Mercury accumulation and metallothionein expression from aquafeeds by *Litopenaeus vannamei* Boone, 1931 under intensive aquaculture conditions

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(With 3 figures)

Abstract

This study describes the accumulation of Hg and metallothionein gene expression in *Litopenaeus vannamei* Boone, 1931 with aquafeeds as the major source of Hg. Trials were conducted under controlled conditions in experimental tank facilities with high (indoor tanks) and low (outdoor tanks) Hg aquafeeds concentrations. Aquafeeds were the sole source of Hg for the shrimps and concentrations varied from 5.4 to 124 ng.g^{-1} d.w.. In the three animal fractions analysed; muscle (6,3 – 15,9 ng.g^{-1}); hepatopancreas (5,1 – 22,0 ng.g^{-1}) and exoskeleton (3,0 – 16,2 ng.g^{-1}), Hg concentrations were significantly lower in the outdoor trials submitted to Hg-poor aquafeeds. Maximum shrimp muscle Hg concentrations were low (36.4 ng.g^{-1} w.w.) relative to maximum permissible concentrations for human consumption and Hg content in muscle and hepatopancreas were significantly correlated with Hg content in aquafeeds. Highest Hg concentrations in the exoskeleton of animals exposed to Hg-richer aquafeed, suggested that a detoxification mechanism is taking place. On the other hand the metallothionein suffered no variation in its relative expression in any of the experiments, meaning that the contact with feed containing the observed Hg concentrations were not sufficient to activate gene transcription. It was not possible, under the experimental design used, to infer Hg effects on the biological performance of the animals.

Keywords: mercury, shrimp farming, aquafeed, bioaccumulation, metallothionein, PCR.

Acumulação de mercúrio presente em rações e expressão de metalotioneínas em *Litopenaeus vannamei* Boone, 1931 sob condições de cultivo intensivo

Resumo

Este estudo descreve a acumulação de Hg e a expressão gênica de metaloproteínas em *Litopenaeus vannamei* Boone, 1931 tendo a ração como única fonte de Hg. Os experimentos ocorreram sob condições experimentais controladas comparando-se tanques receptores de rações ricas em Hg (tanques interiores) e pobres em Hg (tanques exteriores). As concentrações de Hg nas rações variaram de 5,4 a 124 ng.g^{-1} em peso seco. Nas três frações dos animais analisados; músculo (6,3 – 15,9 ng.g^{-1}); hepatopâncreas (5,1 – 22,0 ng.g^{-1}); e exoesqueleto (3,0 – 16,2 ng.g^{-1}), as concentrações de Hg foram significativamente menores nos experimentos em tanques exteriores submetidos a rações com menores teores de Hg. As maiores concentrações de Hg medidas na musculatura dos camarões ao final do experimento (34,6 ng.g^{-1}) foram muito baixas em relação às concentrações legais máximas permitidas para consumo humano e as concentrações em musculatura e hepatopâncreas foram significativamente correlacionadas às concentrações nas rações. As maiores concentrações de Hg medidas no exoesqueleto de animais expostos a rações de maior conteúdo de Hg, sugerem a ocorrência de um mecanismo de detoxificação. Por outro lado, a expressão de metaloproteínas não apresentou variação entre as diferentes concentrações de Hg presentes na ração, sugerindo que a concentração utilizada não foi capaz de induzir a transcrição gênica responsável pela produção de metaloproteínas.

Palavras-chave: mercúrio, aquacultura, bioacumulação, metalotioneína, PCR.

1. Introduction

The necessity of large scale supply of animal protein for a growing population simultaneously to a sharp decrease in natural fisheries occurring during the past two decades has resulted in a worldwide spread of aquaculture of many aquatic species. Shrimps are one of the most successful aquaculture products with a well established technology for large scale intensive and semi-intensive cultivation. The high productivity achieved in shrimp aquaculture is dependent on a large supply of aquafeeds (5 to 10 $t.ha^{-1}.year^{-1}$) with a resultant productivity of 2 to 6 $t.ha^{-1}.year^{-1}$. Chemical composition of aquafeeds is variable, concentrations of major nutrients, protein and fat contents are relatively controlled. However, minor components, including trace metals mostly originally present in aquafeeds as natural constituents of the original raw materials, are not controlled (Boyd and Massaut, 1999; Tacon and Forster, 2003; Usydus et al., 2009). For example, concentrations of Cd in shrimp aquafeeds can vary up to one order of magnitude (4.8 to 36.9 $\mu g.g^{-1}$) (Macedo et al., 1991). Concentrations of Cu ranging from 13.1 to 79.0 $\mu g.g^{-1}$ were reported in aquafeeds from intensive shrimp culture in northeastern Brazil, resulting in a total load of 194.5 $gCu.ha^{-1}.cycle^{-1}$ (Lacerda et al., 2006).

Among the trace metals eventually present in aquafeeds, Hg is of high significance not only due to its ubiquitous presence but to its toxicity to the shrimps proper since it affects the osmoregulation capacity, fundamental for estuarine organisms (Kraus and Weis, 1988; Choi and Cech, 1998; Amand et al., 1999). Excessive Hg concentrations in water or aquafeeds caused an inhibition of oxygen consumption of over 52% and an increase of 217% in ammonium excretion in *Farfantepenaeus brasiliensis* (Barbieri et al., 2005). Other effects on gill morphology and loss of hepatopancreas R cells have also been described for *L. vannamei* (Frías-Espericueta et al., 2008). Therefore, Hg exposure may negatively affect aquaculture activities located in estuarine areas, in general receiving anthropogenic loads of Hg.

Most studies on Hg toxicity to reared shrimps were based on the metal being present in water. No study, to our knowledge, discussed the importance of aquafeeds as the major Hg source to the animals; although Hg in aquafeeds is known to adversely affect growth and physiology of reared marine fish (Berntssen et al., 2004). Also in reared fish, changing Hg concentrations in fish aquafeed resulted in changing final muscle Hg concentrations (Nakao et al., 2009).

In most marine invertebrates metal toxicokinetics relays on the chelating proteins, of which the ubiquitous small cystein rich metallothioneins (MT) are the best example. The active transport of divalent cations by epithelial cell membrane ATPase and precipitation with negatively charged sulfate, phosphate or oxalate insoluble complexes within lisosome, or as amorphous granules, are other reported detoxification mechanisms (Rainbow, 1997). In crustaceans ecdysis is also an important detoxification strategy, due to their efficiency in pumping metals elements across the epithelial membrane to the exoskeleton (Keteles and Fleeger, 2001). Despite the suggestion that non essential metals such as Cd^{2+} and Hg^{2+} are not regulated in crustaceans (Frías-Espericueta et al., 2001) concentrations in the primary targets for Hg bioaccumulation in *L. vannamei*, such as hepatopancreas and muscle, presents concentrations as high as in most other marine invertebrates (Ruelas-Inzunza et al., 2004).

The majority of toxicity tests is conducted in laboratory and can evaluate the acute response of a biomarker, either concentration of the chemical or the response time. However, under aquaculture conditions, it should be more important to find out the whether a resistance mechanisms are initiated (with great damage to the organism and therefore to the farm's productivity) or new equilibrium is reached within tolerance limits, which would allow continuation of the culture. Thus, the present study was developed to describe the accumulation of Hg and MT gene expression in *L. vannamei*, with aquafeeds as the major source of Hg, in trials conducted under controlled conditions in the experimental tank facilities at the Instituto de Ciências do Mar (UFC), Ceará, Brazil.

2. Material and Methods

2.1. Trials description

In order to assess Hg accumulation by *L. vannamei*, two controlled culture systems consisting of a total of 10 indoor and 10 outdoor tanks (1.02 m and 0.57 m) were assigned for the trials. The outdoor system has green water conditions and is continually exposed to weather changes and was stocked with 40 $animals.m^{-2}$. Comparatively, indoor tanks have clear water conditions (no availability of natural food), are sheltered from significant weather changes and water is sand filtered for removal of solid wastes. Shrimp density in indoor tanks was 100 $animals.m^{-2}$. Both systems are provided with constant aeration supplied from mechanical blowers. In both the indoor and outdoor systems, water quality parameters were kept within levels considered adequate for rearing of *L. vannamei*. No statistical differences could be detected in water salinity, pH and temperature among treatments in both the indoor and outdoor systems ($p > 0.05$; ANOVA). For the indoor tanks, mean observed values were 28 ± 2.2‰ water salinity ($n = 2,339$), 7.87 ± 0.11 water pH ($n = 2,398$) and 28.3 ± 0.5 °C water temperature ($n = 2,339$). In outdoor tanks, mean observed values were 27 ± 3.6 ‰ water salinity ($n = 1,590$), 7.46 ± 0.32 water pH ($n = 1,590$) and 29.5 ± 1.1°C water temperature ($n = 1,590$).

The trials took 70 days of cultivation. After this period, the shrimps with approximately 5.0 cm in length were collected and carefully dissected in muscle, exoskeleton and hepatopancreas for analysis.

No additional Hg, except the natural content of aquafeeds, was added to the tanks in both trials. In the external tanks, some Hg may be present in the phytoplankton grown during the trial, but since the only Hg source for the cells were the natural Hg content of the seawater used, concentrations although not monitored are probably much lower than those found in the aquafeeds.

2.2. Hg determinations

Tissues from a composite sample of 5 individuals were mixed with an acid solution of H_2SO_4:HNO_3 (1:1) and left to rest for 16 hours under ambient temperature. After this period, hydrogen peroxide (H_2O_2) was added to prevent the re-complexing of Hg to the existing fat in the samples and left to rest for one hour under ambient temperature. After this stage, samples were warmed in a hot bath for one hour at 70-80 ºC. Aquafeed samples were digested with 50% *aqua regia* ($4H_2O$: 3HCl: $1HNO_3$) for one hour at 70-80 ºC (Adair and Cobb, 1999). After extraction, Hg was quantified by cold vapor atomic fluorescence spectroscopy in a PSA Millenium Merlin 10.025 equipment. The calibration curves were made between 0 and 1.000 $ng.L^{-1}$ from of successive dilutions of a commercial standard of 1.000 ± 1 $\mu g.L^{-1}$ (MERCK®). The operational detention limit was 0.1 $ng.g^{-1}$ (0.03 - 0.1 $ng.g^{-1}$).

2.3. MT gene expression

For each treatment, in each trial, hepatopancreas and abdominal muscle from 25 individuals were dissected and pooled in groups of 5 organs to optimize all further procedures. Total RNA was extracted using Trizol®, and first-strand cDNA was synthesized using M-MLV Reverse Transcriptase and Oligo (dT)18-20 (all reagents, Invitrogen, Carlsbad, USA; processed according to manufacturer instructions). Primers for Real Time PCR were designed after Metallothionein (GI: 11037992) and Actin (gi: 11038080) sequences available for *L. vanamei* in NCBI Genbank. Identity was confirmed by sequencing of the standard PCR products. Real time PCR was performed in 25 µL final volume reactions of 1× QuantiTect™ SYBR® Green (Qiagen – Valencia, USA.), with 4 µl of cDNA and 0.3 Mm of primers MT forward: ATGCCAGGCCCCTGCTGCAATGAGA and reverse: GGACAGCACTTGCATGGCTTGGTGC), or Actin forward: CGAGCTGTGTTCCCCTCCAT and reverse: CGATGCCAGGGTACATGGTG; in 96 wells optic plates in a ABI PRISM 7000 thermocycler (both Applied Biosystems – Foster City, USA.), following standard protocol with 40 cycles of denaturation (60 seconds@95 ºC), annealing (45 seconds@60 ºC) and elongation (90 seconds@72 ºC). Data was acquired at 84 ºC. Each sample was amplified in triplicate and in each plate there was standard curve to ensure PCR efficiency.

Relative gene expression was calculated according to Muller et al. (2002) as the inverse rate of mean MT to mean ACT threshold amplification cycle (CT) in each triplicate sample (for every biological replicate). Permutation analysis (Cade, 2005) was used to test difference between gene expressions in treatments (aquafeeds).

3. Results and Discussion

The two trials resulted in different biological performance of *L. vannamei*. A detailed description of these results is not the objective of the present study and is available elsewhere (Nunes, 2009). In summary, shrimp performance in the two trials showed the indoor experiment average survival rate lower (59.8%), when compared to the outdoor (83.3%). Final average individual shrimp biomass was significantly higher in the outdoor trial (16.3 g) compared to the indoor trial (5.8 g), resulting in significantly different yields (214 and 597 $g.m^{-2}$, in the indoor and outdoor trials respectively) ($p < 0.05$; One-way ANOVA). Since environmental conditions and initial animal densities between the two trials were different, it is difficult to relate the differences in biological performances to Hg exposure. However, it cannot be ruled out.

Mercury concentrations measured in aquafeeds and shrimp organs after 70 days in both trials are presented in Figure 1. Concentrations in aquafeeds were 2.9 times higher and showed larger variation in the indoor experiments (15 - 124 $ng.g^{-1}$, average 45 $ng.g^{-1}$) than in the outdoor trials, where Hg concentrations were significantly (paired *t*-test $p < 0.05$, $n = 10$) lower and less variable (9 - 34 $ng.g^{-1}$, 16 $ng.g^{-1}$). These results show that Hg is a non-controlled component of aquafeeds. Commercial aquafeeds analysed by Choi and Cech (1998) and Ikem and Egilla (2008), two of the very few studies presenting Hg content in aquafeeds, showed similar concentrations and variability (35 to 90 $ng.g^{-1}$ and 30 to 80 $ng.g^{-1}$, respectively), also highlighting the absence of control of Hg concentrations in aquafeeds. Final Hg concentrations in aquafeeds are a function of the original raw material used, mostly the amount of fish meal, which is the major source of Hg in aquafeeds. Also of significance are the fish species and individual fish size, as well as the trophic status of the fish species used to produce fish meal and oil (Choi and Cech, 1998; Berntssen et al., 2004).

Concentrations in shrimps were also significantly higher in the indoor trial compared to the outdoor (Figure 1) (paired *t*-test, $n = 10$, $p < 0.05$, for muscle and $p < 0.01$ for hepatopancreas and exoskeleton). Hg concentrations in muscle were very low in all trials varying from 15.9 ± 11.2 $ng.g^{-1}$ in the indoor trial and 6.3 ± 5.2 $ng.g^{-1}$ in the outdoor trial. Muscle concentrations (maximum 36.4 $ng.g^{-1}$) are 20 to 50 times lower then the maximum allowed concentrations posing human consumption risks (ANVISA, 1998), meaning that there is no exposure risk due to shrimp consumption by humans. Hepatopancreas Hg concentrations were higher than muscle concentrations in the indoor trial (22.0 ± 11.3 $ng.g^{-1}$)

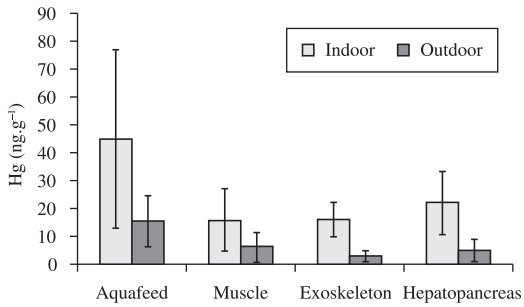

Figure 1. Mercury concentrations ($ng.g^{-1}$ d.w.) measured in aquafeeds and *L. vannamei* organs (muscle, hepatopancreas and exoskeleton) in the indoor and outdoor trials rearing conditions.

than in the outdoor trial (5.1 ± 3.8 ng.g^{-1}), again suggesting the importance of aquafeeds as the major Hg source to the animals. Berntssen et al. (2004) showed that Hg body burden in reared fish increases with increasing concentrations of organic-Hg present in aquafeeds, but not with inorganic-Hg. Organic-Hg is the major form of Hg present in fish meal constituent of aquafeeds, therefore *L. vannamei* behaves similarly to the reported results obtained with fish. In our trials Hg concentrations were 2.5; 4.3 and 5.4 times higher in muscle, hepatopancreas and exoskeleton in the indoor trial compared to the outdoor, confirming aquafeeds as the major source of Hg to the animals.

Although muscle Hg concentrations do not pose a threat to human consumption it may eventually affect the biological performance of the animals. Hg concentrations in the exoskeleton were significantly higher than in muscle in the indoor experiment and significantly lower in the outdoor trial (16.2 ± 6.2 ng.g^{-1} and 3.0 ± 1.7 ng.g^{-1}, respectively). Transfer of metals to the exoskeleton and loss with ecdysis is a known mechanism of metal detoxification in crustaceans (Khan et al., 1989; Reinfelder and Fisher, 1994; Smokrowski et al., 1998; Lacerda et al., 2009), and in our study the higher concentrations in shrimp exoskeleton in the indoor trials may already signify a response to the higher Hg concentrations available in the aquafeeds.

Table 1 shows Spearman correlation coefficients between Hg concentrations in the different shrimp organs and aquafeeds analysed. In the indoor trial, only muscle and aquafeed and muscle and exoskeleton Hg concentrations were significantly correlated, suggesting that internal detoxification mechanisms are influencing Hg in shrimps, including Hg transfer to the exoskeleton, as a response to the higher Hg concentrations present in aquafeeds. In the outdoor trial, however, the exoskeleton Hg concentrations, as well as all other compartments, are significantly correlated with the concentrations of the aquafeeds, suggesting no detoxification mechanism taking place.

Figure 2 shows the relationship between Hg concentrations in shrimp organs in the indoor and the outdoor trials. In both trials there were significant positive correlations between Hg concentrations in aquafeeds and in muscle and the exoskeleton. Correlation between Hg in aquafeed and in hepatopancreas, however, was only significant in the outdoor trial. Comparing the obtained curves, other differences occur between the two trials. At Hg concentrations smaller than 20 ng.g^{-1}, there is very small, if any, change in Hg content in shrimp organs, clearly shown by the curves obtained in the outdoor trials, where most aquafeed Hg concentrations were below this limit. However, in the outdoor ones with concentrations varying between 20 and 40 ng.g^{-1}, shrimp concentrations increased exponentially. In the indoor trials where most

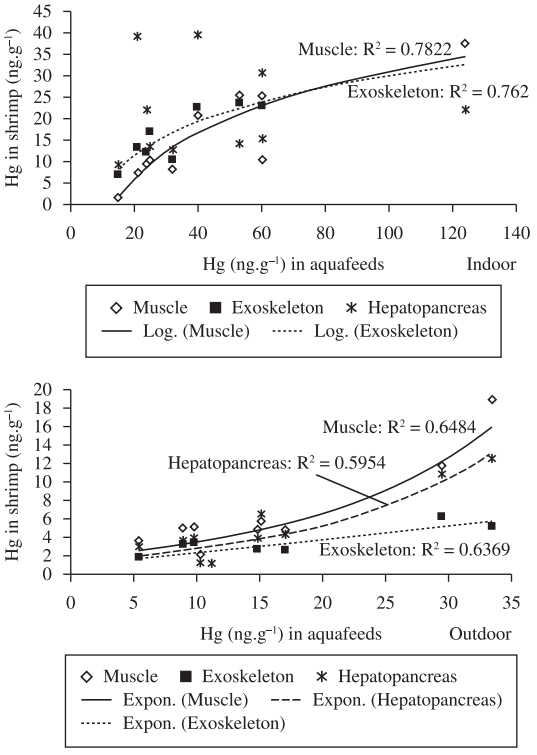

Figure 2. Mercury accumulation by different organs of *Litopenaeus vannamei* Boone, 1931 supplied with aquafeeds with different Hg concentrations.

Table 1. Spearman correlation coefficients between Hg concentrations in the different shrimp organs and aquafeeds.

Indoor trial				
	Aquafeed	**Muscle**	**Hepatopancreas**	**Exoskeleton**
Aquafeed	1			
Muscle	0.837*	1		
Hepatopancreas	–0.002 (ns)	0.375 (ns)	1	
Exoskeleton	0.610 (ns)	0.819*	0.224 (ns)	1
Outdoor trial				
	Aquafeed	**Muscle**	**Hepatopancreas**	**Exoskeleton**
Aquafeed	1			
Muscle	0.905*	1		
Hepatopancreas	0.922*	0.957*	1	
Exoskeleton	0.798*	0.847*	0.930*	1

* $p < 0.01$; ns = non-significant; n = 10.

aquafeed Hg concentrations were much higher, the animals' response was logarithmic and only significant for those organs typically accumulating organic-Hg (muscle and exoskeleton), suggesting a proportional and rapid transfer of Hg from aquafeeds to theses tissues.

MT gene expression values were similar in both hepatopancreas and muscle (although sometimes higher in the former) (Figure 3). Relative MT expression values below 1.0 are lower than the Actin constitutive expression levels. There was no significant difference in MT gene expression in neither muscle nor hepatopancreas in relation to the two trials (different rearing conditions and Hg content of aquafeeds). Despite some outlier values in the graphics, overall variability in MT expression was low (less than 20%) in the majority of the samples. There was no significant correlation between MT expression and Hg content neither in muscle or hepatopancreas, nor aquafeeds.

Molecular analyses by real-time PCR have shown that MT gene expression remains at basal levels after 70 days exposure to Hg in the diet. The relative expression levels were bellow that of Actin, which was expected given the higher production of Actin for proper cytoskeleton activity. In comparison to other works with marine invertebrates, MT constitutive expression depends on the gene isoform, but it is always lower than Actin expression, increasing following metal exposure and decreasing after several days, depending on species and metal involved, but is usually higher than the non-exposure levels (Viarengo, 1989). Therefore, the MT expression levels found in *L. vannamei* after exposure are not representative of a metal induction response, even in the indoor trial where some aquafeed exhibited relatively high mercury content (~140 $ng.g^{-1}$). Hg has higher affinity for MTs and higher toxicity than most other trace metals. In the mussel *Mytilus galloprovincialis*, LC50 for Hg^{2+} is two orders of magnitude lower than for the other high toxicity trace metal Cd, for example. However, exposure to sub lethal concentrations just below LC50 (200 $\mu g.L^{-1}$ Cd and 0.15 $\mu g.L^{-1}$ Hg) gives a 10-fold higher MT induction for Cd than for Hg (Dondero et al., 2005). In the fish *Dicentrarchus labrax* it takes more than twice the amount of Hg (100 $ng.g^{-1}$ Cd^{2+} and 250 $ng.g^{-1}$ Hg^{2+}) in peritoneal injections to increased MT expression in the same levels as Cd (Jamel et al*.,* 2008).

Somehow similar results were observed by Wu and Chen (2005) who analyzed MTs-like proteins in *L. vannamei* exposed to 100 $\mu g.L^{-1}$ Cd^{2+} and found dose-response relationship until the 56th day of exposure, with significant decrease at the 86th. The response changes when concentration is doubled (200 $\mu g.L^{-1}$ Cd^{2+}) and MT concentration increased rapidly until the 28th day, stabilizing until the end of the experiment. Since exposure to similar Cd and Hg concentrations gives lower MT response for Hg, we could expect that at the 1.000-fold smaller concentrations observed in our trials (up to 140 $ng.g^{-1}$ Hg^{2+}), *L. Vannamei* would exhibit the same or even lower MT induction and response. In the zebra fish (*D. rerio*) it takes 10 to 100-fold higher concentrations (5 to 13.5 $\mu g.g^{-1}$ Hg^{2+}) than the highest concentration observed in our experiments (140 $ng.g^{-1}$ Hg), to raise up MT expression from 3 to 10-fold after 63 days exposure (González et al., 2005).

Induction is the base for an efficient detoxification mechanism and without it basal concentrations would soon saturate leaving the organism unprotected. Unlike regular toxicity evaluation of biomarkers, aimed to assess which conditions turns on MT induction, our goal with this experiment was to found out whether the stress of

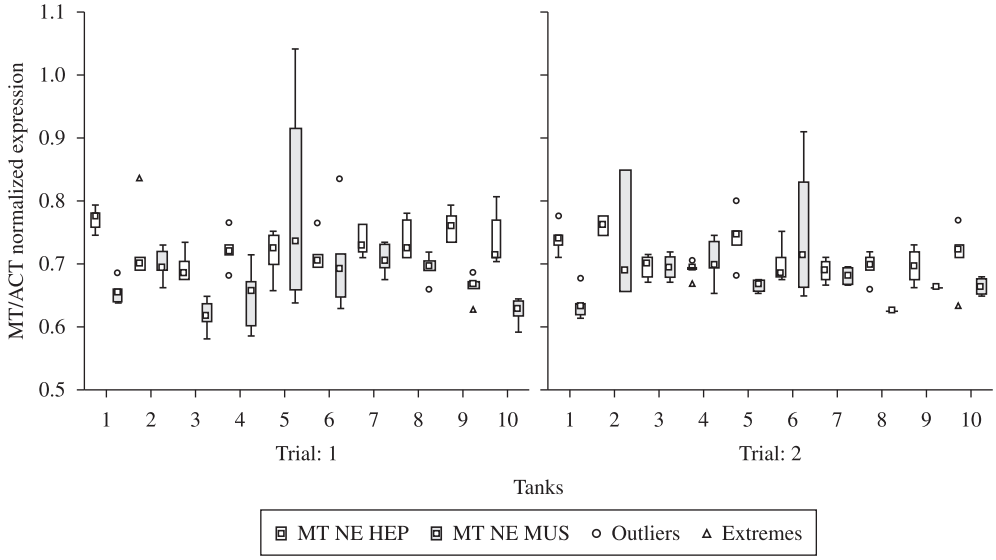

Figure 3. Metallothionein expression (MT), normalized by Actin expression, in the hepatopancreas (empty bars) and muscle (grey bars) of *L. vannamei* in the indoor (trial 1) and outdoor (trial 2) aquacultures conditions. Central squares are the median MT values (measured in triplicate) of 5 biological replicates. Bars stands for 25 and 75% quartiles and error bars upper and lower limits. Outliers (higher than 2-fold median variability) and extreme (higher than 3-fold median variability) values are represented by circles and stars.

Hg contaminated aquafeeds would lead *L. vannamei* in intensive aquaculture conditions to deviate energy to a MT protection mechanism for long time periods (70 days), which could compromise its ability to fully grow. Our results have shown that is not the case and MT expression is not high at the end of the term with the reported organ and aquafeed Hg concentrations. In fact, MT expression in *L. vannamei* harvested in aquaculture ponds at different growth stages (larvae and adult of 40, 90, 105 and 130 days old) was also low, repeating the results observed in the controlled trials (data not shown).

It is important to have in mind, however, that MT is not the only detoxification system present in crustaceans. It is also possible that in crustaceans such as *L. vannamei*, the lack of MT induction is related to the strong ability to transport divalent cations to the exoskeleton and intracellular vacuoles using protein membranes such as Ca^{2+}ATPase and Cu^{+}ATPase, as seems to be the case in the present study. Notwithstanding, it is very difficult to relate Hg concentrations present in aquafeeds to the lower biomass and yield verified in the indoor experiments, since environmental conditions and stock densities, in particular, are well recognised as the most important control of shrimp performance under aquaculture. To ensure that *L. vannamei* ability to grow was not compromised by the Hg content in aquafeeds, a more comprehensive analysis of biomarkers as well as repeating the work under real farm conditions would be necessary.

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