strongest naturally occurring carbonic anhydrase (CA) inhibitors⁴. Metals such as Ag^+ , Cd^{2+} , Cu^{2+} and Zn^{2+} are known to be inhibitory to CA activity *in vitro*, and to CA-related physiological processes *in vivo*. For example, Ag^+ is known to inhibit both gill CA and ion transport in freshwater fish^{4,5}, as well as intestinal ion transport in marine fish⁶. Gill CA from an estuarine crab (*Chasmagnathus granulata*) is highly sensitive to Cd^{2+} , with an IC (inhibitory concentration) value of $2.15 \mu\text{M}$ 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 Na^+ / K^+ ATPase¹. Other studies with crabs showed that cadmium exposure similarly affected intracellular calcium concentration^{3,8}. Therefore, it is possible that toxic metal exposure in estuarine organisms can inhibit multiple ion transport processes both individually and/or through the inhibition of gill CA. 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 (MT)^{8–10}. These low molecular weight proteins are extremely important to aquatic organisms, leading

to increased tolerance to some toxic metals during exposure¹¹. In general, metallothioneins play an important role in homeostasis and control of essential metals, mainly Cu, Cd and Zn^{12,13}. Studies with mollusks in Cd contaminated regions reveal an increase of MT in the cytosol of gill cells, acting as an important stress marker¹⁴. Another stress marker of environmental pollution is lipid peroxidation (LPO), also associated with excess of metals^{15–18}. Several studies with vertebrates and invertebrates showed that with rising pollution levels (particularly with respect to cadmium), there is an increase in the antioxidant systems, including LPO¹⁹.

Environmental changes caused by human activities also interfere with biological membranes, transferring external signals to cell internal milieu²⁰. In experiments with crabs (*Carcinus maenas*), mobilization of fatty acids from the hepatopancreas was a response to toxic metal contamination²². Long chain polyunsaturated fatty acids (PUFA) play a role in osmoregulation, nutrient assimilation and ion transport. Furthermore, changes in the proportion of monounsaturated fatty acids (MUFA) modulate protein activity. Reduction in some proteins and ionic channels are correlated with a reduction in PUFA and an increase in MUFA, and both can influence the permeability of the cell membrane and Na⁺ / K⁺ ATPase activity²¹. In this work, the breakdown of fatty acids was used as energy for detoxification and excretion of toxic metals²².

Estuarine crab gills are usually divided into the anterior gill, that is a typical gas exchange epithelium made up of thin cells, and posterior gill, that is essential for the transport of ions²³. The hepatopancreas is characterized by four different cell types: Embryonic (E) cells, which originate the other cell types; Resorptive (R) and Fibrillar cells (F), both important for absorption of nutrients; and Blister (B) cells, responsible for digestion and storage of nutrients^{23,24}. In addition, these organs exchange ions and metals with the environment through calcium channels, exchangers and zinc 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 this study was to compare the physiological responses of a mangrove crab

Ucides 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 (see 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 systems²⁷. Studies verified the presence of cadmium in this estuarine system, mainly in the river Piaçaguera, which receives effluents from fertilizer industries in Cubatão. Also they found high levels of cadmium in sediments of that river, indicative of a possible source of this metal linked to local industries²⁷.

The Juréia-Itatins Ecological Station is located in the South Coast of São Paulo (24°26'7.43"S - 47° 4'46.12"W). It has an area of approximately 80,000ha covering land belonging to the districts of Itariri, Miracatu, Pedro de Toledo, Iguape and Peruíbe. This is an important biodiversity²⁸ reserve of the Atlantic Forest still preserved and considered a Conservation Unit (CU). The CUs 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 humans²⁸. The CU Juréia-Itatins Ecological Station was created in 2006. In the same data, The Juréia-Itatins Ecological Station was transformed into a protected area mosaic, and the Barra do Una Sustainable Development Reserve²⁸.

Animals

Species of mangrove crab *Ucides cordatus* Linnaeus (Decapoda, Ucididae) were captured on March 2013 and 2014, at polluted and unpolluted

regions. Water and sediment (n=3 each) were collected from three 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 also placed into labeled flasks. The animals were brought to Laboratory of Physiology at University of São Paulo (USP), where they were acclimatized for seven days in the vivarium. The 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 to animals. Photoperiod (12:12) was constant, as well as the temperature ($22\pm 3^{\circ}\text{C}$). Only intermolt males were used in order to avoid different metabolic influences. For each set of experiments, four animals were used from each mangrove area.

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

Cellular dissociation

After acclimatization for seven days, gills (anterior and posterior) were submitted to cell dissociation³ using an enzymatic method, where 10 ml of the extraction solution (NaCl 395 mM; KCl 10 mM; NaHCO_3 2.5 mM; NaH_2PO_4 2.5 mM; Hepes 3.75 mM; glucose 1 mM and EDTA 0.9 mM; pH buffered to 7.8) were mixed with 200 μl of Trypsin²⁹ (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 $115 \times g$ at 5°C). After centrifugation, the pellet was re-suspended in fresh extraction solution and kept on ice.

The protocol for hepatopancreatic dissociation and other procedures²⁹ 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 $30 \mu\text{m}$, centrifuged (5 min., $115 \times g$), and stored for separation in the sucrose gradient (SG). The final volume of cell suspension was 2 mL. The different cell types were collected using a sucrose gradient²⁹. 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 ($115 \times g$ for 5 min). Next, the layers containing cells and sucrose were collected with a Pasteur pipet 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 ($405 \times g$ for 5 min.) and then washed in the extraction solution, without EDTA. The cell suspension was distributed in Elisa plates (180 μL per well that correspond at 23×10^4 cells) in series with CdCl_2 concentrations of 0.15, 0.25, 0.5, 0.75 and 1.0 mM. Cellular transport was analyzed in a fluorimeter (MODELO Biotek®), with emission of 525 nm and excitation of 495 nm with measurements of each 90 s in real time. A calibration curve was prepared to transform arbitrary fluorescence units into intracellular Cd^{2+} concentrations³⁰. At the end of the experiment, 180 μL of cells non-exposed to cadmium were added to Elisa plates with 50 μL of Triton X-100 20% for 15 min. To obtain the minimum fluorescence (F_{min}), we added 50 μL of HEDTA (N-hydroxyethyl-ethylenediamine-triacetic acid 1 mM). For the maximum

fluorescence (F_{max}), we added 50 μ L of Triton X-100 20 % for 15 min and 50 μ L of $CdCl_2$ (5 mM) in different batch of non-exposed cells. The transport of cadmium was interpreted through the fluorescence increase that originated a hyperbolic curve that follows Michaelis-Menten equation. Intracellular cadmium for every experiment was calculated according to the equation (Eqn 1):

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

where $[Cd^{2+}]_i$ is the intracellular free cadmium concentration (nM); Kd 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 fluorescence³.

Lipid and fatty acid analysis

Lipids and fatty acids (FA) analyses were performed in quadruplicate³¹. The 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}. The lipid extracts were separated into polar (phospholipids) and neutral lipids (triglycerides) via an activated silica column³⁴. Methylation of the polar fraction was performed using acetyl chloride (5% HCl in methanol)³⁵, and fatty acid composition was determined from the methyl ester content via a Varian Model 3900 gas chromatograph (GC), coupled to a flame ionization detector (FID). Identification of the fatty acids was obtained by comparing retention times using known methyl ester standards (FAME) (Supelco, 37 components, Sigma-Aldrich-and Mixture Me93, Qualmix PUFA fish M, and Menhaden Oil, Larodan). The FAME were analyzed using a capillary column CP Wax 52 CB, 0.25 μ m 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 (FID) temperatures were 250 and 260°C, respectively. At the end of the experiment, we have percentages of saturated fatty acids (SFA), which decrease the membrane fluidity; monounsaturated fatty acids (MUFA), which increase the membrane

fluidity; and polyunsaturated fatty acids (PUFA) which increase even more the membrane fluidity.

Measurement of Lipid Peroxide with spell out FOX (LPO)

The standard assay for measuring lipid peroxidation in tissue extracts was performed as follows^{15,36,37}. Frozen tissue samples were rapidly weighed and homogenized in 100% cold (5°C) HPLC grade methanol (1:9 w:v). Homogenates were centrifuged (1,000 × g), and then supernatants were removed. For the standard assay, the following reagents were added sequentially in an Elisa plate: 0.25 mM FeSO₄, 25 mM H₂SO₄, 0.1 mM xylene 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 (1h), 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 (CHP) equivalents.

Metallothionein assay (MT)

Metallothionein was measured as described³⁸ 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-HCl buffer pH 8.6, containing 0.5 mM PMSF 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.), and followed by the 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 (6,000 × g for 10 min., at 4°C). The 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 (6.000 x g for 10 min.). The pellets were washed with ethanol:chloroform:homogenization buffer (87:1:12), centrifuged again (6.000 x g for 10 min.) and suspended in 300 µL of 5 mM Tris-HCl, 1 mM EDTA, pH 7. The concentration of metallothionein was estimated by concentration of GSH³⁸ and quantified in a spectrophotometer using Ellman's reagent (2 M NaCl, DTNB in 0.43 M and 0.2 M phosphate buffer pH 8.0) at 412 nm.

Cadmium location in gills and hepatopancreatic cells

Glass microscope slides were previously immersed in an aquarium for 24 h, in order to allow the formation of a biofilm and improve cell adhesion. Subsequently, the 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 previously described, 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 Cd³⁹. The preparation was incubated with 1 mM CdCl₂ solution and observed with a fluorescence microscope (Zeiss®) with excitation at 495 nm, emission at 525 nm. Photographs were taken immediately before and after incubation with CdCl₂, by checking the fluorescence change in each organ studied, during 5 min. Variation of the fluorescence was calculated through the 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 Fig. 9 and Fig. 10.

Statistical analysis

For the tests performed, we used parametric analysis through the analysis of variance (ANOVA), testing for normality and equality of variance. For the MT and LPO analysis, we used the Tukey post hoc test. In the experiment

with phospholipids, we separated crabs as CF and CL to compare some physiological differences that could appear during the acclimatization processes, using ANOVA. Analyses were performed using Sigma Stat® for Windows® V. 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 that were described in the Material and Methods. In sediment samples, we observed the presence of cadmium in contaminated areas. The sediment had 0.50 µg/g of Cd, while all the other samples, the concentrations were bellow the detection limit.

It was observed a significant fluorescence increase and a higher cadmium transport (Fig. 1) only in the posterior gills of animals from polluted areas compared to the posterior gills of unpolluted areas (ANOVA, $P=0.019$ for V_{max} and $P=0.035$ for K_m).

Figure 1: Cd transport from anterior and posterior gills from polluted and unpolluted areas (Itanhaém and Juréia, respectively). Mean $\pm$ s.e. (N=3) for the total experiment.

For hepatopancreatic cells (Fig. 2), R, F and E 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), unlike B cells.

Figure 2: Cd transport from hepatopancreatic cells (R, F, E and B cells) from polluted and unpolluted areas (Itanhaém and Juréia, respectively). Mean $\pm$ s.e. (N=3). * Significant difference between Km or Vmax among groups. ^{ab}Different letters mean significant differences among groups ($p < 0.05$).

In the gill cells, only monounsaturated fatty acids of anterior gills from CF showed higher values compared to animals of unpolluted regions (Fig. 3) (ANOVA, $P=0.009$). The posterior gills of CF from polluted regions showed a decrease of omega-6 compared to CF from unpolluted areas (Fig. 4) (ANOVA, $P=0.018$). And anterior and posterior gills of CF 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 CL, 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 (Fig. 5) (ANOVA, $P=0.03$).

Figure 3: Fatty acids (%) from phospholipids. Saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA), in anterior and posterior gills of crabs from field (CF) and crabs lab held (CL) from polluted and unpolluted mangrove areas (Itanhaém and Juréia, respectively). Mean $\pm$ s.e. (N=4). * Significant difference between areas ($P<0.05$).

Figure 4: Percentage of omega 3 (n3) and omega 6 (n6) fatty acids from phospholipids in anterior and posterior gills of crabs from field (CF) and crab lab held (CL) from polluted and unpolluted mangrove areas (Itanhaém and Juréia, respectively). Mean $\pm$ s.e. (N=4). * Significant difference between areas ($p < 0.05$).

Figure 5: Percentage of fatty acids of phospholipids in anterior and posterior gills of crabs from field (CF) and crab lab held (CL) from polluted and unpolluted mangrove areas (Itanhaém and Juréia, 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). Mean $\pm$ s.e. (N=4). * Significant difference between areas ($p < 0.05$).

In hepatopancreas, the percentage of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) in phospholipids from CF and CL crabs, belonging to polluted and unpolluted

mangrove areas is presented in Fig. 6. CF of polluted region showed an increase in MUFA (ANOVA, $P=0.004$) and decrease in PUFA (C18 n3, short chain of fatty acids) (ANOVA, $P=0.022$) and n3 (omega 3) (ANOVA, $P=0.001$) compared to animals of unpolluted region. In CL, crabs from polluted areas presented a decrease in C18 n3 (short chain) in hepatopancreas compared to animals from unpolluted regions (ANOVA, $P=0.014$).

Figure 6: Fatty acids (%) from polar fraction of hepatopancreas phospholipids. A and B) Saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA); C and D) omega 3 (n3) and omega 6 (n6) PUFA; E and F), 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). Crabs from field (CF) and crab lab held (CL) from polluted and unpolluted mangrove areas (Itanhaém and Juréia, respectively). Mean $\pm$ s.e. (N=4). * Significant difference between areas ($p < 0.05$).

Higher lipid peroxidation levels were detected in anterior gills from polluted regions compared to unpolluted ones (Fig. 7). In addition, posterior gills showed the highest LPO in polluted compared to unpolluted regions. The same was found for hepatopancreas (ANOVA, $P=0.0001$ for anterior and posterior gills and hepatopancreas).

Figure 7: Lipid peroxidation (Eq CHP.g⁻¹) in gills (anterior and posterior) and hepatopancreas of crabs from polluted and unpolluted mangrove areas (Itanhaém and Juréia, respectively). Mean $\pm$ s.e. (N=4).^{ab}Different letters mean significant differences among groups ($p < 0.05$).

The highest concentration of metallothioneins in relation to GSH (Fig. 8) was detected in hepatopancreas compared to gills (0.24 ± 0.05 against 0.13 ± 0.01 $\mu\text{mol GSH} \cdot \mu\text{g protein}^{-1}$; 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 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 (Fig. 9 and Fig. 10) (ANOVA, $P=0.003$).

Figure 8: Concentration levels of metallothionein ($\mu\text{mol GSH} \cdot \mu\text{g protein}^{-1}$) for hepatopancreas and gill tissues from animals collected from polluted and unpolluted regions. Asterisk show significant differences between polluted and unpolluted regions in each tissue.

Figure 9: Fluorescence variation (RFU) in the cytoplasm in anterior gills (AG), posterior gills (PG) and hepatopancreas, E cells (E) and R cells (R) of animals from polluted (Itanhaém) and unpolluted areas (Juréia). The images show E cells labeled with FluoZin 3 before (A) and after (B) the injection of Cd (1 mM). The arrows show the fluorescence variation in the cytoplasm of E cells before (A) and after (B) the injection of Cd (1 mM). Mean $\pm$ s.e. (N = 4). * Significant differences between areas ($p < 0.05$).

Figure 10: Fluorescence variation (RFU) in the organelles in anterior gills (AG), posterior gills (PG) and hepatopancreas, E cells (E) and R cells (R) of animals from polluted (Itanhaém) and unpolluted areas (Juréia). The images show PG cells labeled with FluoZin 3 before (C) and after (D) the injection of Cd (1 mM). The arrows show the fluorescence variation in the organelles of PG cells before (A) and after (B) the injection of Cd (1 mM). Mean $\pm$ s.e. (N = 4). * Significant differences between areas ($p < 0.05$).

Discussion

Organisms are known to display physiological and biochemical mechanisms of tolerance to environmental chronic contamination^{1,40,41}. Therefore, our first approach to study populations of *U. cordatus* from polluted

and unpolluted environments was the measurement of Cd transport in gills and 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 regions. Similarly, the same occurs in the E, R and F 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 found in gills of animals from polluted areas when compared to non-polluted areas¹. Another interesting finding for *Eriocheir sinensis* submitted to acute and chronic exposure to cadmium, was a decrease of the Na^+ / K^+ ATPase activity during acute exposure and no effect in chronic exposure^{42,43}. In fish, an increase of Na^+ / K^+ ATPase was confirmed when some species were exposed chronically to copper (e.g., trout *Oncorhynchus mykiss*)⁴⁴, or in chronically contaminated environments (e.g., black fin *Goodea atripinnis*)⁴⁵.

Our results suggest that a greater transport of Cd in posterior gills could be linked to an increased activity of the Na^+ / K^+ ATPase in animals from polluted areas, favoring a higher Cd entry. Other studies⁴⁶ 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 *Carcinus maenas*. In a study with *Ucides cordatus*³, ouabain, a Na^+ / K^+ ATPase inhibitor, affected Cd transport in anterior and posterior gills of *Ucides cordatus*. According to these authors, a change in Na^+ equilibrium could alter the exchanger $\text{Cd}^{2+} / \text{Ca}^{2+}$ (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 the Na^+ / K^+ ATPase.

After this, we investigated how cadmium 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 Cd³⁹. 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. The

results are shown in Figs. 9 and 10. 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 realized by X-ray microprobe analysis, toxic metals can also be stored in vacuoles of different types: type A (phosphate and zinc); B (sulfur groups, binding cadmium, copper, mercury and silver); C (iron through ferritin groups); and D, which is extracellular and filled with calcium concretions⁴⁷. 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 here. This fact suggests the importance of metallothionein in the hepatopancreas cytoplasm as the main site for detoxification of toxic metals, unlike gill cells^{13,48}. 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 granules¹³, similar to our results.

Environmental contamination may also influence biological membranes, especially in organs having direct contact with the environment²⁰. With regard to gills, a higher percentage of MUFA was found in anterior gills of crabs from polluted regions when compared to non-polluted ones. According to the literature, MUFA can be used as an energy source for physiological repair processes^{20,49}. In vertebrates, studies show the action of an enzyme, stearyl-CoA $\Delta 9$ desaturase, which adds an unsaturation between carbons 9 and 10, converting them into monounsaturated⁵⁰. A hypothesis for the higher presence of MUFA in anterior gills of *Ucides cordatus* from contaminated regions would be the presence of a similar desaturase enzyme, in order to provide more energy for physiological processes such as respiration, which is carried out mainly in the anterior gills of this species^{29,51}. For CF from unpolluted region,

our results showed an increase in the long chain fatty acids C18 n3 and C20-22 n6 in anterior and posterior gills. This fact contributes to an increased membrane fluidity⁵²⁻⁵⁴, important for osmoregulatory mechanisms. In addition, there was lower PUFA in plasma membrane of gill and hepatopancreatic cells of crabs from polluted regions, which decrease membrane fluidity, a possible physiological regulation 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 O₂ concentration, that can produce free radicals and therefore increase the lipid peroxidation process^{17,55}. The hepatopancreas, in turn, is an organ for detoxification. This process occurs in order to decrease the concentration of toxic metals. With the accumulation of metals, there is the 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 peroxidation⁵⁵. Therefore, an increase in lipid peroxidation in gills and hepatopancreas lead to a decrease in PUFA (C18 n3 and C20-22 n6) and an increase in MUFA.

We found larger amounts of MT in hepatopancreas and gills of animals from unpolluted regions. Comparison between CF and CL did not show statistically significant difference. It is possible that the acclimatization process did not affect the MT levels. Studies with invertebrates showed that MT have an important role in detoxification as a fast process when animals are exposed to metals, and are also critical in cellular homeostasis^{13,48}. The induction of MT 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 MT, as well as an inverse pattern^{13,48}. For example, in oysters translocated from an unpolluted region to a polluted one⁵⁶, the researchers verified lower levels of MT than specimens from more contaminated areas¹³. One hypothesis suggested here would be that animals from polluted regions could present physiological modifications that permit their survival in a

contaminated environment, and an increase in the production of MT would be unnecessary. Also, there are tissue-related variations in MT concentrations that reflect their physiological role in metal handling strategies. In addition, the influence of contamination factors limits the possibility of using MT concentration as a biomarker of metal exposure¹³.

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 amount of MUFA, decreased PUFA and higher LPO in both gills and hepatopancreas. The animals from non-polluted environments, on the other hand, had a higher percentage of PUFA, together with greater amounts of MT. 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, while for hepatopancreas Cd binds to metallothionein present in the cell cytoplasm.

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DISCUSSÃO GERAL

As brânquias e o hepatopâncreas apresentam um importante papel na osmorregulação e na detoxificação de poluentes, respectivamente^{1,2}. Por serem os primeiros sítios de contato com o ambiente, estes órgãos são afetados pela poluição local, sendo possível o acúmulo e, em última instância, a perda da efetividade de suas funções^{3,4}. Contudo, organismos presentes em regiões contaminadas e expostos a poluentes por longos períodos, podem apresentar modificações fisiológicas relacionadas ao processo de contaminação crônica, permitindo a sua sobrevivência nestas regiões⁵.

Para entender melhor os mecanismos fisiológicos que envolvem a persistência de populações de caranguejos em regiões contaminadas, planejou-se um estudo sistemático sobre o transporte de um metal não essencial. Primeiramente, foi caracterizado o transporte de Cd em brânquias anteriores e posteriores (cap. 1), juntamente com o hepatopâncreas (cap. 2). Os experimentos de transporte realizados com brânquias foram executados apenas separando-se as brânquias anteriores e posteriores. Ou seja, nas brânquias anteriores havia a presença dos tipos celulares hemócitos (células da hemolinfa) e principais (células voltadas para o processo respiratório), enquanto que nas brânquias posteriores haviam ionócitos (células relacionadas à troca iônica e osmorregulação) e células pilares (células de sustentação), principalmente. Cada tipo celular não foi separado através do gradiente de sacarose, como no experimento do hepatopâncreas, devido ao baixo número de células encontrado em cada camada do gradiente, tornando inviável a quantificação do processo de transporte. Nos experimentos realizados com as brânquias, verificou-se uma maior entrada de cádmio em brânquias posteriores para animais provenientes de regiões poluídas, sendo essa entrada significativa em maiores concentrações externas de Cd (mM) e de maneira cumulativa. Este fato ocorreu devido à maior abundância das células ionócitos e pilares nas brânquias posteriores, sendo os ionócitos as células responsáveis pelos processos de trocas iônicas e osmorregulação⁶.

Comparando-se os transportes de Cd realizado em células de brânquias e de hepatopâncreas (Tabela 1), notou-se diferenças quanto ao Km e V_{máx}. As células E apresentaram um Km 3,5x maior em comparação com as células das brânquias anteriores, e 10,2x maior em comparação com as células das brânquias posteriores. Em relação ao V_{máx}, as células E apresentaram V_{máx} similar ao das brânquias anteriores e posteriores. As células R apresentaram V_{máx} 2 a 3x maior e Km 8 a 22x maior, respectivamente em relação às brânquias anteriores e posteriores. Por sua vez, as células F e B apresentaram menor V_{máx} em relação às brânquias, sendo ao redor de 3 a 4x maior para a brânquia posterior e anterior, respectivamente; e um Km maior, sendo em torno de 3 a 7x maior em relação às brânquias anteriores, e em torno de 7 a 19x maior em relação às brânquias posteriores. Este fato realça o maior transporte de Cd que ocorre em células E e R, células estas responsáveis pelo crescimento, osmorregulação e troca iônica realizado pelo hepatopâncreas¹. Vale ressaltar a maior afinidade no transporte de Cd nas células branquiais em relação às células do hepatopâncreas.

Tabela 1: Valores de V_{máx} (Velocidade máxima da reação) e Km (constante de Michaelis Menten) dos tipos celulares hepatopancreáticos e das células de brânquias anteriores e posteriores durante o transporte de Cd.

Tipos celulares	V _{máx}	Km
E	2,91 ± 1,04	1,84 ± 0,98
R	5,36 ± 3,75	3,94 ± 3,43
F	0,57 ± 0,12	1,21 ± 0,44
B	0,89 ± 0,26	3,36 ± 1,25
Brânquias Anteriores	2,24 ± 0,03	0,52 ± 0,15
Brânquias Posteriores	1,77 ± 0,04	0,18 ± 0,17

Com a ação dos inibidores de transporte, pode-se perceber que o influxo de cádmio ocorreu principalmente através de trocadores $\text{Na}^+/\text{Ca}^{2+}$ e canais de cálcio³. Em brânquias anteriores, a ação da nimodipina causou uma diminuição do $V_{\text{máx}}$ e K_m em 3 e 2x, respectivamente; e o verapamil, em brânquias posteriores, causou uma diminuição do $V_{\text{máx}}$ e aumento do K_m em 2x. Estudos realizados com *Carcinus maenas* determinaram que o influxo de cádmio aumentava devido à entrada accidental do metal via proteínas transportadoras de cálcio. Em peixes, o cádmio afeta a homeostase do cálcio, sendo que ambos competem pela entrada nas células de cloreto. Aparentemente, esta competição se dá via Ca-ATPase, na região basolateral e via canais de cálcio na região apical^{3,7}. Da mesma maneira, podemos identificar que, em brânquias de *Ucides cordatus*, o cádmio utiliza canais de cálcio e trocadores de cálcio para entrar nas células, além do sódio e do cálcio intracelulares exercerem um papel fundamental no sistema de transporte de cádmio, onde maiores concentrações de cálcio ou sódio intracelulares poderiam influenciar no maior influxo de cádmio via canais ou trocadores de cálcio (cap. 1).

Com relação ao transporte de cádmio em células hepatopancreáticas, observou-se maior transporte em células E e R, possivelmente devido às funções de absorção e secreção enzimática desenvolvida por esses tipos celulares^{1,8}. Com a ação dos inibidores, verificou-se que, em células E, houve uma diminuição no K_m e $V_{\text{máx}}$ em 5x, de maneira significativa, na presença de nimodipina, vanadato, cafeína e ouabaína, indicando um influxo de cádmio principalmente através dos canais de cálcio tipo L, ou canais lentos de cálcio. Da mesma maneira, o cálcio intracelular também influenciou no transporte de cádmio para o interior celular^{3,9}. Já as células R apresentaram, na presença de vanadato, uma diminuição no K_m ; na presença de nimodipina e verapamil, o K_m e $V_{\text{máx}}$ diminuíram 8x em relação ao controle, corroborando com a hipótese da entrada de cádmio através dos canais de cálcio tipo L ou canais lentos de cálcio e trocadores de sódio. Além disso, houve um aumento significativo do transporte de cádmio na presença de ouabaína e cafeína, e uma diminuição do transporte na presença de BAPTA, sugerindo uma relação entre o cálcio intracelular e o transporte de cádmio^{3,9}. As células F também

apresentaram um comportamento similar às outras células estudadas, com um Km 2x menor na presença de nimodipina, um maior transporte de cádmio na presença de cafeína e ouabaína e um influxo menor do metal na presença de verapamil, com $V_{máx}$ e Km diminuídos^{4,7,8}. As células B, por sua vez, apresentam um menor influxo de cádmio na presença de nimodipina, vanadato e verapamil. Com relação aos inibidores cafeína e ouabaína, somente nesse tipo celular, houve diminuição na entrada de Cd em presença de cafeína, sugerindo que a liberação de Ca intracelular diminui a entrada de Cd, diferente do que ocorreu para as brânquias e todos os outros tipos celulares do hepatopâncreas, ressaltando aqui a importância do cálcio intracelular em levar a uma diminuição do influxo de cádmio na célula^{3,10}. Em estudos realizados com brânquias de *Ucides cordatus*, os autores verificaram que os canais de cálcio, assim como os trocadores Na^+/Ca^{2+} poderiam estar relacionados com o transporte de cálcio³. Da mesma maneira, estudos realizados com hepatopâncreas de lagostas relataram a presença dos mesmos canais e trocadores para a realização do transporte de metais⁴. Esses estudos suportam ainda mais a hipótese de transporte de cádmio através de canais de cálcio e trocadores Na^+/Ca^{2+} . Além disso, de maneira geral, nota-se que o cálcio extracelular apresentaria um papel fundamental no influxo de cádmio (cap. 2). A tabela a seguir (Tabela 2) resume o transporte para cada tipo celular do hepatopâncreas e das brânquias anteriores e posteriores.

Tabela 2: Resumo do transporte de cádmio em diferentes tipos celulares de hepatopâncreas e em brânquias anteriores e posteriores, na presença dos diferentes inibidores. O efeito negativo no transporte de cádmio está indicado com o símbolo (-), o efeito positivo está indicado através do símbolo (+) e quando o transporte de cádmio não foi afetado pelo inibidor, utilizou-se o termo SE (sem efeito).

Tipo Celular	Inibidores					
	Nimodipina	Vanadato	Verapamil	BAPTA	Ouabaina	Cafeína
E	-	-	-	SE	+	+
R	-	-	-	-	+	+
F	-	SE	-	SE	+	+
B	-	-	-	SE	-	-
Brânquia Anterior	-	-	-	-	+	+
Brânquia Posterior	-	-	-	-	+	+

Com o objetivo de comparar a fisiologia do transporte de Cd em animais de regiões contaminadas com animais de regiões não contaminadas, foram realizados testes de transporte de cádmio em brânquias e hepatopâncreas de animais provenientes de locais com diferentes níveis de contaminação por Cd (cap. 3). Assim, notou-se que animais provenientes de regiões poluídas e não poluídas apresentaram um sistema de transporte em brânquias e em hepatopâncreas diferenciado. As células das brânquias posteriores dos animais das regiões poluídas transportaram e acumularam mais Cd que as brânquias posteriores dos animais de regiões não poluídas, possivelmente devido ao aumento da atividade da $\text{Na}^+ / \text{K}^+ \text{ATPase}$, e, conseqüentemente, uma maior entrada do metal^{8,11-15}. O mesmo ocorreu com as células E, R e F do hepatopâncreas, as quais também transportaram e acumularam mais Cd intracelular em animais de regiões poluídas quando comparados com animais de regiões não poluídas^{12,16}, corroborando com a hipótese de aumento da atividade da $\text{Na}^+ / \text{K}^+ \text{ATPase}$ e maior entrada de cádmio. Em estudos realizados com *Ucides cordatus* e *Callinectes danae* provenientes de regiões poluídas e não poluídas, pode-se notar que, em animais de regiões poluídas, houve um aumento da concentração de cádmio em seus tecidos (brânquias e

hepatopâncreas), além de um aumento na atividade da Na^+ / K^+ ATPase e nas concentrações de Na^+ na hemolinfa, sendo considerada uma resposta fisiológica em relação aos mecanismos osmorregulatórios em regiões contaminadas¹².

Assim como o cádmio, outros metais tóxicos também podem ser transportados para o interior celular, sendo verificados diversos mecanismos de detoxificação pelo animal. Dentre esses processos encontram-se: mecanismos de regulação fisiológica através da excreção, mecanismos de sequestro intracelular de metais envolvendo proteínas como as metalotioneínas e processos de sequestro intracelular envolvendo vacúolos e organelas¹⁰. Em células epiteliais, como no caso de células branquiais e hepatopancreáticas, os íons metálicos, além de serem sequestrados por metalotioneínas, podem ser transportados para organelas como as mitocôndrias, lisossomos e retículos. Além das organelas, vacúolos e grânulos de metal também podem atuar na detoxificação¹⁰. Os vacúolos e grânulos apresentam enxofre, o qual pode se ligar aos metais tóxicos, principalmente zinco, cobre, cádmio e ferro^{10,17,18}. Dessa maneira, investigamos como o cádmio se acumula em células branquiais e hepatopancreáticas através da observação do Cd no meio intracelular, usando marcadores fluorescentes (cap. 3). Foi possível verificar que o acúmulo de cádmio se deu principalmente em organelas como as mitocôndrias e lisossomos, vacúolos e grânulos, em células das brânquias posteriores, onde havia maior transporte de Cd. Os resultados sugerem o auxílio das organelas das brânquias posteriores na detoxificação de metais tóxicos, sendo estes acumulados principalmente em lisossomos, retículos endoplasmáticos e mitocôndrias, estando metabolicamente disponível. Em hepatopâncreas, por sua vez, as organelas, vacúolos e/ou grânulos aparentemente não acumularam Cd, sugerindo a importância do papel das metalotioneínas do citoplasma, para a detoxificação de metais tóxicos, estando biologicamente detoxificado^{19,20}.

As mudanças ambientais e a contaminação local também podem influenciar nas membranas biológicas, principalmente em órgãos que estão em contato direto com o meio-ambiente contaminado¹⁶. No caso das células de brânquias e hepatopâncreas, a membrana foi estudada através de alguns

fatores, como: porcentagem de ácidos graxos dos grupos saturados (SFA), monoinsaturados (MUFA) e polinsaturados (PUFA); nos grupos polares, a porcentagem de ômega 3 (n3) e 6 (n6); e cadeias longas e curtas presentes nos grupos polares (cap. 3). Nos experimentos realizados, notou-se uma maior porcentagem de MUFA nas brânquias anteriores e no hepatopâncreas de caranguejos de regiões contaminadas, quando comparados aos caranguejos de regiões não contaminadas, sugerindo a utilização deste grupamento como fonte de energia^{21,22,16}. Em vertebrados, estudos apontam a ação da enzima esteroil-CoA $\Delta 9$ desaturase, que age acrescentando uma insaturação entre os carbonos 9 e 10 da cadeia de ácidos graxos saturados, convertendo-os em monoinsaturados¹⁶, formando mais MUFA, podendo, portanto, ser utilizados como fonte de energia. Uma hipótese para a maior presença de MUFA em brânquias anteriores e em hepatopâncreas de animais de regiões contaminadas seria a ação desta ou de uma enzima semelhante, que permitiria tal evento, sendo possível disponibilizar mais energia para os processos fisiológicos, como a respiração ou a síntese de enzimas, processos estes que são realizados principalmente na brânquia anterior e no hepatopâncreas^{3,6,23,24}. As brânquias posteriores e o hepatopâncreas de animais de regiões não poluídas apresentaram uma maior concentração de ômega 6 (n6) e ômega 3 (n3), respectivamente, caracterizando uma alimentação a base de detritos de folhas e animais²⁵. Além disso, as brânquias anteriores, posteriores e o hepatopâncreas de caranguejos de regiões não poluídas apresentaram um aumento nas cadeias longas de ácidos graxos, no caso, C20-22 n6 e C18 n3, contribuindo para um aumento na fluidez da membrana^{24,26,27}, garantindo uma estabilidade da mesma. Em resumo, há uma menor concentração de PUFA na membrana plasmática de brânquias e hepatopâncreas de caranguejos de regiões poluídas.

Em brânquias e hepatopâncreas, verificou-se também uma maior peroxidação lipídica, principalmente em animais provenientes de regiões poluídas. Esta maior lipoperoxidação poderia ocorrer devido às características dos órgãos. Em brânquias, região mais voltada para a respiração celular, encontra-se a maior concentração de O₂. O contaminante, em presença de O₂, poderia originar radicais livres que acarretariam em um aumento do processo

de peroxidação lipídica^{28,29}. O hepatopâncreas, por sua vez, atua em processos de detoxificação. Com o acúmulo de metais, poderia ocorrer o estímulo para a formação de radicais hidroxil, os quais poderiam ocasionar o estresse oxidativo através da reação destes radicais com macromoléculas, levando a danos celulares, como a apoptose e a peroxidação lipídica²⁹. Em estudos realizados com brânquias de *Sinopotamon henanense*, observou-se altos níveis de metalotioneínas durante 12 horas de acúmulo de cádmio, levando à ativação do sistema antioxidante, e, conseqüentemente, a diminuição de cádmio livre. Porém, com o aumento do período de exposição (após 72 horas), a toxicidade de cádmio excedeu a capacidade de detoxificação por metalotioneínas, o que resultou em um aumento dos níveis de LPO e de danos celulares²⁸. Dessa maneira, podemos entender que os níveis de LPO e de metalotioneína estão ligados entre si, onde, baixos níveis de LPO resultam da eficiência das metalotioneínas e, altos níveis de LPO resultam da incapacidade de ligação das metalotioneínas aos metais.

Outros processos conhecidos que permitem a detoxificação é a presença de pequenas proteínas conhecidas como metalotioneínas, as quais podem se ligar aos metais através de seus sítios de cisteína²⁹. Estudos realizados em invertebrados demonstrou que as metalotioneínas também são fundamentais na homeostase celular^{19,20}. A indução de metalotioneínas, dessa maneira, não dependeria somente de elevados níveis de metais poluentes, mas também de qual metal está mais presente em um determinado ambiente contaminado, dos fatores ambientais como alterações em salinidade, da espécie em questão e dos seus processos fisiológicos. Assim, animais em ambientes poluídos poderiam apresentar baixos níveis de metalotioneínas e vice-versa^{19,20}.

Estas proteínas estão presentes principalmente nas brânquias e no hepatopâncreas de animais de regiões contaminadas, porém, de acordo com os resultados obtidos aqui, notou-se uma maior presença de metalotioneínas em animais de regiões não contaminadas, e ainda com um maior aumento no hepatopâncreas, independente do processo de aclimatização. Foi observado uma maior concentração da proteína no hepatopâncreas, sugerindo grande importância desta nos processos de detoxificação^{19,20}. Além disso, observou-se

que as brânquias apresentam um maior acúmulo de metais nas organelas, sendo, possivelmente, estas responsáveis pela detoxificação de metais, principalmente através de mitocôndrias, retículos endoplasmáticos e lisossomos¹⁰. Uma hipótese seria que, animais de regiões contaminadas poderiam apresentar um ajuste fisiológico em relação ao ambiente, acumulando metais em organelas ou vacúolos, não sendo necessário a maior produção de metalotioneínas. Além disso, estudos realizados com ostras, notou-se baixos níveis de metalotioneínas em animais presentes em ambientes contaminados³⁰. Sabe-se que ocorre um polimorfismo em relação às metalotioneínas em invertebrados marinhos e que o método empregado nesse trabalho não permite medições de metalotioneínas de diferentes isoformas. Em mexilhões, por exemplo, a isoforma MT-20 parece ser mais sensível ao cádmio que a isoforma MT-10, envolvida na homeostase de metais essenciais, como zinco e cobre, e presente em níveis basais em vários organismos³⁰. Assim, os baixos níveis encontrados de metalotioneína podem ser de diferentes isoformas, que não são sensíveis ao cádmio. Uma outra possibilidade seria a degradação do conjunto metalotioneína e cádmio pelos lisossomos, o que também resultaria em baixos índices da proteína em animais de ambientes poluídos. Já em animais de regiões não poluídas, qualquer alteração ambiental por metais tóxicos, mesmo que de pequena magnitude, poderia ser mais perceptível ao animal, sensibilizando-o e causando um aumento nos níveis de metalotioneína.

Dessa maneira, é possível observar que brânquias e hepatopâncreas de animais de ambientes contaminados apresentam modificações fisiológicas que possibilitam a sua sobrevivência em áreas poluídas, e, portanto, este trabalho contribuiu de maneira significativa para posteriores estudos em toxicologia e fisiologia.

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CONCLUSÕES

Conclusões

Após a realização dos experimentos de caracterização e transporte de cádmio em epitélios de troca de crustáceos, pode-se concluir que ocorre significativo transporte de cádmio tanto em células de brânquias como de hepatopâncreas. Em brânquias, juntamente com a ação de inibidores, o transporte ocorreu principalmente através de canais de cálcio e trocadores sódio/cálcio. Em hepatopâncreas, também foi possível observar que todos os tipos celulares, na presença de inibidores, transportaram cádmio através de canais de cálcio, trocadores de sódio e através da relação com o cálcio intracelular. Foi verificado também que as células B (blister), especificamente, apresentaram uma diminuição no transporte de cádmio através da diminuição da fluorescência. Esse fato poderia sugerir um processo de acúmulo de metais através de vacúolos intracelulares ou por meio da ação das metalotioneínas.

Com relação à comparação entre animais provenientes de regiões poluídas e não poluídas do litoral de SP, pode-se concluir que, tanto em células de brânquias quanto em células de hepatopâncreas, animais de regiões mais poluídas apresentaram um maior transporte de cádmio em comparação aos animais de regiões não poluídas, possivelmente devido a um aumento da atividade da $\text{Na}^+ / \text{K}^+ \text{ATPase}$. Com esse aumento, poderia ocorrer um maior influxo de Na^+ , que, através de trocadores $\text{Na}^+/\text{Ca}^{2+}(\text{Cd}^{2+})$, levaria a um aumento na entrada do metal na célula. Verificou-se também que as células branquiais apresentaram um maior acúmulo de Cd em suas organelas, enquanto que as células hepatopancreáticas acumularam o metal através da ação de metalotioneínas em maior quantidade no citoplasma dessas células.

Em experimentos de quantificação de LPO, conclui-se que animais de regiões poluídas, por apresentarem maiores índices de lipoperoxidação, são afetados diretamente pela poluição local, acarretando em estresse fisiológico. Em relação à quantificação de metalotioneínas, animais de regiões não poluídas apresentaram maiores índices dessa proteína, principalmente em hepatopâncreas, concluindo assim que as metalotioneínas exercem um papel importante na regulação fisiológica, quelando o Cd^{2+} . Em animais de regiões poluídas, a regulação fisiológica poderia apresentar-se de maneira

diferenciada, com o acúmulo de Cd^{2+} em organelas ou vacúolos, sendo visto principalmente em brânquias posteriores, e maior ação das metalotioneínas no processo de detoxificação em hepatopâncreas. Por último, verificamos os ácidos graxos presentes em brânquias e hepatopâncreas e observou-se que, em brânquias anteriores e hepatopâncreas de animais de regiões contaminadas, a elevada porcentagem de MUFA está relacionada com a possível utilização deste grupamento como fonte de energia. Por outro lado, em brânquias anteriores, posteriores e hepatopâncreas de animais de regiões não poluídas verificou-se uma maior porcentagem de ácidos graxos polinsaturados, PUFA, principalmente C18 n3 e C20-22 n6, indicando uma maior fluidez na membrana. Em brânquias anteriores de animais de regiões poluídas, notou-se uma maior porcentagem de C18 n6, que também permite uma maior fluidez na membrana, porém a fluidez é menor quando comparado com o C20-22 n6. Observou-se também que os animais apresentaram tanto ômega 3 (n3) quanto ômega 6 (n6), o que pode explicar a alimentação onívora nesses animais.

De maneira geral, podemos concluir que animais de regiões poluídas apresentaram ajustes fisiológicos, que permitiriam que estes animais pudessem sobreviver em ambientes poluídos. Não se pode afirmar, porém, que se trata de tolerância ou adaptação em relação ao meio, no entanto, este estudo pode clarificar a existência de modificações fisiológicas e bioquímicas que animais de regiões contaminadas desenvolvem, e que permitiriam a sua existência nesses locais.

Animais presentes em regiões de maior contaminação apresentam diferenças fisiológicas, quando comparados com animais de regiões menos contaminadas, provavelmente permitindo a sua sobrevivência em ambientes poluídos. Assim, o objetivo da tese foi caracterizar o transporte de cádmio em brânquias e hepatopâncreas de *Ucides cordatus*, como também comparar o transporte nestes órgãos, em animais de regiões poluídas (Itanhaém) e não poluídas (Juréia), além de analisar a porcentagem de ácidos graxos na membrana, níveis de metalotioneína (MT), lipoperoxidação (LPO) e mecanismo de acúmulo do metal. Assim, as células de brânquias anteriores (BA), posteriores (BP) e hepatopâncreas (H) foram separadas. Em seguida, realizou-se o transporte de cádmio em BA, BP, e em cada tipo celular do H, com ou sem a utilização de inibidores; o mesmo procedimento foi utilizado para caracterizar o transporte de cádmio em cada região estudada. Também foram analisadas a porcentagem de ácidos graxos, níveis de MT, LPO e mecanismos de detoxificação de cádmio. Observou-se um maior transporte através de canais de cálcio e trocadores $\text{Na}^+/\text{Ca}^{2+}$, em BP e nas células E, R e F de H, pois são células específicas para as trocas iônicas. Em animais de regiões poluídas, observou-se um maior transporte que ocorreu nos mesmos parâmetros, devido aos tipos celulares específicos envolvidos. Em animais de regiões contaminadas verificou-se maior porcentagem de MUFA em hepatopâncreas, acarretando em menor fluidez na membrana, e maior índice de LPO, devido ao estresse causado pela presença do contaminante no meio. Já em animais de regiões não poluídas, observou-se maior porcentagem de PUFA, com maior fluidez na membrana, e maior quantidade de MT, principalmente no hepatopâncreas, resultando na detecção de pequenas alterações ambientais. Nas brânquias, o cádmio se acumula em organelas intracelulares, enquanto em hepatopâncreas se ligam a MTs do citoplasma celular. Portanto, acredita-se que estes animais apresentaram diferentes mecanismos fisiológicos que permitiram a sua sobrevivência na região poluída.

Palavras chaves: transporte de cádmio, brânquias, hepatopâncreas, poluição.

Abstract

Animals resident in regions polluted by metals must have physiological mechanisms for survival, compared with animals from unpolluted regions. Therefore, the objective of this study was to characterize cadmium transport in gills and hepatopancreas of *Ucides cordatus*, and to compare this transport in animals from polluted (Itanhaém) and unpolluted (Juréia) regions. In addition, the animals were analyzed for cell membrane fatty acids composition, metallothionein levels (MT), lipid peroxidation (LPO) and mechanisms of accumulation of intracellular Cd. The anterior gills (BA), posterior gills (BP) and hepatopancreas (H) cells were separated. After, the transport of cadmium was realized in BA, BP and in each cellular type of H, with or without various inhibitors present. The same approach was utilized to characterize cadmium transport from crabs of polluted and unpolluted regions: fatty acids composition, MT levels, LPO and cadmium detoxification mechanisms were also analyzed. An increase in the transport of cadmium was observed through calcium channels and $\text{Na}^+/\text{Ca}^{2+}$ exchangers, in BP and in E, R and F cells of H, probably because they are specific cells for ion transport. In animals from polluted regions, we observed an increase in cell cadmium transport when compared with animals from unpolluted regions. Animals from polluted environments also presented more MUFA in H, leading to lower membrane fluidity, and higher LPO, probably due to the stress caused by pollution. In animals from unpolluted regions, we observed more PUFA in the plasma membrane, resulting in higher membrane fluidity, and more MT levels, mainly in H. In gills, intracellular cadmium accumulation occurred in organelles, whereas in H, Cd binding to MT in cellular cytoplasm was more evident. Therefore, we believe that animals from metal polluted environments present the above-described physiological mechanisms to deal with Cd, allowing their survival in this environment.

Key words: cadmium transport, gills, hepatopancreas, pollution by metals.

SÚMULA CURRICULAR

PRISCILA ORTEGA

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Ano	Título ou atividade	Instituição
2008	Licenciada em Ciências Biológicas	Universidade Presbiteriana Mackenzie – São Paulo - Brasil
2009	Bacharel em Ciências Biológicas	Universidade Presbiteriana Mackenzie – São Paulo - Brasil
2012	Mestre em Fisiologia Geral	Universidade de São Paulo – São Paulo – Brasil
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2) Histórico profissional

2007-2009: Universidade Presbiteriana Mackenzie

Empresa Júnior de Biologia Mackenzie (EJBio).

Experiência com Biologia molecular, extração de DNA, PCR e eletroforese.

2007-2009: Universidade Presbiteriana Mackenzie

Iniciação Científica.

Projeto: Viabilidade de células branquiais de caranguejo de mangue *Ucides cordatus*.

Experiência com espectrofotômetro, câmara de Neubauer e microscopia.

2009-2009: Universidade Presbiteriana Mackenzie

Iniciação Científica

Projeto: Caracterização do transporte de cobre e histologia das células epiteliais do hepatopâncreas de *Ucides cordatus*.

Experiência com espectrofluorímetro, microscopia de fluorescência e cromatografia a gás.

2009 (agosto – dezembro): Monitora pela Universidade Presbiteriana Mackenzie na área de Biologia Celular.

2009-atual: Grupo de Pesquisa CRUSTA

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2010 (julho – setembro): University of North Florida

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Projeto: 3H-L-leucine transport by the promiscuous crustacean dipeptide-like cotransporter

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2013 (julho): Curso complementar em *Biologia Molecular*

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2015 (agosto-dezembro): Monitoria em *Bioquímica Básica. IQ – USP.*

3) Artigos completos publicados em periódicos

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Mestrado

Projeto: Transporte de cádmio em células branquiais do caranguejo de mangue *Ucides cordatus*.

Orientador: Flávia Pinheiro Zanotto.

Universidade de São Paulo, São Paulo, Brasil.

CAPES: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (2013 – 2016).

Doutorado

Projeto: Caracterização do transporte de cádmio em células epiteliais de populações do caranguejo de mangue *Ucides cordatus* provenientes de áreas com diferentes níveis de poluição.

Orientador: Flávia Pinheiro Zanotto.

Universidade de São Paulo, São Paulo, Brasil.

ANEXOS

Anexo 1: Artigo referente ao doutorado, publicado na revista Aquatic Toxicology 157, 21-29, 2014. Titulado Characterization of cadmium plasma membrane transport in gills of a mangrove crab *Ucides cordatus*

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Characterization of cadmium plasma membrane transport in gills of a mangrove crab *Ucides cordatus*

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ABSTRACT

Membrane pathway for intracellular cadmium (Cd^{2+}) accumulation is not fully elucidated in many organisms and has not been studied in crab gill cells. To characterize membrane Cd^{2+} transport of anterior and posterior gill cells of *Ucides cordatus*, a hypo-hyper-regulating crab, a change in intracellular Cd^{2+} concentration under various experimental conditions was examined by using FluoZin, a fluorescent probe. The membrane Cd^{2+} transport was estimated by the augmentation of FluoZin fluorescence induced by extracellular application of CdCl_2 and different inhibitors. Addition of extracellular calcium (Ca^{2+}) to the cells affected little the fluorescence of FluoZin, confirming that Cd^{2+} was the main ion increasing intracellular fluorescence. Ca^{2+} channels blockers (nimodipine and verapamil) decreased Cd^{2+} influx as well as vanadate, a Ca^{2+} -ATPase blocker. Chelating intracellular Ca^{2+} (BAPTA) decreased Cd^{2+} influx in gill cells, while increasing intracellular Ca^{2+} (caffeine) augmented Cd influx. Cd^{2+} and ATP added at different temporal conditions were not effective at increasing intracellular Cd^{2+} accumulation. Ouabain (Na^+/K^+ -ATPase inhibitor) increased Cd^{2+} influx probably through a change in intracellular Na and/or a change in cell membrane potential. Routes of Cd^{2+} influx, a non-essential metal, through the gill cell plasma membrane of crabs are suggested.

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1. Introduction

Estuarine and coastal areas are regions affected by discharge of heavy metals, mainly cadmium. Cadmium (Cd) plays no role as a trace element and is considered a toxic metal with unknown physiological function for animals. One exception, however, is shown by some diatoms, and in the absence of Zn^{2+} , use Cd^{2+} in the active site of their carbonic anhydrase (Lane and Morel, 2000; Thévenod, 2010). It is known that crabs exposed to Cd up-regulate several antioxidant enzymes, and chronic exposure affect more extensively the metabolic and protein expression profiles than acute exposure to Cd (Silvestre et al., 2006). Cd also causes detrimental effects in respiratory and osmoregulatory functions (see Silvestre et al., 2006).

Membrane pathway for intracellular cadmium transport is not fully elucidated in many organisms (Bridges and Zalups, 2005; Gagnon et al., 2007; Ojo and Wood, 2008; Thévenod, 2010; Verheyen et al., 2012; Kwong and Niyogi, 2012; Li et al., 2012a,b)

and has not been studied in crab gill cells, apart from work on perfused gills (Pedersen and Bjerregaard, 1995; Lucu and Obersnel, 1996; Bondgaard and Bjerregaard, 2005). At the cellular level, it is assumed that Cd^{2+} is taken up by cells through transporters for other essential elements as a consequence of low specificity of most membrane transporters (Rainbow and Luoma, 2011). Work with mammals, adopting methodologies for Cd^{2+} transport using radio-tracers, or sensitive dyes, has shown unequivocally that Cd^{2+} can enter cells through divalent metal transporters (DMT1), Zn related protein transporters (ZIP) and through mediated receptor endocytosis as a Cd-metallothionein complex, using cell energy (see review by Thévenod, 2010). Other modes of transport such as Ca^{2+} channels are still controversial in mammals (Thévenod, 2010).

For aquatic organisms, studies using whole animals with radioactive tracers show that Cd^{2+} entry in gill epithelium occurs probably via calcium transport routes in fish (Perry and Flik, 1988; Wicklund Glynn et al., 1994), mollusks (Roesijadi and Unger, 1993) and crustaceans (Borowitz and McLaughlin, 1992; Pedersen and Bjerregaard, 1995; Lucu and Obersnel, 1996). Nørum et al. (2005) observed a postmolt increase in cadmium influx in the crab *Carcinus maenas* due to increased transport of cadmium, occurring at least partly, by accidental uptake via calcium transporting proteins. In fish, Cd^{2+} affect Ca^{2+} homeostasis, as both ions compete at the chloride cells of the gills (Lock et al., 1987; Matsuo et al., 2005), and

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

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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 ($\text{Na}^+/\text{K}^+-\text{ATPase}$) [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.

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Characterization of cadmium transport in hepatopancreatic cells of a mangrove crab *Ucides cordatus*: the role of calcium.

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Anexo 4: Apresentação de Pôster referente à tese de doutorado no Fourth Latin American Meeting on Biological Inorganic Chemistry – IV LABIC and Fifth Workshop on Bioinorganic Chemistry V WOQUIBIO, 2014. (Congresso). “Cadmium transport in epithelial cells of a hypohyperregulator crab *Ucides cordatus*”.

Anexo 5: Apresentação de Pôster como co-autora no Fourth Latin American Meeting on Biological Inorganic Chemistry – IV LABIC and Fifth Workshop on Bioinorganic Chemistry V WOQUIBIO, 2014. (Congresso). “Iron transport in hepatopancreatic cells of the mangrove crab *Ucides cordatus*: use of different iron compounds of pharmacologic interest”.

Anexo 6: Apresentação de Pôster como co-autora no IBIS Biennial World Meeting, 2015. (Congresso). “Water soluble magnetite nanoparticles Fe₃O₄ – oleic acid tween 80: stability, redox activity and transport in hepatopancreatic cells of the mangrove crab *Ucides cordatus*”.

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WATER SOLUBLE MAGNETITE NANOPARTICLES Fe₃O₄ – OLEIC ACID-TWEEN 80 : STABILITY, REDOX ACTIVITY AND TRANSPORT IN HEPATOPANCREATIC CELLS OF THE MANGROVE CRAB *UCIDES CORDATUS*

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Introduction

Biomodels are used to assess the consequences of human activity on animals and their habitat. The edible crab *Ucides cordatus*, endemic to the mangrove, one of the most populated and degraded ecosystems in Brazil, is used as a bioindicator of toxic metal contamination. In this study, hepatopancreatic cells of *U. cordatus* were exposed to water-soluble nanoparticles (NP) with three different sizes (5, 8, 10 nm), coated with oleic acid-detergent, with the objective to evaluate the transport of this metal to hepatopancreatic cells of the animal model. The correlation of metal incorporation with hepatopancreatic cell type, NP stability and NP redox activity was tested.

Experimental

Nanoparticles Synthesis

Pro-oxidant Activity

Determination of free iron

Transport of iron in cell

The animals were collected at Itanhaém, São Paulo coast, and acclimatized in the vivarium of the University of São Paulo.

For the experiment, the hepatopancreas were separated from crab. The organ was used for the dissociation of hepatopancreatic cells through mechanical method for the dissociation was used.

Hepatopancreatic cells were further separated using the sucrose gradient method.

The hepatopancreatic cells was then labeled with CAL-AM. The cellular transport was measured each 90 seconds in real time using a spectrofluorometer.

Results and discussion

Table 1. Concentrations and percentage (as compared to total iron) of redox-active iron in 10 µM samples of NPs (average ± s.d. of 4 measurements)

Nanoparticles of Magnetite	Redox Activity (µM)	%
5 nm	6.66 ± 0.23	10.7
8 nm	5.71 ± 0.48	14.3
10 nm	5.63 ± 0.16	14.1

Our results indicate that the NPs are very stable against different fluorescent chelators, probably because iron is not readily available from the mineral core and/or beneath the outer layer of oleic acid. Despite this stability, we found the equivalent to 5.63 - 7.16 µM of redox-active iron, which could be a surface phenomenon.

Figure 2. Iron transport in hepatopancreatic cells of *Ucides cordatus* represented as fluorescence (a.u.) in relation to time (s), during 90 s. The dashed line represents the addition of iron compound (800 µM, final concentration 200 µM), where letters represent significant statistic difference (ANOVA, P=0.05, n=5).

Compound	Iron Free	5 nm	8 nm	10 nm
Cell E	1.27±0.12	0.86±0.04	0.90±0.03	0.67±0.05
Cell R	1.05±0.02	0.61±0.02	0.55±0.01	0.43±0.01
Cell F	1.54±0.02	0.54±0.02	0.67±0.04	0.44±0.01
Cell B	1.88±0.01	0.64±0.03	0.84±0.02	0.66±0.01

Table 2. Concentrations of intracellular iron after treatment with the iron metalloids and ferrous derivatives (100 µM, average ± s.d. of 5 measurements)

The transport of NPs to the interior of cells will depend on the size, nature and charge of its surface [5]. Iron loading to E (0.67 - 0.90 µM) and R (0.66 - 0.84 µM) cells were similar because their main function is absorption of nutrients. On the other hand F (0.43 - 0.61 µM) and B (0.44 - 0.67 µM) cells, responsible for digestion and storage of nutrients, displayed lower load and therefore was not detected by the fluorescent probe in large amounts [6].

Conclusions

In this work three small magnetic NPs were synthesized and displayed similar stability in physiological buffered medium. Iron loading to hepatopancreatic cells of *U. cordatus* depends on the type of cell, and is related to their function and developmental stage.

Acknowledgements

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