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Research Paper

# 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 of crustaceans and its route into the cell is unknown. It is possible that occurs by calcium channels or calcium transporters. 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 AM 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 from hepatopancreas and is partially explained by the function the cells perform in this organ. Embryonic (E) and Resorptive (R) cells transported more $Cd^{2+}$ 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 and in a stronger way. B cells were the least responsive to $Ca^{2+}$ channel inhibitors and, unlike the other cells, showed a competition of $Cd^{2+}$ with 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 could be used as a mitigating factor for Cd accumulation in crabs' hepatopancreas.

## 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 ones 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 are known to reduce the adverse effects of toxic metals in the circulation (Kang et al., 2012). At the cellular level, it is already known that $Cd^{2+}$ is taken up by gill cells in crabs, an osmoregulatory organ, through transporters for other essential ions (). Therefore, we expect that $Cd^{2+}$ can be transported by hepatopancreatic cells through membrane transport processes in a similar way.

For other aquatic organisms, studies using radioactive tracers showed that $Cd^{2+}$ can enter the gill epithelium by calcium transport routes in fish and mollusks (Perry and Flick, 1988; Wicklund Glynn et al., 1994; Roesijadi and Unger, 1993). This transport 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. In relation to hepatopancreas, 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 $2Na^{+}/1H^{+}$ antiporter and, inside the cells, the metal can be stored in vesicles and detoxified. One other possibility is the transport through $Ca^{2+}$ channels or through competition between $Ca^{2+}$ and $Cd^{2+}$.

To date, the only study in isolated hepatopancreatic cells and metal transport in crustaceans looked at $Cu^{2+}$ transport (Chavez-Crooker

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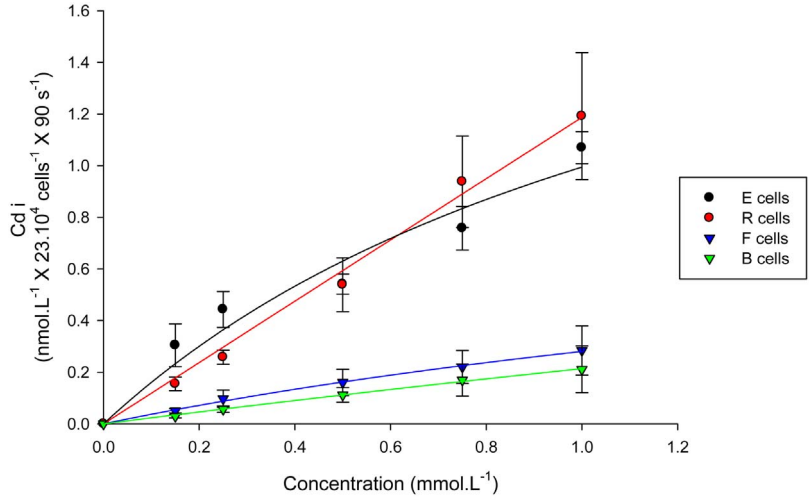



| Cell | Km | Vmax |
|---|---|---|
| E | 1.36 ± 0.93 (*) | 2.35 ± 1.05 (*) |
| R | 1.49 ± 0.35 (*) | 1.77 ± 0.42 (*) |
| F | 2.71 ± 0.72 | 1.04 ± 0.21 |
| B | 3.36 ± 1.25 | 2.40 ± 0.26 |

**Fig. 1.** Cadmium transport (nmol $L^{-1}$ × $23.10^4$ $cells^{-1}$ × 90 $s^{-1}$) in hepatopancreatic cells of a mangrove crab *Ucides cordatus* at Cd concentrations of 0.15, 0.25, 0.5, 0.75 and 1.0 mmol $L^{-1}$ added to the different cells. N = 4, P < 0.05.

et al., 2001). Copper uptake by epithelial cells of lobster hepatopancreas occurred in the presence of calcium. $Ca^{2+}$ appeared to affect the kinetics constant for $Cu^{2+}$ transport, suggesting that both metal binding and its rate of transport across the membrane were influenced by calcium. Zinc was a competitive inhibitor of $Cu^{2+}$ 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. Interestingly, Vitorino et al. (submitted, 2017) measured cell iron transport, mainly in E cells from crab hepatopancreas, reflecting these cells demand for ions to allow cell replication and differentiation. The authors noted that $Fe^{2+}$ internalization occurred by divalent metal transporters, for free iron in solution, or by endocytosis or passive diffusion, in case of iron provided as nanoparticles.

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

## 2. Materials and methods

### 2.1. Animals

Male crabs, in the intermolt 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 of salinity, gravel, water filter and pieces of brick for the emersion of the animals. Photoperiod was constant (12:12) and temperature (22 ± 3 °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 × *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 × *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 AM (1 μL) during 1 h 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 and 1.0 mmol $L^{-1}$, 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 mmol $L^{-1}$) 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 mmol $L^{-1}$) in a different batch of cells. Intracellular cadmium was calculated using the following equation:

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

where $[Cd^{2+}]i$ is the intracellular free cadmium concentration (nmol $L^{-1}$); Kd is the dissociation constant of FluoZin-3 AM (1.88); F is the actual fluorescence measured; $F_{min}$ is the minimum fluorescence

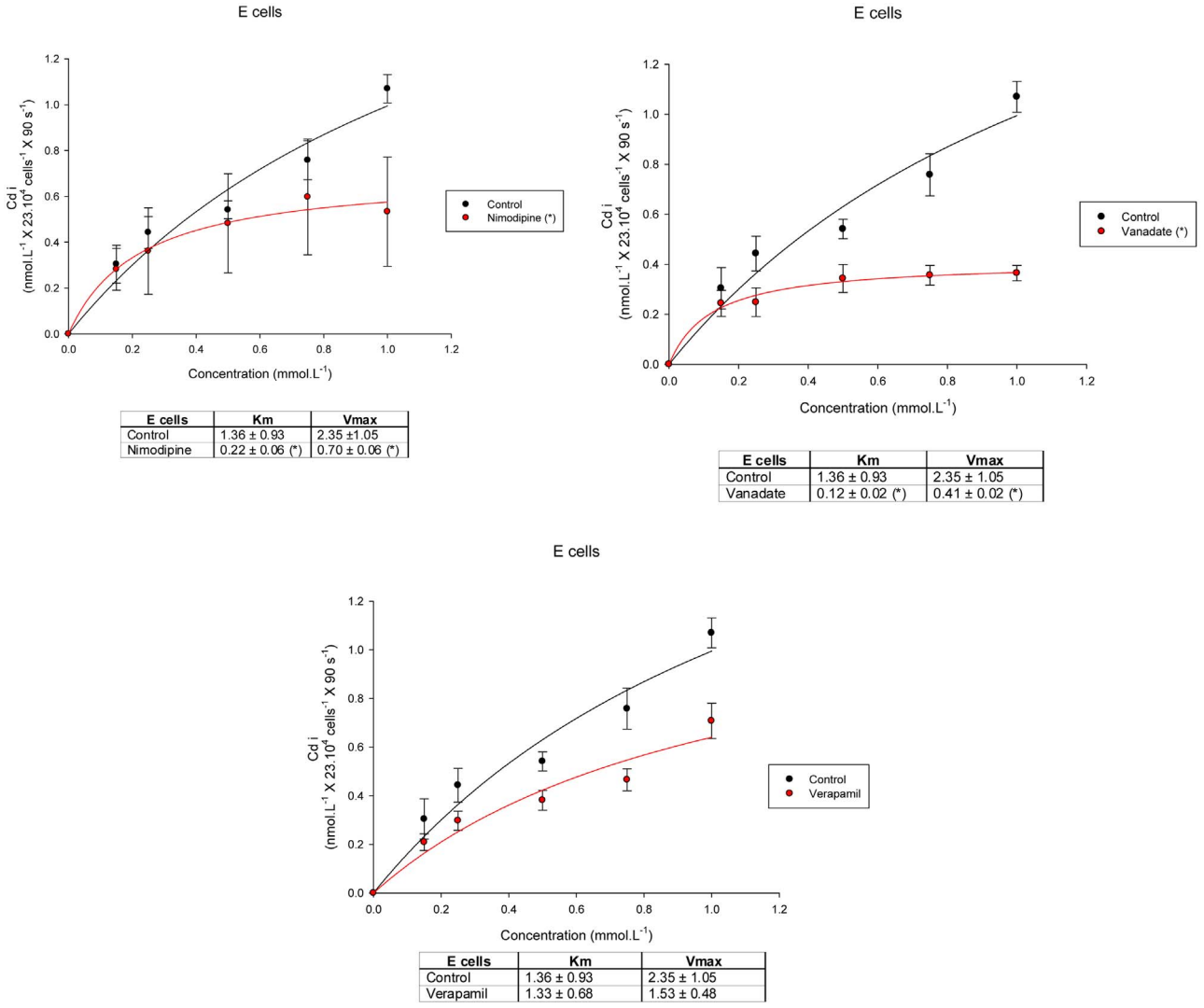



**Fig. 2.** Cadmium transport (nmol $L^{-1}$ × $23.10^4$ $cells^{-1}$ × 90 $s^{-1}$) in E cells from hepatopancreas of a mangrove crab *Ucides cordatus* at Cd concentrations of 0.15, 0.25, 0.5, 0.75 and 1.0 mmol $L^{-1}$ in the presence of nimodipine (111 mmol $L^{-1}$), vanadate (100 µmol $L^{-1}$) and verapamil (1 mmol $L^{-1}$ mM), added to the cells. N = 4. $P < 0.05$.

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 µmol $L^{-1}$) and nimodipine (111 mmol $L^{-1}$), blockers of slow calcium channels and L-type voltage-dependent calcium channels, respectively (Ahearn and Franco, 1993; Gavazzo et al., 2005): (b) ouabain (2 mmol $L^{-1}$), a $Na^+/K^+$- ATPase inhibitor (Zare and Greenaway, 1998): (c) vanadate (100 µmol $L^{-1}$), a $Na^+/K^+$ and $Ca^{2+}$-ATPase inhibitor (Zare and Greenaway, 1998): (d) BAPTA (2 mmol $L^{-1}$) a calcium intracellular chelator and (e) caffein (20 mmol $L^{-1}$), responsible for releasing intracellular calcium from endoplasmic reticulum.

For experiments using BAPTA, ouabain and caffein, specifically, each inhibitor was incubated with hepatopancreatic cells and FluoZin-3 AM during 1 h. After, cellular cadmium transport was measured through time (90 s), using 1 mmol $L^{-1}$ of $CdCl_2$.

### 2.5. Data analysis

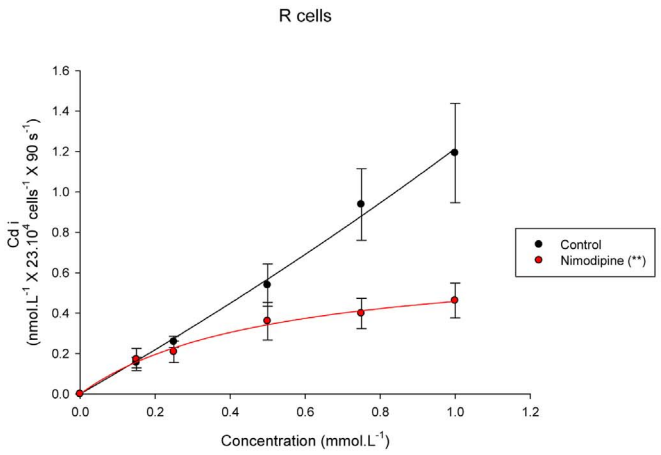
One-Way ANOVA was used to compare different inhibitors (treatments) against control. Cadmium transport was also compared between treatments using the kinetic variables, Km and Vmax, for data displayed as $Cd^{2+}$ transport against $Cd^{2+}$ concentration and/or time, after verifying the kinetic parameters fit using SigmaPlot software. We used hyperbola with diffusion fit for the cells, according to the best $R^2$ fit.

The statistical package used was SigmaStat and the data were verified for normality and homogeneity of variance. Data are displayed as means ± SE.

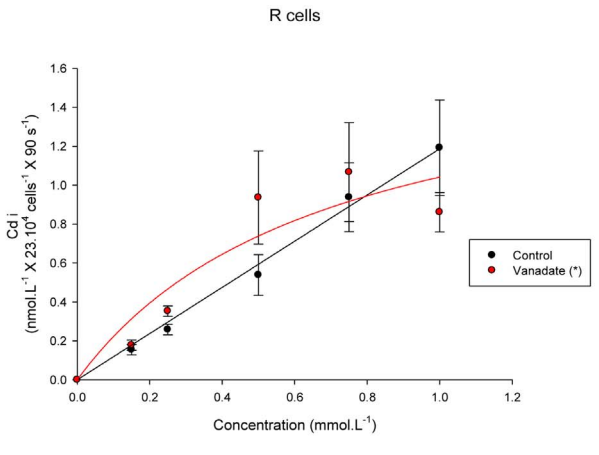
## 3. Results

The results demonstrated differences in cadmium influx, depending on the cell type from hepatopancreas. There was a cadmium influx at all $Cd^{2+}$ concentrations, and E cells, R cells, F cells and B cells transported $Cd^{2+}$ through a hyperbola kinetic fit with diffusion. Overall, R and E cells transported $Cd^{2+}$ more efficiently than F and B cells ($P < 0.05$; Fig. 1). These last two cell types showed similar transport velocities between them.

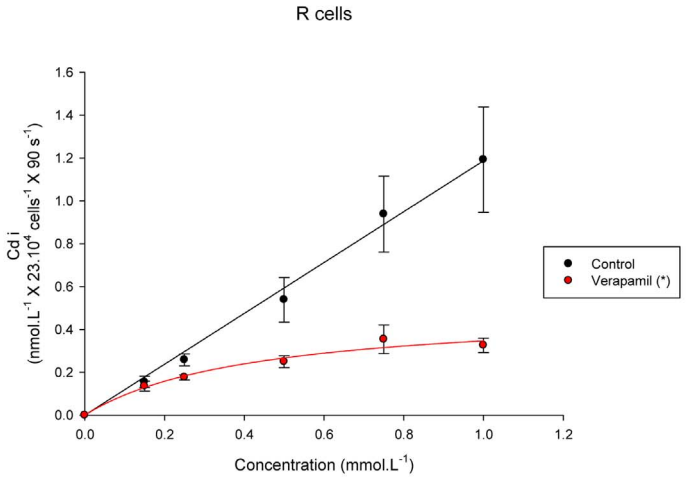
For E cells (Fig. 2), cadmium transport was statistically different between control and using the inhibitors nimodipine and vanadate. Vmax was lower in the presence of both inhibitors ($P < 0.05$). R cells, (Fig. 3) showed differences for $Cd^{2+}$ transport compared to control: nimodipine change Km and Vmax compared to control (decrease); for vanadate, there was a decrease in Km; and for verapamil, a decrease in Km and Vmax. These differences were significant ($P < 0.05$ and $P < 0.001$; Fig. 3). F cells (Fig. 4) showed a difference in transport




| R cells | Km | Vmax |
|---|---|---|
| Control | 1.49 ± 0.35 | 1.77 ± 0.42 |
| Nimodipine | 0.50 ± 0.08 (*) | 0.68 ± 0.05 (**) |




| R cells | Km | Vmax |
|---|---|---|
| Control | 1.49 ± 0.35 | 1.77 ± 0.42 |
| Vanadate | 0.69 ± 0.39 (*) | 1.76 ± 0.86 |




| R cells | Km | Vmax |
|---|---|---|
| Control | 1.49 ± 0.35 | 1.77 ± 0.42 |
| Verapamil | 0.42 ± 0.14 (*) | 0.49 ± 0.06 (*) |

**Fig. 3.** Cadmium transport (nmol $L^{-1}$ × $23.10^4$ $cells^{-1}$ × 90 $s^{-1}$) in R cells of a mangrove crab *Ucides cordatus* at Cd concentrations of 0.15, 0.25, 0.5, 0.75 and 1.0 mmol $L^{-1}$ in the presence of nimodipine (111 mmol $L^{-1}$), vanadate (100 µmol $L^{-1}$) and verapamil (1 mmol $L^{-1}$), added to the cells. N = 4, $P < 0.05$; $P < 0.001$.

($P < 0.05$) using the inhibitors nimodipine, vanadate and verapamil, with a decrease in Km and Vmax. Finally, B cells (Fig. 5) were not affected by the presence of nimodipine, vanadate and verapamil, and the transport of cadmium in the presence of inhibitors was similar to control curve.

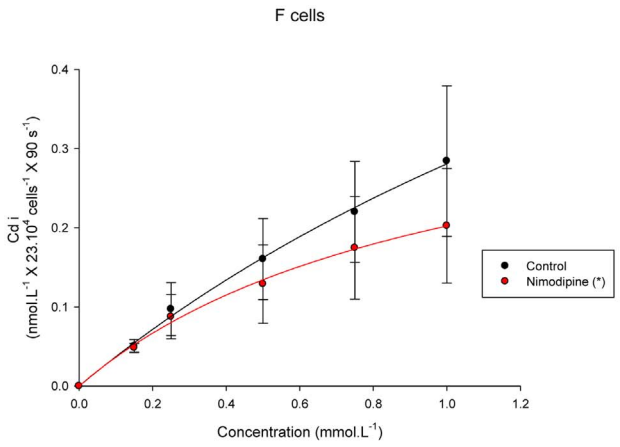
Next, in the presence of ouabain (inhibitor of $Na^+/K^+$-ATPase) and caffein (responsible for $Ca^{2+}$ efflux from sarcoplasmic reticulum) we observed that R and E cells showed a significantly higher transport compared to control cells (Fig. 6A, B). F cells also transported more $Cd^{2+}$ in the presence of ouabain and caffein (Fig. 6C). For B cells, on the other hand, results showed the opposite, a decrease of $Cd^{2+}$ transport in the presence of ouabain and caffein (Fig. 6D). In contrast, the inhibitor BAPTA interferes with cadmium transport for E, R, F and B cells, decreasing Km (affinity increased) in the presence of BAPTA. For B cells, on the other hand, Km for $Cd^{2+}$ transport was increased (affinity decreased) in the presence of BAPTA (Fig. 6A–D).

Table 1 shows an integrative view of cadmium influx for each cell type from hepatopancreas in the presence of all inhibitors used. Overall, $Cd^{2+}$ enters cells by carrier-mediated transport and, in addition, part of the transport occurs by diffusion, probably through calcium channels (both inhibited by nimodipine and verapamil) and possibly by $Ca^{2+}$/$2Na^+$ exchanger. The transport is also affected by intracellular calcium for all cell types.

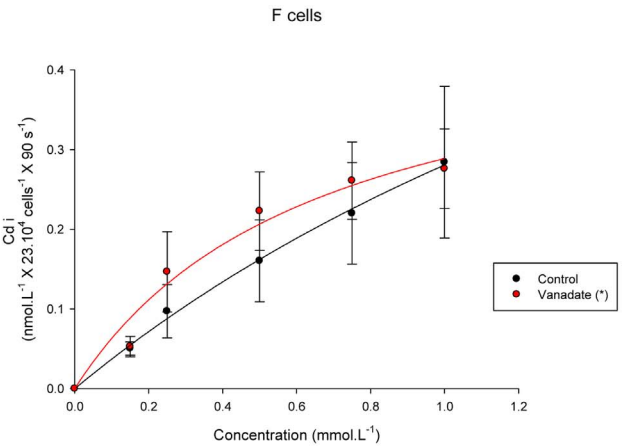
## 4. Discussion

Cadmium transport occurs differently for each hepatopancreatic cells because of different functional characteristics of each cell, reflecting the routes a toxic metal such as $Cd^{2+}$ could follow inside these cells. In general, the different cell types from hepatopancreas presented a transport of cadmium in association with saturable transporters, as well as through protein channels. However, the transport of cadmium via calcium channels and/or sodium/calcium exchanger was more expressive for all cells studied. E and R cells showed higher $Cd^{2+}$ transport compared to F and B cells. In addition, R and F cells were highly affected by $Ca^{2+}$ inhibitors (nimodipine, verapamil and vanadate) compared to E cells. All cell types (except B cells) showed increased affinity for $Cd^{2+}$ when intracellular $Ca^{2+}$ was manipulated. B cells, in addition, were not affected by plasma membrane $Ca^{2+}$ inhibitors, and were affected in the opposite manner compared to the other cell types, decreasing $Cd^{2+}$ transport when intracellular $Ca^{2+}$ was manipulated.

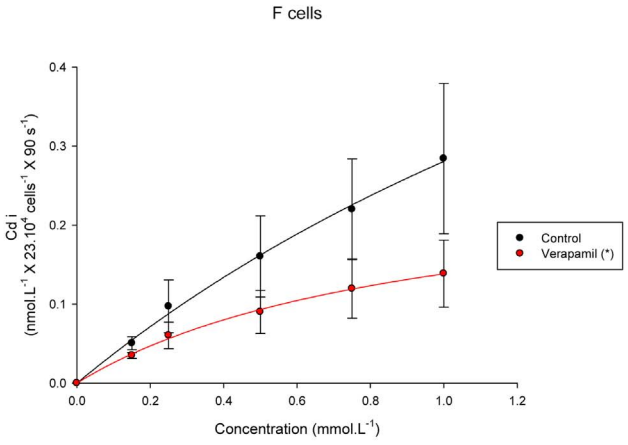
Cadmium is a toxic metal, non-essential to animals and, in high




| F cells | Km | Vmax |
|---|---|---|
| Control | 2.71 ± 0.72 | 1.04 ± 0.21 |
| Nimodipine | 1.02 ± 0.16 (*) | 0.40 ± 0.04 (*) |




| F cells | Km | Vmax |
|---|---|---|
| Control | 2.71 ± 0.72 | 1.04 ± 0.21 |
| Vanadate | 0.66 ± 0.27 (*) | 0.47 ± 0.10 (*) |

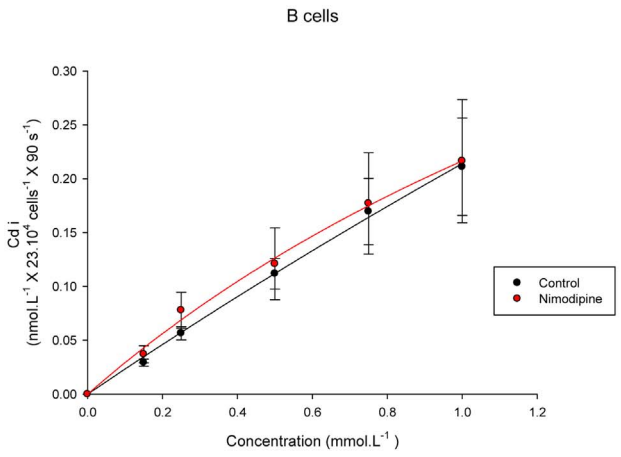



| F cells | Km | Vmax |
|---|---|---|
| Control | 2.71 ± 0.72 | 1.04 ± 0.21 |
| Verapamil | 0.93 ± 0.11 (*) | 0.26 ± 0.01 (*) |

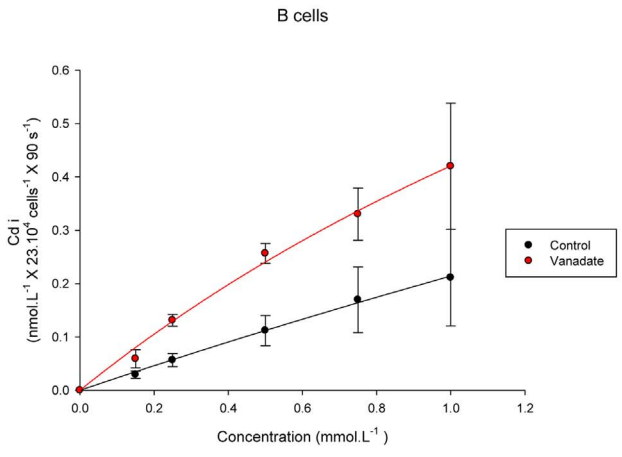
**Fig. 4.** Cadmium transport (nmol $L^{-1}$ × $23.10^4$ $cells^{-1}$ × 90 $s^{-1}$) in F cells of a mangrove crab *Ucides cordatus* at Cd concentrations of 0.15, 0.25, 0.5, 0.75 and 1.0 mmol $L^{-1}$ in the presence of nimodipine (111 mmol $L^{-1}$), vanadate (100 μmol $L^{-1}$) and verapamil (1 mmol $L^{-1}$ mM), added to the cells. N = 4, P < 0.05.

concentrations, cause cell damage (Kang et al., 2012). Work with a freshwater crab, *Sinoptamon henanense*, showed that $Cd^{2+}$ accumulates in the hepatopancreas, known as an important detoxification organ (Wu et al., 2013). $Cd^{2+}$ is believed to enter cells via $2Na^+/Ca^{2+}$ 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). We observed in another study using gill cells of *Ucides cordatus* that cells present in posterior gills had an apparent, but not significant, 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., 2014a,b). Likewise, the hepatopancreatic R-cells studied here are well known for ion transport and absorption in this organ, having a greater amount of mitochondria and ion exchangers (Chavez-Crooker et al., 2001; Kang et al., 2012; Liu et al., 2013; Ortega et al., 2014a,b) compared to the other cell types. Therefore, cadmium transport was higher in R cells probably due to its major transport role in hepatopancreas. Moreover, for hepatopancreatic R cells, Vmax was similar to $Cd^{2+}$ transport in gill cells (Ortega et al., 2014a,b). On the other hand, the transport affinity for $Cd^{2+}$ was higher in gill cells compared to what we saw here for all hepatopancreatic cells. Interestingly, studies conducted by Wheatly et al. (2002) on crayfish, gill cells transported $Ca^{2+}$ 5x more than cells from hepatopancreas, and showed greater affinity for calcium.

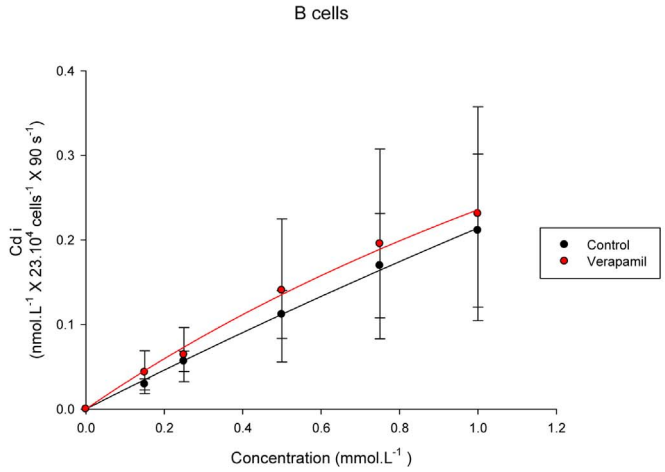
For Embryonic (E cells), a high mitotic activity is present in these cells, which are constantly replicating. This could explain the higher $Cd^{2+}$ transport observed here compared to F and B cells (Vogt, 1994; Chavez-Crooker et al., 2001; Mandal et al., 2005; Kang et al., 2012; Liu et al., 2013; Ortega et al., 2014a,b). 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 $Cd^{2+}$ transport 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




| B cells | Km | Vmax |
|---|---|---|
| Control | 3.36 ± 1.25 | 2.40 ± 0.26 |
| Nimodipine | 2.48 ± 0.80 | 0.75 ± 0.18 |




| B cells | Km | Vmax |
|---|---|---|
| Control | 3.36 ± 1.25 | 2.40 ± 0.26 |
| Vanadate | 2.98 ± 1.27 | 1.67 ± 0.26 |

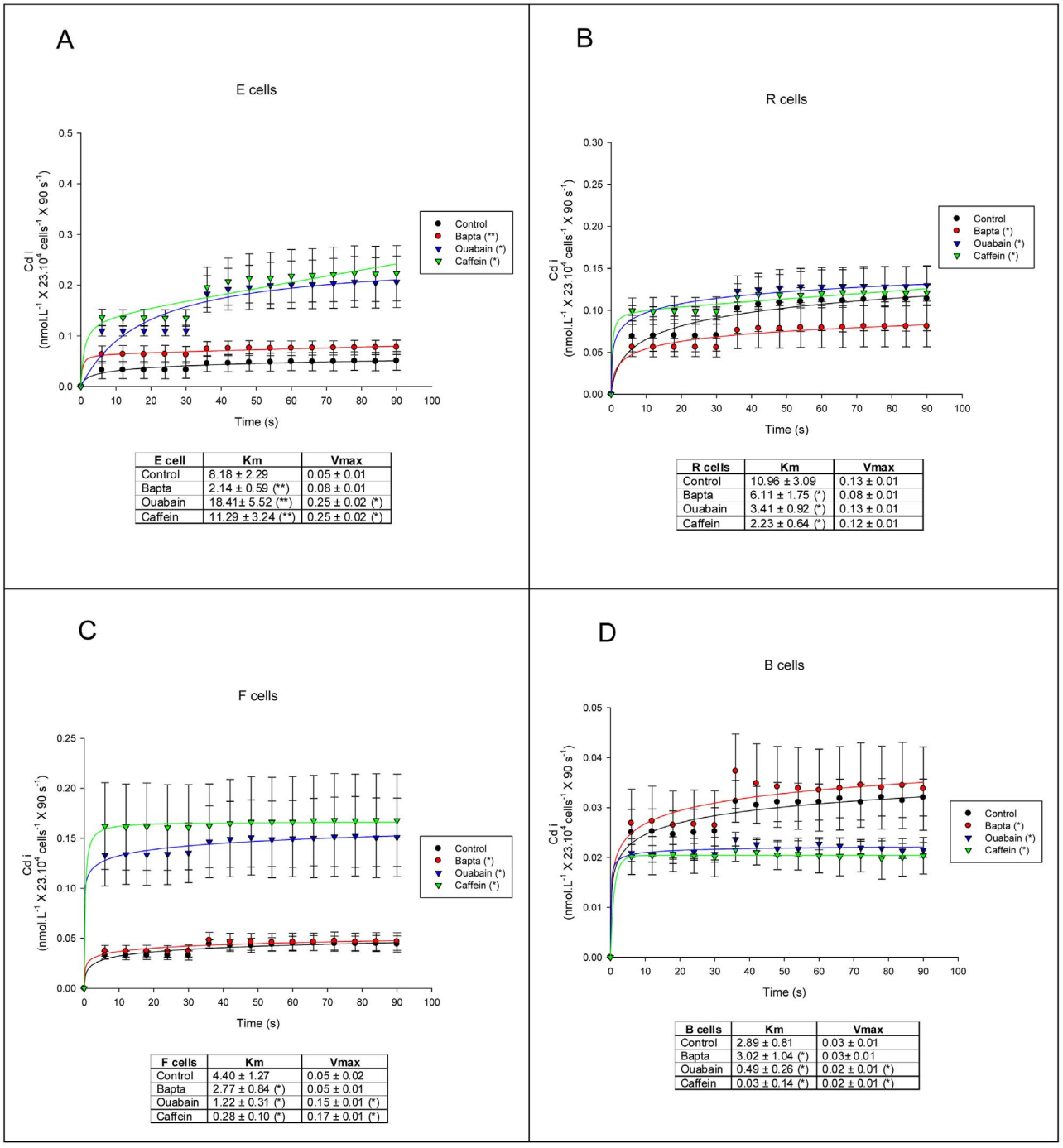



| B cells | Km | Vmax |
|---|---|---|
| Control | 3.36 ± 1.25 | 2.40 ± 0.26 |
| Verapamil | 2.85 ± 0.99 | 0.90 ± 0.24 |

**Fig. 5.** Cadmium transport (nmol $L^{-1}$ × $23.10^4$ $cells^{-1}$ × 90 $s^{-1}$) in B cells of a mangrove crab *Ucides cordatus* at Cd concentrations of 0.15, 0.25, 0.5, 0.75 and 1.0 mmol $L^{-1}$ in the presence of nimodipine (111 nmol $L^{-1}$), vanadate (100 µmol $L^{-1}$) and verapamil (1 nmol $L^{-1}$), added to the cells. N = 4.

used for B cells. These cells have a primary function of metal detoxification, and have vacuoles which occupy most of the cell. After entering the cell, $Cd^{2+}$ could be stored, or bind to metallothionein, leading to a similar decrease in intracellular cell fluorescence (Chavez-Crooker et al., 2001; Kang et al., 2012; Liu et al., 2013; Ortega et al., 2014a,b). We saw earlier through fluorescence imaging that hepatopancreatic cells usually immobilize $Cd^{2+}$ through metallothionein binding, while gills cells store excess $Cd^{2+}$ in intracellular compartments (Ortega et al., 2016).

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 $Cd^{2+}$ transport. Thus, it can be suggested that cadmium transport in R cells occurs, predominantly, 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 increased $Cd^{2+}$ transport in the presence of ouabain and caffein, and a decrease in the presence of BAPTA. This fact suggests a positive relationship between intracellular calcium and cadmium transport (Ahearn et al., 1999; Ahearn et al., 2004; Ortega et al., 2014a,b). R cells contain microvilli in the apical region (Vogt, 1994; Chavez-Crooker et al., 2001; Ortega et al., 2014a,b). This specific cellular region is responsible for transport, and allows more influx of $Cd^{2+}$, that can exchange with intracellular $Ca^{2+}$. Some studies revealed that there is a $Ca^{2+}/2Na^{+}$ 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). Therefore, our results strongly suggested that a $Ca^{2+}/2Na^{+}$ 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 are two orders of magnitude higher than for $Ca^{2+}$ (Verbost et al., 1987; Ortega et al., 2014a,b). So, $Cd^{2+}$ could affect intracellular $Ca^{2+}$ homeostasis due to the extreme sensitivity of the $Ca^{2+}$-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 dependence for intracellular $Ca^{2+}$ stores. The use of caffein, responsible for releasing $Ca^{2+}$ from sarcoplasmic reticulum (Mandal et al., 2005), affected the transport of $Cd^{2+}$




| E cell | Km | Vmax |
|---|---|---|
| Control | 8.18 ± 2.29 | 0.05 ± 0.01 |
| Bapta | 2.14 ± 0.59 (**) | 0.08 ± 0.01 |
| Ouabain | 18.41± 5.52 (**) | 0.25 ± 0.02 (*) |
| Caffein | 11.29 ± 3.24 (**) | 0.25 ± 0.02 (*) |

| R cells | Km | Vmax |
|---|---|---|
| Control | 10.96 ± 3.09 | 0.13 ± 0.01 |
| Bapta | 6.11 ± 1.75 (*) | 0.08 ± 0.01 |
| Ouabain | 3.41 ± 0.92 (*) | 0.13 ± 0.01 |
| Caffein | 2.23 ± 0.64 (*) | 0.12 ± 0.01 |

| F cells | Km | Vmax |
|---|---|---|
| Control | 4.40 ± 1.27 | 0.05 ± 0.02 |
| Bapta | 2.77 ± 0.84 (*) | 0.05 ± 0.01 |
| Ouabain | 1.22 ± 0.31 (*) | 0.15 ± 0.01 (*) |
| Caffein | 0.28 ± 0.10 (*) | 0.17 ± 0.01 (*) |

| B cells | Km | Vmax |
|---|---|---|
| Control | 2.89 ± 0.81 | 0.03 ± 0.01 |
| Bapta | 3.02 ± 1.04 (*) | 0.03± 0.01 |
| Ouabain | 0.49 ± 0.26 (*) | 0.02 ± 0.01 (*) |
| Caffein | 0.03 ± 0.14 (*) | 0.02 ± 0.01 (*) |

**Fig. 6.** Cadmium transport (nmol $L^{-1}$ × $23.10^4$ $cells^{-1}$ × 90 $s^{-1}$) in hepatopancreatic cells of a mangrove crab *Ucides cordatus* in relation to time, using Cd concentration at 1.0 mmol $L^{-1}$, in the presence of BAPTA (2 mmol $L^{-1}$), ouabain (2 mmol $L^{-1}$) and caffeine (20 mmol $L^{-1}$) added to the cells. N = 4. $P < 0.05$.

**Table 1**
Summary of cadmium transport in different cell types of hepatopancreas, such as embryonic (E), absorptive (R), fibrillar (F) and blister (B) cells. The negative effect on the transport of cadmium is indicated by the negative symbol (−) and the positive effect is indicated by the positive symbol (+), NE (no effect).

| Celular type | Inhibitors | | | | | |
|---|---|---|---|---|---|---|
| | Nimodipine | Vanadate | Verapamil | BAPTA | Ouabaine | Caffein |
| E | − | − | NE | − | + | + |
| R | − | − | − | − | + | + |
| F | − | − | − | − | + | + |
| B | NE | NE | NE | + | − | − |

positively, suggesting that is necessary the presence of intracellular $Ca^{2+}$ for $Cd^{2+}$ 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) and vanadate (inhibitor of $Na^+/K^+$-ATPase and $Ca^{2+}$-ATPase). It can be suggested that cadmium transport in E cells occurs, predominantly, 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 caffein. 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., 2014a,b). 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 $Cd^{2+}/Ca^{2+}(2Na^+)$ exchanger in hepatopancreatic cells (Ortega et al., 2014a,b). 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, vanadate and verapamil. According to these results, we suggest that $Cd^{2+}$ input occurs, predominantly, via an L-type calcium channel, slow calcium channels and $Ca^{2+}$-ATPase transporter (Ahearn et al., 1999; Ahearn et al., 2004). F cell showed an increased transport in the presence of ouabain and caffein. 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., 2014a,b).

Finally, $Cd^{2+}$ transport in B cells was not affected by nimodipine, verapamil and vanadate. A channel therefore is not relevant for $Cd^{2+}$ transport. B cell was 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 caffein. We suggest that there is a competition between cadmium and intracellular calcium in these cells (Ortega et al., 2014a,b). These cells also play a strong role in detoxification and metal accumulation (Vogt, 1994; Ortega et al., 2014a,b), and $Cd^{2+}$ could enter these cells and be rapidly sequestered by vacuoles or chelating proteins like metallothionein. In a study by Ahearn et al. (2004), the authors suggested that 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). A $Ca^{2+}$ −stimulating $Cd^{2+}$ uptake inside lysosomes could explain why $Cd^{2+}$ was low in B cells cytoplasm when intracellular $Ca^{2+}$ increased. Studies developed by Ahearn et al. (2004) with lobster lysosomes *Homarus americanus* revealed the presence of a calcium-stimulating ATPase on the organelle membrane, showing high affinity transport for copper and cadmium. Thus, high intracellular concentrations of calcium could stimulate the entry of $Cd^{2+}$ into the organelle.

## 5. Conclusions

Cadmium transport occurs differently in each cell type and is partially explained by the function each cell performs in the hepatopancreas. The different cell types can transport cadmium by carrier-mediated transporters and channels. E and R cells have higher $Cd^{2+}$ transport compared to F and B cells. R and F cells were more affected by $Ca^{2+}$ channel inhibitors, compared to E cells, and all cells type responded positively to increased intracellular $Ca^{2+}$. B cells, unlike the other cells, were not affected by $Ca^{2+}$ channel inhibitors and responded negatively to increased intracellular $Ca^{2+}$. Therefore, the diversity of response of each hepatopancreatic cell to $Cd^{2+}$ transport and the diversity of responses to the presence 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 cells. In addition, knowledge about cell metal transport can, although in a small scale, help us to understand how $Ca^{2+}$ mitigate $Cd^{2+}$ and other non-essential metals accumulation.

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