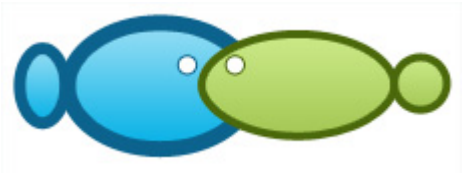

Metallothionein levels and heavy metals in Caspian Sea gammarid, *Pontogammarus maeoticus* (Crustacea, Amphipoda, Pontogammaridae)

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Abstract. Metallothioneins are a family of proteins that have a low molecular weight and are rich in cysteine capable of attaching to metals. Biosynthesis of metallothionein could be influenced by several factors such as season and gender. This study was carried out to assess the effect of sex and season on metallothionein, total protein and some heavy metal concentration in *Pontogammarus maeoticus*. The concentration of metallothionein (MT) was in the range of 1.41-2.82 nmol mg^{-1} protein. MT in female gammarus was significantly higher than in male ($p < 0.05$). The amount of MT in summer was recorded higher than spring in both genders. There was observed a based gender difference for MT levels and essential metals values. Females indicated higher level of MT and metals concentrations (Cu and Zn) than males. Significant difference between Cd and Pb with MT in the both gender was not observed through the seasons ($p > 0.05$). Also, the trend of total protein was steady during season in both genders. Therefore metallothionein and essential metals such as Cu and Zn are influenced by season and gender in pontogammarus maeoticus.

Key Words: metallothionein (MT), *Pontogammarus maeoticus*, heavy metals, total protein.

Introduction. Heavy metals are well known as dangerous pollutants in the marine environment that bio accumulate in different parts of aquatics organisms. These metals cause toxicity to aquatic organism by influencing their physiological and vitals processes. Accumulation of them among aquatic organisms through food chain cause bio magnification and even transference of these contaminants can impact human health (Rainbow 2007; Zhou et al 2008). Ecotoxicology studies on pollutants mainly involve chemical monitoring (measurement of pollutants in the environment such as water, sediments, and soil) and biological monitoring (using biomarkers and bioindicators) in natural ecosystems. Chemical monitoring of pollutants does not provide enough information about bioavailability of aquatic organisms to them (Geffard et al 2005). On the other hand, natural environments are subject to dramatic changes of governing physiochemical factors, leading to significant differences between the data obtained from chemical monitoring of pollutants. Biomonitoring that is carried out using suitable biomarkers is a safer method that provides more acceptable information about bioavailability of contaminants and their potential effect (Amiard et al 2006). Measuring the biological impact of contaminants needs the use of early warning biomarkers such as metallothionein (MT) for metal exposure (Kovarova & Svobodova 2009). MTs are non-enzymatic proteins with low molecular weight and are rich in cysteine. Thiol groups (-SH) of the residuals of cysteine enable MTs to bond with special heavy metals. Proteins like MTs or real MTs have been reported in a variety of vertebrates including fish (Roeva et al 1999), aquatic invertebrates (Roesijadi & Fowler 1991), Mollusca (Langston et al 1998; Isani et al 2000) and crustaceans (Roesijadi 1992; Engel & Brouwer 1993; Barka et al 2001). The significance of MTs is related to their interference with metal homeostasis,

free radicals, protection against damages, and regulation of the body's metabolism (Cosson 2000; Isani et al 2000; Amiard et al 2006).

MT is synthesized in response to heavy metals contamination such as mercury, cadmium, copper, and zinc in the body of vertebrates and invertebrates (Sarkar et al 2006). Numerous studies have shown that there is a direct relationship between the levels of synthesis of this protein with the concentration of heavy metals in tissues of aquatics organism especially mussels (Ivanković et al 2005; Geffard et al 2005; Amiard et al 2006).

Studies on different species of invertebrates such as *Corbicula fluminea* (Hamza-Chaffai et al 1999), *Mytilus edulis* (Geffard et al 2005), *Gammarus locusta* (Correia et al 2004), *Gammarus pulex* (Geffard et al 2005) indicate that the main difference of the MT level is related to physiological conditions of organism and especially reproductive status. Most of studies indicate that abiotic factors such as temperature fluctuation and biotic factors such as age, sex and reproduction influence the level of expression of these proteins (Geffard et al 2007; Dragun et al 2007). The gammarids are a good candidate for ecotoxicological studies, because this group of Crustacea has a wide distribution and is often valuable with a high frequency and easy for identification (Sroda & Cossu-Legguille 2011). Another important factor is their important as live food for fish and some strains of amphibians (Welton1979; MacNeil et al 2000). The aim of this study is to investigate the concentration of heavy metals including cadmium, lead, and copper in the body of *P. maeoticus* in Caspian Sea and their relationship with the concentration of MT, to evaluate the possibility of using it as a biomarker in Sahelghoo region in the southwest of the Caspian Sea.

Material and Method. *P. maeoticus* was sampled with hand net in Sahelghoo, south west of Caspian Sea (37°28'N, and 58°49'E) between spring to summer 2014. They were transported to the laboratory at Islamic Azad University, Branch of Lahijan and were sorted as male and female according to gnathopod size. Three pools of 10 gammarids were sampled and frozen at -80°C until experimental time. Physicochemical parameters such as temperature, pH, conductivity and dissolved oxygen were measured in the field with WTW multi set measurement (GmbH, Weilheim, Germany).

Metallothionein quantification. MT analysis was performed based on Viarengo et al (1997) method. Each pool of *P. maeoticus* was homogenized on ice in buffer solution (20 mM tris, 1.5 µM phenylmethyl sulfonyl, 10^{-5} mM β-mercaptoetanol and 0.5 M sucrose) in 1:3 (w/v) ratio range using a hand-held glass-grinder. Following that, the homogenate was centrifuged at 25000 g for 60 min at 4°C. Cold pure ethanol (1.05 mL at -20°C) and 80 µL chloroform were added to 1 mL of supernatant. After centrifugation at 6000 g for 10 min at 4°C, 40 µL of 37% HCl and 3 fold sample volume of ethanol were added.

After protein precipitation (storing samples at -20°C), they were centrifuged at 6000 g for 10 min. The pellets were washed in homogenizer buffer containing 87% ethanol and 1% chloroform. The samples were again centrifuged at 6000 g for 10 min at 4°C and pellet dried under a nitrogen gas stream for 10 min. The dry pellets were added to 150 µL NaCl (0.25 M) and 150 µL HCl (1 N) containing 4 mM EDTA. Sodium chloride solution (2 M) containing 43% DTNB (5,5'-dithiobis-2-nitrobenzoic acid, Ellman's reagent) was mixed with a buffer of Na-phosphate (pH = 8) and added to the samples at the room temperature. At the end, the samples were centrifuged at 3000 g for 5 min. The absorbance of the samples was measured at 412 nm and the concentration of metallothionein was determined using reduced glutathione as the reference standard (Tanguy et al 2002).

Metal determination. Aliquot of the whole body of *P. maeoticus* homogenate were digested in concentrated nitric acid (HNO_3) and heated at 90°C for 5 hours. After cooling the samples at room temperature, metal concentration (Cd, Cu, Pb and Cd) were determined by atomic absorption spectrophotometry (280FS AA, Agilent, USA) according to the method of Zhou et al (2008). Metal concentrations are explained on dry weight basis. Total protein was measured in whole body of both genders of *P. maeoticus*

according to Bradford (1976) method using Bovine Serum Albumin (BSA) (Sigma-Aldrich) as standard and reading absorbance at 595 nm using a Jenway spectrophotometer (6305 Jenway, UK).

Data analysis. All data were presented as the average ± standard deviation of replicate measurements (n = 3). Statistical analyses of data were carried out with SPSS 17.0 software package. Significance of results was tested by an analysis of variance (ANOVA) and Duncan's Multiple-Range Test. Significance of differences was defined at $p < 0.05$.

Results and Discussion. Seasonally recorded physico-chemical parameters are shown in Table 1. Temperature was the most variable parameter among the different parameters and ranged between 18 and 31°C from spring to summer. All other parameters showed stable trend over two seasons.

Table 1

Water parameters measured in the Sahelghoo at each sampling season as well as standard deviation (SD)

Season	*Temperature (C°)*	*O_2 (mg L^{-1})*	*Conductivity (µS cm)*	*pH*
Spring	18 ± 0.02^{a}	8.51 ± 0.54^{a}	279 ± 15.25^{a}	8.17 ± 0.06^{a}
Summer	31 ± 1.24^{b}	8.12 ± 0.12^{a}	1441 ± 16.22^{a}	8.44 ± 0.01^{a}

Different letters indicate significant differences ($p < 0.05$)

Heavy metal concentrations in *P. maeoticus* are summarized in Table 2. Cu and Zn concentrations varied between 1.18-2.42 µg g^{-1} and 1.05-2.71 µg g^{-1} respectively. Significantly the higher levels of Cu and Zn were observed in female during summer. Concentrations of the other metals (Cd and Pb) were lower and lowest value was recorded in Cd in both genders and seasons ($p < 0.05$). Pb concentration showed a steady trend in both sexes and there was no significantly different in spring and summer ($p > 0.05$).

Table 2

Heavy-metal concentrations (mean value ± SD) (µg g^{-1} d w) in whole body of male and female *Pontogammarus maeoticus* in the Sahelghoo at sampling seasons

Season	*Sex*	*Cu*	*Zn*	*Cd*	*Pb*
Spring	Male	1.18 ± 0.08^{aA}	1.79 ± 0.09^{aB}	0.05 ± 0.01^{aA}	0.49 ± 0.07^{aA}
	Female	2.13 ± 0.11^{bA}	2.43 ± 0.11^{bA}	0.09 ± 0.01^{bA}	0.39 ± 0.05^{aA}
Summer	Male	1.68 ± 0.66^{aA}	1.05 ± 0.45^{aA}	0.07 ± 0.03^{aB}	0.44 ± 0.05^{aA}
	Female	2.42 ± 0.14^{bB}	2.71 ± 0.05^{bB}	0.09 ± 0.04^{bA}	0.45 ± 0.06^{aA}

Different letters indicate significant differences ($p < 0.05$).

MT concentration ranged from 1.41 nmol mg^{-1} protein (spring) to 2.82 nmol mg^{-1} protein (summer) (Figure1). The higher levels of MT observed in female than male in both seasons and significantly highest level of MT was recorded in summer. The values of total protein of *P. maeoticus* is shown in Figure 2. No significant difference of total protein was observed in both genders in spring ($p > 0.05$), but significantly higher level of protein was observed in female in summer ($p < 0.05$).

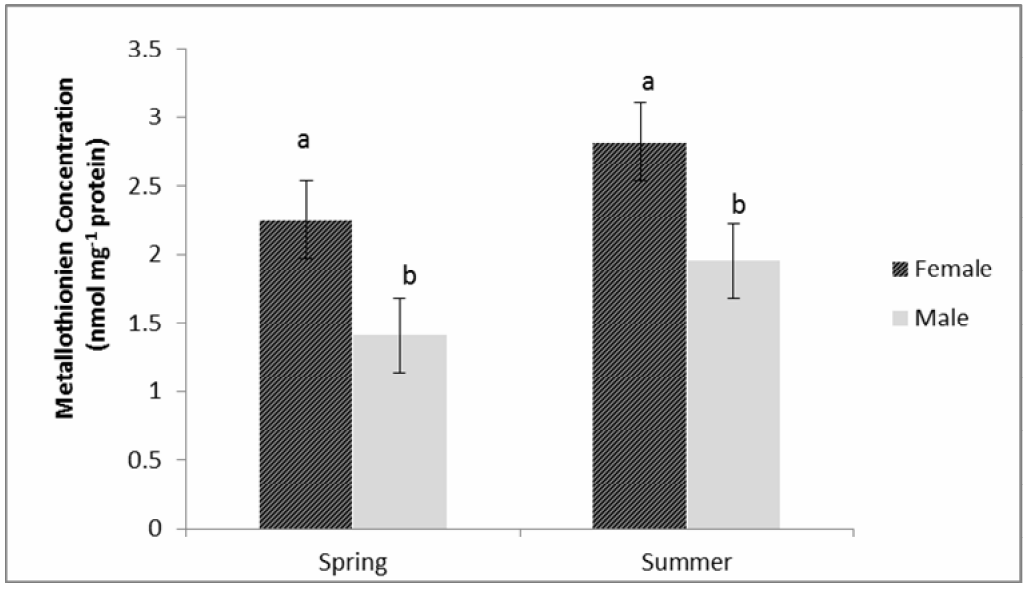

Figure 1. MT levels in the Sahelghoo at sampling seasons in both genders of *P. maeoticus*. Different letters indicate significant differences ($p < 0.05$).

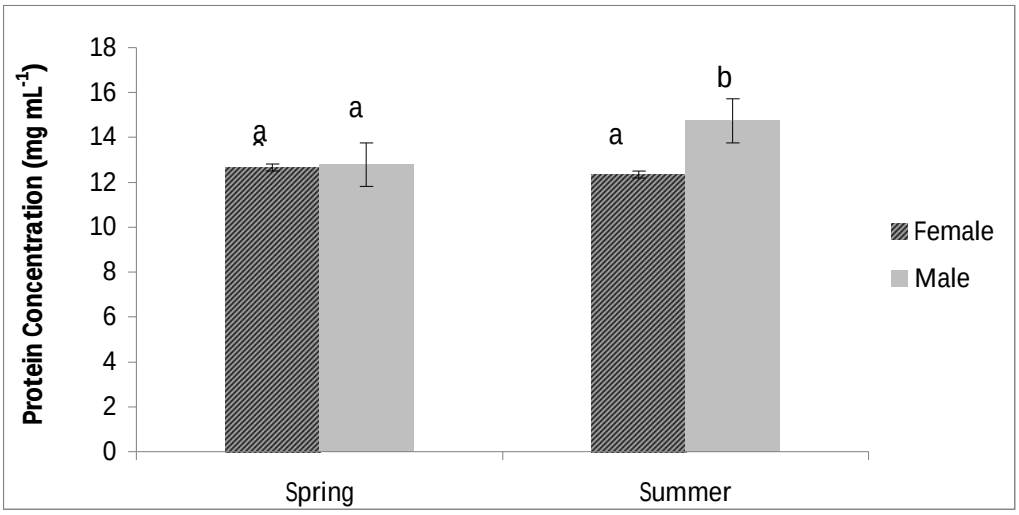

Figure 2. Total protein levels in the Sahelghoo at sampling seasons in both genders of *P. maeoticus*. Different letters indicate significant differences ($p < 0.05$).

There was observed quit well trend in metal concentration in *P. maeoticus.* Concentration of Cu and Zn increased from spring to summer, and the others (Cd and Pb) were not shown any changes in seasons in both sexes. So, toxic (Cd, Pb) and essential metals (Cu, Zn) indicated a different pattern with both genders in *P. maeoticus*. Metal concentrations were higher in female compared with male should be attributed to higher metabolitic rates of female. The fluctuations in the levels of these two elements can be related to the molting and sexual maturity, where these fluctuations were also observed in *G. locusta* (Correia et al 2004). Cu is stored in hepatopancreas during molting which is released through synthesis of new hemocyanin in the molting and pre-molting stages. Such a system prohibits the potentially toxic accumulation of Cu in the hepatopancreas cells. Therefore, due to this mechanism in the body of these crustaceans, lack of correlation between the amounts of Cu and Zn and the levels of metallothionein during different developmental stages and different gender is not far from mind (Caussy et al 2003; Chen & Chen 2003; Correia et al 2004). MT levels in *P. maeoticus* varied from 1.41 nmol mg^{-1} to 2.82 nmol mg^{-1} protein during our study. Geffard et al (2007) and Correia et al (2004) found similar levels in two other gammarid specices, *G. pulex* and *Gammarus locusta*, with values 4.4 to 13.8 and of 1.3 to 2.3 mg g^{-1} dry weight respectively. MT levels in *P.*

maeoticus were impacted by organism sex. The amount MT was higher in female than male. According to our results, similar observation is recorded of gammarids species, *G. locusta* (Correia et al 2004). Conversely, Geffard et al (2007) and Legras et al (2000) found opposite results in other gammarids, *G. pulex* and in two crustacean species, *Pachygrapsus marmoratus* and *Carcinus maenas* (green crab) respectively. Seasonal variations impact MT synthesis in gammarids species directly with higher and lower temperature. Many studies recorded temporal changes of MT levels in aquatic organism (Baudrimont et al 1997; Hamza-Chaffai et al 1999; Bebianno et al 2000; Geffard et al 2001, 2005) The physiological conditions and reproduction stages of organism were related to MT levels. In our study higher levels of MT were observed in summer and also in winter (data was not shown). In the same way, Geffard et al (2005, 2007) and Raspor et al (2004) suggested higher MT values during gametogenesis period that does not correspond to the warmest period. Moreover higher level of MT in winter may be related to essential metals need after the reproductive period (Geffard et al 2007).

Conclusions. Our findings show that the gender related changes in MT status, the level in females being higher than males in *P. maeoticus*. MT levels and metal concentrations are increased in both genders during summer. Perhaps, increasing levels of MT link with essential metals which need during metabolic processes.

Acknowledgements. The authors thank Islamic Azad University Lahijan Branch for the financial support.

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Received: 29 November 2015. Accepted: 01 January 2016. Published online: 04 January 2016.
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How to cite this article:
Ghasemian S., Karimzadeh K., Zahmatkesh A., 2016 Metallothionein levels and heavy metals in Caspian Sea gammarid, *Pontogammarus maeoticus* (Crustacea, Amphipoda, Pontogammaridae). AACL Bioflux 9(1):1-7.