Ecdysis as an auxiliary route for the removal of heavy metals in crustaceans: Laboratory analysis with native mangrove specimens

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Research Article

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Abstract

Fiddler crabs (*Minuca burgersi*) were kept under controlled conditions in an experimental laboratory to assess their ability to secrete metallic contaminants by ecdysis. They were fed different copper (Cu) and lead (Pb) concentrations, with subsequent measurement of heavy metals concentration in the released and renewed exoskeletons. Our results show that these crabs can purify both metals during ecdysis, demonstrating to be an efficient way to decontaminate these toxic components, besides accrediting this native species as a strong indicator of contamination in estuarine environments. Our results also reinforce the need for attention to the contamination characterization in crustaceans before and after ecdysis to avoid underestimation.

1. Introduction

Different studies in ecotoxicology, which use reference specimens to measure contaminants concentrations in different environments, have been described for years. The use of these reference specimens, which are generally not native to the environment, can generate underestimated responses to contamination's real impact. Therefore, recent publications advocate using native species for such measurements since toxicological responses may more accurately reflect environmental conditions (Do Hong et al. 2004; Takenaka et al. 2006, 2007; Okumura et al.2007).

Among the different types of contaminants of anthropic origin, metallic compounds cause concern worldwide since they cause profound adverse effects on the organisms of the most diverse ecosystems and human populations (Ramos et al. 2010; Vilhena et al. 2013).

Among these ecosystems, the estuarine environment is one of the most contaminated by metallic compounds, which seriously affect the physiology of the organisms exposed to these contaminants (Capparelli et al. 2017; Portner 2010; Veríssimo et al. 2013). Organisms, such as crustaceans, exposed for a long time to these metallic contaminants, when they survive, end up accumulating them directly or indirectly through contact with contaminated water, substrate, and food intake (Boyd 2010; Oliveira et al. 2013; Rumisha et al. 2017). As the bioaccumulation process is continuous, this will provide the absorption of heavy metals by different tissues of native crustaceans, accrediting them to sensitive indicators of environmental degradation (Bergey & Weis 2007; Kannan et al. 2016; Capparelli et al. 2016, 2017)

However, these species have detoxification mechanisms to deal with these contaminants, such as the production of metal granules linked to metallothionein, performed by the hepatopancreas, or the secretion of metal salts by osmosis made by the gills (Capparelli et al. 2017; Martins et al. 2011; Mazzei et al. 2014; Pinheiro et al. 2012).

Although less studied, another way for crustaceans to contain and reduce the bioaccumulation process by heavy metals is through the exchange of their exoskeleton (ecdysis). This mechanism proved to be efficient and essential for the detoxification process because it occurs frequently and naturally, in which exoskeletons containing a portion of the contaminants end up being discarded in the environment during the growth of the animal. Through the process of ecdysis, a portion of the contaminants present in the hemolymph ends up migrating to the exoskeleton, providing for its elimination when discarded (Bergey & Weis 2007).

However, establishing baseline metal contamination levels in studies with specimens collected directly from the natural environment is challenging to measure. Therefore, experimental studies where the native crustaceans samples are kept in laboratory conditions free from contamination by metals before evaluating their secretion capacity are necessary. Therefore, under such controlled experimental conditions, we quantify the elimination of metallic contaminants, copper, and lead, from the ecdysis process, in native specimens typical of estuarine ecosystems, *Minuca burgersi* (Holthuis 1967).

Under controlled laboratory conditions, we seek to demonstrate that heavy metals' clearance on crustaceans occurs at specific time intervals linked to ecdysis processes. In this way, it would be possible to establish a more precise sampling protocol for these organisms to ascertain the environment's real degradation rates in the event of impacts with metallic contaminants.

2. Methods

2.1. Animal acclimatization

In this experiment, 36 adult male guitarist crabs of the species *Minuca burgersi* (Holthuis 1967), with carapace ranging from 18 to 20 mm, were used. These animals were collected in the Lameirão mangrove, located in the city of Vitória, Espírito Santo, Brazil (20°17 '0.27"S and 40°18' 20.87"O).

These crustaceans were equally divided into six acrylic containers with 27,000 cm^3 containing sand sediment and partially immersed in 24‰ saltwater, forming six groups (Fig. 1).

After testing with different food offerings, e.g., mangrove plant leaves, shrimp, and fruit pieces, the pineapple, without heavy metal contamination, was selected as a nutritional source in the whole experiment due to better acceptance by the crustaceans.

These individuals were kept in these conditions for 90 days for acclimatization and to allow the animals to perform ecdysis to release the exoskeletons formed in the natural environment, ensuring that the next exoskeleton contained the smallest possible traces of contaminants acquired in the natural environment. It is worth mentioning that under laboratory conditions, the average ecdysis time was three months.

2.2. Crab contamination procedure and arrangement of experimental groups

For food contamination, we follow indications of works published by Overnell & Trewhella (1979) and Overnell (1984), where 5 mg of $CuSO_4.5H_2O$ and 10 mg of salts $Pb(NO_3)_2$ (Sigma-Aldrich Inc., USA) were added to each gram of pineapple, reaching Cu concentrations between 3.2 to 5.9 mg and Pb concentrations between 2.0 to 8.0 mg, per gram of pineapple.

Three groups were established. Group A was fed daily with pineapple without contamination by metals, and the other two groups fed daily with contaminated amounts of pineapple until reaching the daily concentration of 5 mg/g (Group B) and 10 mg/g (Group C).

After the ecdysis was carried out in the acclimatization period, the crabs were fed, as already mentioned, until they carried out the first ecdysis, approximately 30 days after the experiment began. The exoskeletons were preserved for the later analysis, being considered as a released exoskeleton.

After the first molting, all groups' crabs started to receive uncontaminated pineapple until the next ecdysis occurred, which also occurred approximately 30 days after the beginning of this second nutrition procedure, whose exoskeletons were also preserved for later analysis and were considered as a renewed exoskeleton.

The exoskeleton samples were dried for three days at 60°C, with subsequent grinding, 1 g of each crushed sample was digested in a 3:1 acid solution of $HCl + HNO_3$ (both from Sigma-Aldrich Inc., USA). These samples were then kept at 60°C for 48 h, filtered through 0.45 µm filters (Analítica Ltd., Brazil), Cu and Pb measurements were present in the samples. They were evaluated by atomic absorption spectrometry as previously described (Carvalho et al. 1991), using the ICE 3000 atomic absorption spectrometer (Thermo Scientific Inc., USA). All data are presented in µg/g of dry exoskeleton. Our measurements were validated after analysis of certified samples (Table 1) obtained from *Mytilus edulis* tissue (ERMCE-278K) and estuarine sediment (BCR-667), both purchased from Sigma-Aldrich Inc., USA. Whose research inputs were partially financed by the “Espírito Santo Research and Innovation Support Foundation”, FAPES (grant nº 0461/2015), and “Coordination for the Improvement of Higher Education Personnel”, CAPES.

Table 1
Recovery percentages of certified reference material. Cu = copper; Pb = lead

Reference material	**Reference values (mg.kg^{-1})**		**Found values (mg.kg^{-1})**		**Recovery (%)**	
	[Cu]	**[Pb]**	**[Cu]**	**[Pb]**	**[Cu]**	**[Pb]**
Mytilus edulis	6.0	2.2	6.50	1.9	108.7	90.6
Estuarine sediment	60.0	31.9	55.6	26.4	92.6	82.6

Certified samples of *Mytilus edulis* fabric (ERMCE-278K) and estuarine sediment (BCR-667), both purchased from Sigma-Aldrich Inc., USA, were used as reference material for measuring the amounts of copper (Cu) and lead (Pb). The results in mg.kg^{-1} were converted to the percentage recovery of the reference material.

2.3. Statistical analysis

Independent samples Student's t-tests were used to verify statistically significant differences in Cu and Pb concentrations before and after the ecdysis. Six analyzes were applied, one for each element (Cu and Pb) and group (A, B, and C). Before applying the t-tests, the assumptions of normality and homoscedasticity of the data were checked by Shapiro-Wilk and Levene tests, respectively. If the normality assumption were violated, the non-parametric Mann-Whitney U test would be used; and if the normality assumption was not violated, but that of homoscedasticity was, we would use the t-test with Welch's correction. All analysis and graphics were performed at GraphPad Prism software, 5.0 version (GraphPad Software Inc., USA).

3. Results And Discussion

The fiddler crabs *Minuca burgersi* is a crustacean from the intertidal region with wide distribution, occurring in the Western Atlantic of the USA (south of Florida), Mexico to Brazil, and all the Caribbean islands (Thurman et al. 2013). This species inhabits and eats organic debris associated with surrounding sediments, in addition to being considered an ecosystem engineer due to his high bioturbation skills at the sediment-water interface, which also modifies the availability of resources and pollutants such as trace elements (Pillon et al. 2019).

Due to its wide distribution, ease of sampling, high capacity for population recovery, and laboratory management, in addition to the extensive literature on its biological and ecological characteristics, it becomes an adequate bioindicator for these countries.

At the end of the experiment, we verified that there was a reduction, but not significant, in the concentrations of Cu or Pb between the exoskeletons released and renewed in Group A, without contamination, whose average concentrations of Cu and Pb whose variations were 14.72 μg/g and 0.12 μg/g, respectively (Fig. 2A, Table 2).

Table 2

Statistical tests results. Independent samples Student's t-tests were used to verify statistically significant differences in Cu and Pb concentrations before and after the ecdysis. Six analyzes were applied, one for each element (Cu and Pb) and group (A, B, and C).

	Shapiro-Wilk normality test		**Levene's homogeneity of variances test**		**Independent samples Student t-test**	
	W	**p**	**F**	**p**	**t**	**p**
Group A - Cu (0 mg/g)	0.95	0.229	5.06	0.035	1.67	0.109
Group B - Cu (5 mg/g)	0.96	0.361	1.12	0.301	3.66	0.001[a]
Group C - Cu (10 mg/g)	0.96	0.519	13.33	0.001[a,c]	6.71	0.000[b]
Group A - Pb (0 mg/g)	0.96	0.430	0.00	0.977	0.24	0.809
Group B - Pb (5 mg/g)	0.92	0.071	1.19	0.286	5.44	0.000[b]
Group C - Pb (10 mg/g)	0.96	0.359	0.00	0.967	3.60	0.002[a]

[a] $p \leq 0.01$; [b] $p \leq 0.001$; [c] the Welch's correction was applied to the t-test p-value result because of the homoscedasticity assumption violation.

When we evaluated the crabs present in Group B and Group C, we found a significant reduction in the average concentrations of Cu and Pb in the released and renewed exoskeletons, whose variations were 22.05 µg/g and 3.09 µg/g, with 5 mg/g concentration metals in the food, and 80.89 µg/g and 2.12 µg/g, with 10 mg/g concentration metals in the food, respectively (Figs. 2B and 2C, Table 2).

Interestingly, in the renewed exoskeletons of Group B crabs, we found lower levels of concentration of Cu and Pb than renewed exoskeletons in Group A (Figs. 2A and 2B). This indicates that when offering foods containing intermediate concentrations of metals, complementary detoxification processes must have been triggered, such as the action of the hepatopancreas and gills, leading to a lesser migration of metals from the hemolymph to the exoskeleton, leaving a smaller portion of heavy metals available for adsorption in the exoskeleton of this group.

According to Pytharopoulou et al. (2008), Lemus et al. (2016), and Capparelli et al. (2018), metalloneins and similar proteins that bind to metals rich in low molecular weight cysteines are found in increased levels in the digestive gland, hepatopancreas, and gills of marine crustaceans exposed to environmental conditions contaminated with heavy metals, being essential for the pathways of trace elements in the metabolism and sequestration of metals. Thus, they act in the process of bioaccumulation of toxic metals, detoxification, homeostatic regulation of metals, protection against oxidative stress, among others.

It is worth mentioning that some studies have discussed the use of these proteins as specific metallic biomarkers because they are also related to other environmental variations, such as salinity, sex, stages, body size, seasonality, and tissues (Morgan et al. 2004; Ladhar-Chaabouni et al. 2011; Cenov et al. 2018). However, because it is a controlled experiment, this analysis bias can be discarded in our interpretations.

By doubling the concentration of metals in the food, doses of Cu, and Pb in Group C, with 10 mg/g, we found that the metals' migration behavior from the hemolymph released to the exoskeleton occurred differently (Fig. 2C). For Cu, the concentration in this exoskeleton more than doubled in relation to Group B, while for Pb, the concentrations were equivalent. This result may be related to the existence of greater affinity of this metal for the tissues that form the exoskeleton in the stiffening mechanisms during ecdysis, or even the greater availability of Cu in the hemolymph, despite the same concentration of Cu and Pb in the food offered. Several authors, e.g., Rainbow (2002); Kouba et al. (2010); Stanek et al. (2014), reported in their studies that Cu is a component of the respiratory system of crustaceans, forming hemocyanin, causing high concentrations of this metal to exist in their hemolymph when compared to other metals. Thus, it is assumed that there is a greater tendency for the occurrence of a simple migration/diffusion to the tissues that form the exoskeleton.

Despite the difference in the accumulation of metals in the released exoskeleton, it was noticed that the mechanisms of elimination of these toxic components were activated in a similar way to that observed for Group B, generating concentrations of these metals in the renewed exoskeletons, which are also equivalent.

In this way, it can be inferred that different Cu and Pb concentrations do not interfere with the normal clearance physiology of these animals for clearance (Figs. 2B and 2C).

These results indicate that ecdysis is one of the physiological processes by which this species of crustacean purges Cu and Pb concentrations (Fig. 2B and 2C). In agreement with our findings, other authors have described that the mobilization of Cu in the exoskeletons of *Minuca rapax* (other species related to the Fiddler crabs) for the hemolymph is dynamic, occurring whenever the animal needs this metal (Capparelli et al. 2017). For Pb, it was previously described that *Minuca pugnax* raised in environments with high Pb concentrations eliminated Pb by ecdysis, while the same species of crab raised in low concentrations of Pb absorbed it in soft tissues (Bergey & Weis 2007). Ramos et al. (2021) also characterized the process of detoxifying heavy metals in *Ucides cordatus* crabs *in natura* during the period of ecdysis, demonstrating to be an important detoxification route in these invertebrates.

The molecular mechanisms involved in these clearings are unknown, but it was observed that two intracellular enzymes, protein kinase C (PKC) and phosphodiesterase (PDE), were always activated when the crabs were exposed to high concentrations of Cu and Pb; high production of PKC and PDE, and in turn, stimulates the increase of ecdysteroids in the pre-ecdysis phase, which finally stimulates ecdysis (Spaziani et al. 2001).

This work differs from previous studies in that we conducted a controlled experiment in the laboratory, where the dosage of the metallic contaminant could be pre-established and the food removed after ecdysis (Fig. 2B and 2C). Since these animals can re-accumulate these metals due to their ethological behavior (White and Rainbow, 1986), ecdysis in the Cu and Pb purge can be more accurately quantified by controlling other external parameters. This sensitivity is necessary since the renewed exoskeletons' levels of contamination were practically the same observed in renewed exoskeletons of animals fed without contaminated food (Fig. 2B and 2C vs. 2A).

4. Conclusions

Our results indicate that studies with crustaceans to measure the environmental impact in degraded areas must consider the complex physiological and ecological dynamics involved in the biology of the population of native organisms. Also, in ecosystems that continuously receive metallic contaminants, crustaceans can be used at any time of the year because their exoskeletons retain traces of metal.

However, in isolated cases of contamination, e.g., rupture of mining dams, or accidental discharge of effluents with metallic contaminants, the use of crustacean exoskeletons to assess the environmental impact should be performed before ecdysis periods, avoiding underestimation of results and misjudgment of the true environmental impact.

Finally, the use of exoskeleton released in ecdysis as an environmental marker allows the assessment of the degree of contamination of the organism without the need for sacrifice, allowing its reintroduction to its natural environment and minimizing the ecological footprint left by this type of monitoring, then that would be extremely important for studies with endangered species.

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Figures

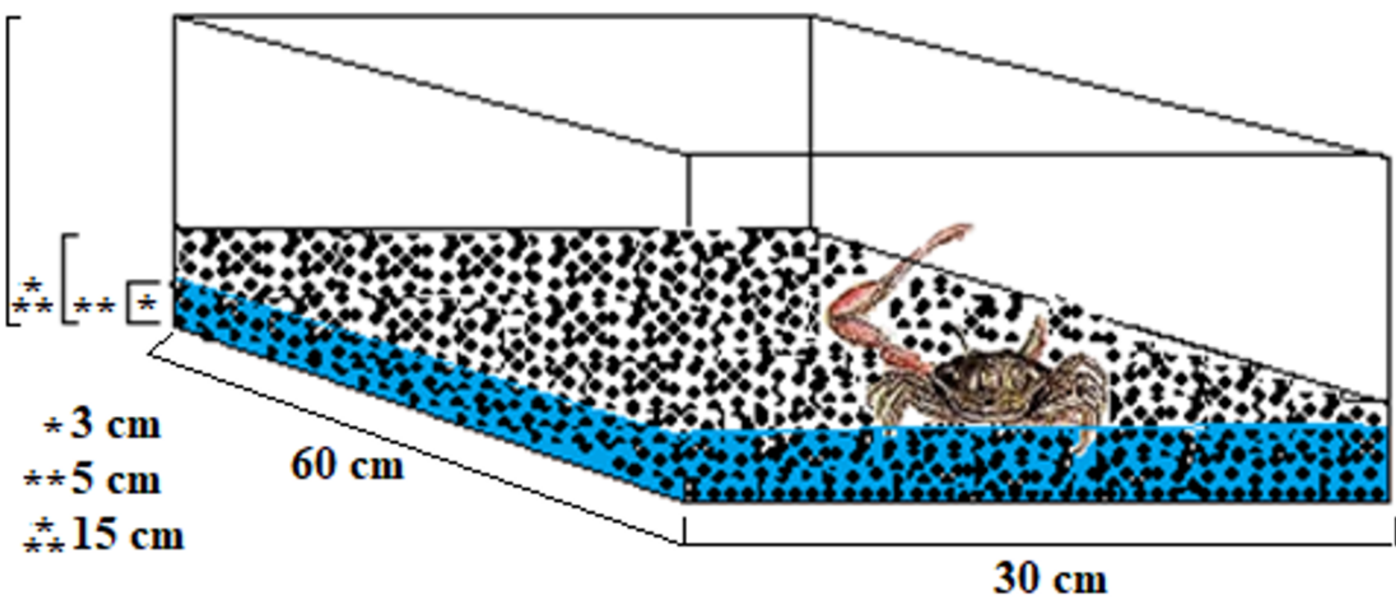

Figure 1

Acclimatization container for crustaceans (27,000 cm3). * height of the saline water layer (24 ‰); ** height of the sandy sediment; *** maximum height of the container.

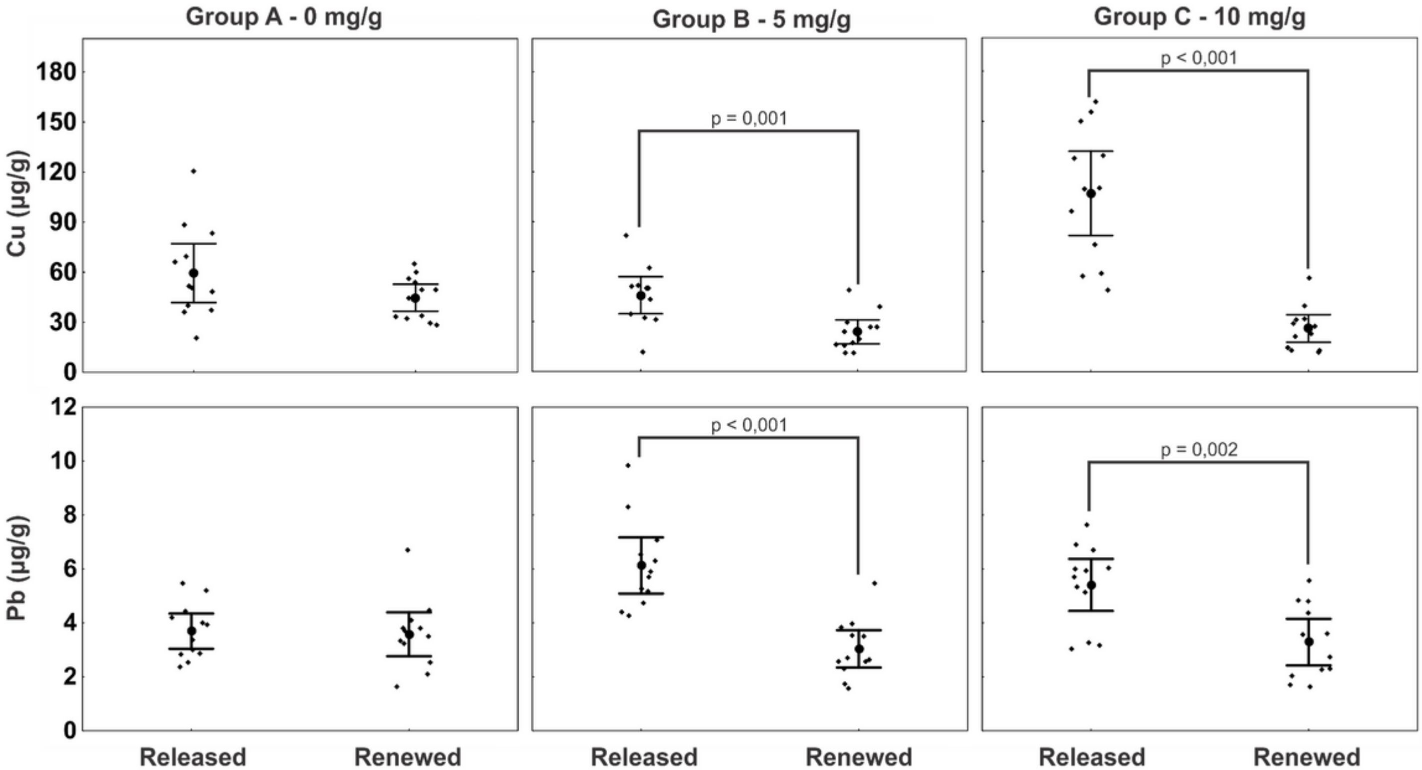

Figure 2

Concentrations of the metals Cu and Pb through ecdysis by the fiddler crabs M. burgersi. Each point represents an individual value for each crustacean. The full circle represents the mean ± 95% confidence interval of all crabs' metal concentrations in each group.