Arch Environ Contam Toxicol (2016) 71:530–540
DOI 10.1007/s00244-016-0310-8

Putative Roles for Metallothionein and HSP70 Genes in Relation with Heavy Metal Accumulation and Parasitic Cymothoid in the Fish *Nemipterus furcosus*

Do Dinh Thinh[1,2] · Mohamad Hafiz Farhan Abdul Rasid[1] · Zulaikha Mat Deris[1] · Noor Azhar Mohamed Shazili[3] · Gudrun De Boeck[4] · Li Lian Wong[1]

Received: 31 March 2016 / Accepted: 24 August 2016 / Published online: 8 September 2016

Abstract To assess stress level induced by multiple stressors in aquatic organism, biomarkers have been adopted as early warning indicator due to their high accuracy, rapidity, and sensitivity. We investigated the effects of ectoparasitic isopod infection on heavy metal bioaccumulation (Fe, Cu, Zn, and Cd) in the fish *Nemipterus furcosus* and profiled the expression of metallothionein (MT) and heat shock proteins 70 (HSP70) genes of the fish host. Sixty individuals (parasitized and nonparasitized with *Cymothoa truncata*) were collected from three sites differing in the levels of anthropogenic activities off the South China Sea. Our results revealed no significant difference in heavy metal concentrations between infected and nonparasitized fish. We observed a positive correlation between heavy metal bioaccumulation in the fish host and anthropogenic activities. Accordingly, expression analysis of MT genes in fish liver showed significant differences in expression level between sampling sites, with lowest level in the least exploited site (Batu Rakit). A reverse pattern in HSP70 gene expression was demonstrated in fish muscle, showing the highest expression at Batu Rakit. While cymothoid infection in *N. furcosus* had no significant impact on fish MT gene expression, it resulted in a reduction of HSP70 level in liver of parasitized fish. These findings highlight the putative roles of MT in heavy metal assessment. Future studies should determine the kinetics of cymothoid infection and other potential stressors in characterizing the HSP70 gene expression profile.

✉ Li Lian Wong
lilian@umt.edu.my

[1] Institute of Aquaculture Tropical, Universiti Malaysia Terengganu, 21030 Kuala-Terengganu, Terengganu, Malaysia

[2] Institute of Marine Environment and Resources, Vietnam Academy of Science and Technology, 246 Danang Street, Haiphong, Vietnam

[3] Institute of Oceanography and Environment, Universiti Malaysia Terengganu, 21030 Kuala-Terengganu, Terengganu, Malaysia

[4] Systemic Physiological and Ecotoxicological Research, Department of Biology, University of Antwerp, Antwerp, Belgium

The discharge of heavy metals into the coastal ecosystem may damage species diversity and ecosystem structure (Dhaneesh et al. 2012). As the representative of the highest levels in the aquatic food-web, fish may accumulate large amounts of heavy metals from food and the aquatic environment (Squadrone et al. 2013). Heavy metal accumulation via polluted water or food may cause oxidative stress, leading to a decreased reproductive ability and increased pathogenic susceptibility in fish (Padmini et al. 2009). In stark contrast to detrimental effects of pathogenic infection, fish infected by a range of parasites showed an inverse correlation with heavy metal bioaccumulation (Sures 2008). Fish infected with intestinal acanthocephalans accumulated less heavy metals in their tissues than uninfected fish (Sures and Siddall 1999), thus potentially reduce adverse effects of heavy metals on fish health. Interestingly, the ability of parasites to absorb essential metals from the fish host has improved the capacity for metal regulation mechanisms in the host. Despite the fact that their effect on host metal accumulation functions have been well described in a diverse group of fish endoparasites (Sures and Siddall 1999; Oyoo-Okoth et al. 2010; Marijić et al. 2013), no research has been conducted on the role of parasitic ectoparasites in this aspect.

Fish are one of the common sentinel organisms, which are important as a biomonitoring device because they can accumulate toxic metal pollutants from the surrounding environment (Ameur et al. 2012). Whereas past environmental assessments heavily relied upon conventional metal analysis of fish tissues, recent development of biomarkers have shown a significant breakthrough in understanding physiological responses of fish towards heavy metal bioaccumulation at the molecular level. Excessive metal could enhance the expression of metal-binding protein, such as metallothionein (MT) (Wang and Rainbow 2010), and stress-induction protein, such as heat shock protein 70 (HSP70), as part of the fish innate immune defense against external stressors (Deane and Woo 2006). Metallothioneins are low molecular weight (6000–7000 Da), cysteine-rich (33 %), heat-resistant cytosolic proteins, universally synthesized by all tissues in an organism (Atli and Canli 2008). They assist in maintaining the homeostasis of metallic ions, regulating the detoxification processes and protect cells against oxidative stress (Monserrat et al. 2007). Considered as metal-specific biomarkers, MTs have been widely employed to indicate the presence of heavy metals (Giguère et al. 2003). On the other hand, the Heat Shock Protein (HSP) family has been identified in various organisms ranging from bacteria to mammals and they have collectively emerged as important molecular chaperones in protein folding and repair mechanisms (Feder and Hofmann 1999; Gupta et al. 2010). HSP70, proven as key component in the first line of immune defense in refolding damaged proteins, is the most sensitive biomarker to be used when organisms are exposed to adverse stress conditions (Scheil et al. 2009).

Considering the possible role of host-parasite interaction into heavy metal bioaccumulation processes and the potential of molecular biomarkers to be used in metal pollutants assessment, we sought to explore these findings in wild-caught demersal fish collected from the South China Sea. Our study represents the first attempt to determine the effects of a parasitic isopod, *Cymothoa truncata* on heavy metal bioaccumulation in its fish host, *Nemipterus furcosus*. We investigated the heavy metal levels in the bioindicator species (fish host) and also put specific emphasis on examining the magnitude of MT and HSP70 genes expression within the liver and muscle tissues in parasitized and nonparasitized fish across three different sampling sites differing in levels of anthropogenic activities. We demonstrated the first gene expression profiles of MT and HSP genes in wild fish infected with the parasitic isopod and suggest a role of these biomarkers in characterizing heavy metal bioaccumulation and the stress level in the fish host.

Materials and Methods

Sample Collection

Fish sampling was conducted at three selected sites as shown in Fig. 1. Site selections were based on the levels of anthropogenic activities along the coastal zones of the South China Sea. Batu Rakit is the least exploited rural area surrounded by lush forest with limited human activities, whereas Kemaman is dominated by petroleum and steel industries. We assumed that Sedili is the most exploited area, because it is a heavily industrialized centre in the Southeastern region of Peninsular Malaysia with large influx of fleet movements through the Straits of Malacca. Fish were collected using trawl net and fish trap and examined for the presence of cymothoids in both gill and buccal cavity. Twenty specimens ($n = 10$ nonparasitized fish; $n = 10$ parasitized fish) from each sampling sites was used in this study. Before dissection, total length (to the nearest mm) and weight (to the nearest gram) of all individuals were measured (Table 1). Tissues samples (liver and muscle) were extracted and stored at −20 °C for heavy metal analysis and preserved in RNAlater (Life Technologies, USA) for gene expression analysis.

Heavy Metal Quantification

Tissues samples were freeze-dried for 48 h in freeze-drying system (Lab-Conco, USA). The microwave digestion system Ethos I (Milestone, Italy) was used to digest the dried fish tissues. Approximately 0.02 g of liver and 0.1 g of muscle were taken and placed into digestion vessels. The dried residues were dissolved in 4 ml HNO_3/H_2O_2 mixture with a ratio of HNO_3:H_2O_2, 3:1 at 200 °C. After completion of digestion process, the samples were diluted with deionized water to a volume of 30 ml, and analyzed for elements in an inductively coupled plasma mass spectrometer (ICP-MS-Perkin Elmer ELAN9000). Accuracy and reproducibility of the reaction were tested by analysis of standard reference material (DOLT-4, National Research Council, Canada) along with each set of triplicate samples (Table 2).

Gene Expression Analysis

Total RNA was extracted from 50 mg of tissue samples using Trizol reagent (Life Technologies, USA), following the manufacturer's instruction. The concentration and purity of total RNA were measured spectrophotometrically using Scandrop 200 (Analytik Jena, Germany). Approximately 0.5 µg total RNA was converted to cDNA using iScript Reverse Transcription Supermix (Bio-rad, USA).

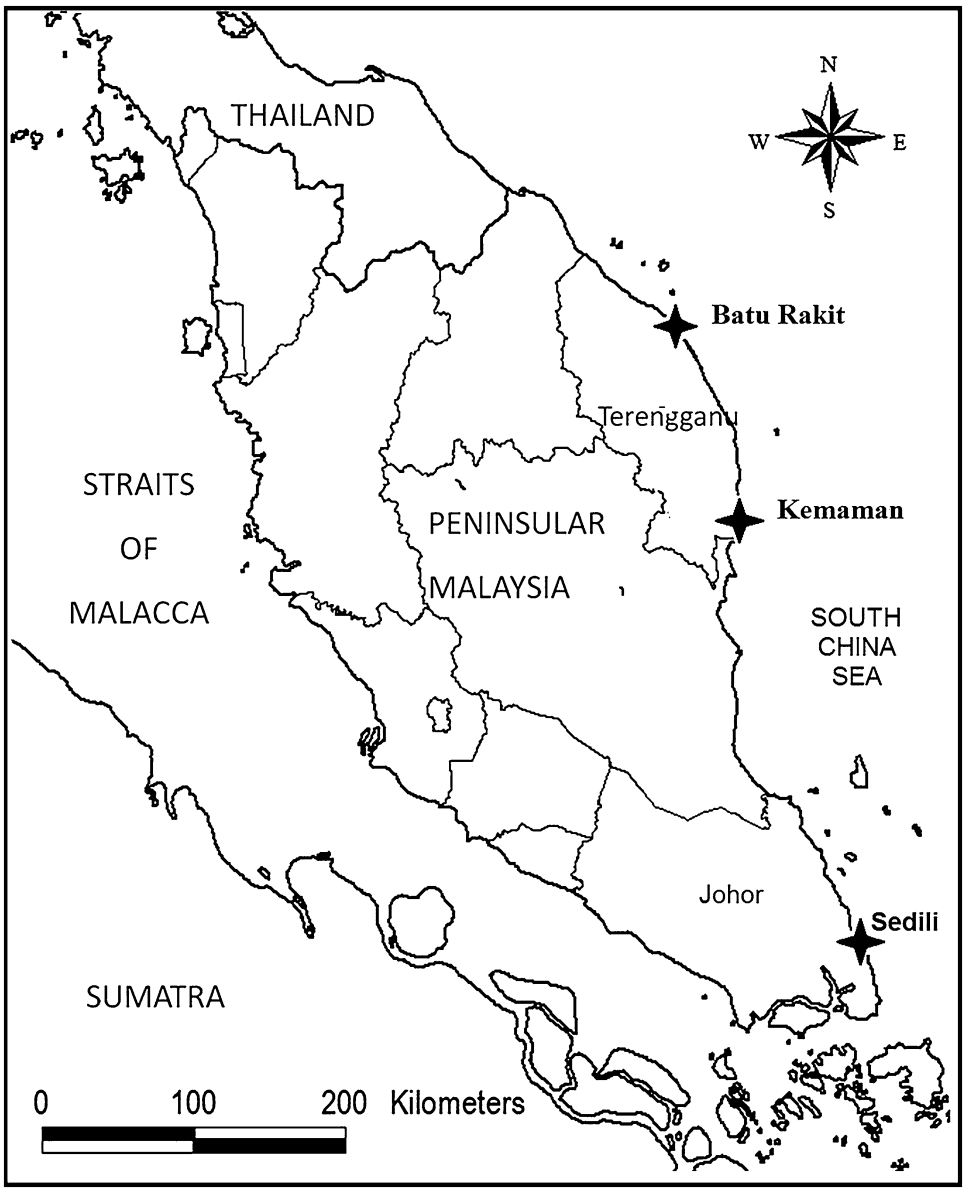

Fig. 1 Sampling sites along the East Coast of Peninsular Malaysia, off the South China Sea. Sampling sites are marked by *stars*

Table 1 Morphometric measurement of *N. furcosus* from all sampling sites

Sampling site	Nonparasitized fish		Parasitized fish	
	Length (cm)[a]	Weight (g)[a]	Length (cm)[a]	Weight (g)[a]
Batu Rakit	21.8 ± 0.9	134.9 ± 17.7	21.4 ± 1.0	130.1 ± 20.3
Kemaman	21.3 ± 0.3	116.4 ± 6.5	20.9 ± 0.7	107.1 ± 11.7
Sedili	20.5 ± 0.6	106.7 ± 9.7	20.2 ± 0.7	100.1 ± 14

[a] Values are mean ± SD

The primer sequences used in this study are listed in Table 3. Real-time PCR was performed using 2× iTaq Universal SYBR Green Supermix (Bio-rad, USA) on MiniOpticon™ System (Bio-Rad, USA). Real-time PCR was performed in a total reaction volume of 10 µl containing 5 µl (1×) of iTaq Universal SYBR Green Supermix (Bio-rad, USA), 0.2 µl (200 nM) of forward primer, 0.2 µl (200 nM) of reverse primer, 0.5 µl of cDNA template, and 4.1 µl of real-time PCR grade water (Life Technologies, USA).

Table 2 Metal concentrations (mean ± SD μg g^{-1}) in the certified standard material (Dolt-4) determined by ICP-MS

Metal	Standard	Certified value	ICP-MS value	Accuracy (%)
Fe	Dolt-4	1833.0 ± 75.0	1795.0 ± 224.5	97.9
Cu	Dolt-4	31.2 ± 1.1	33.4 ± 2.9	107.1
Zn	Dolt-4	116.0 ± 6.0	104.2 ± 8.8	89.8
Cd	Dolt-4	24.3 ± 0.8	23.2 ± 2.4	95.4

We amplified three technical replicates using the following thermal cycling conditions: initial denaturation step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min, and finally 15 s at 95 °C, 1 min at 60 °C, and 15 s at 95 °C. At the end of each reaction, a melting curve analysis was performed from 60 to 95.0 °C to ensure that only one specific amplicon was produced in the reaction. The fluorescence intensities of the real-time PCR products for each gene were measured by quantification cycle (Cq) values. The Cq (y-axis) versus log cDNA dilution (x-axis) was plotted. Fivefold serial dilutions were conducted on cDNA to generate standard curve for estimation of amplification efficiency. The amplification efficiency (Ef) was calculated from the slope of the standard curve using the following formula by Bio-Rad (2006):

Table 3 Primer sequences used for Real-time PCR

Gene	Primer sequence (5′–3′)	Annealing Tm (°C)	References
MT	F: GCGAGTGCTCTAAGACTGGA	60	Man and Woo (2008)
	R: ACTGGCAGCAGCTAGTGTCG		
HSP70	F: AATGTTCTGCGCATCATCAA	60	Guardiola et al. (2014)
	R: GCCTCCACCAAGATCAAAGA		
EF1α	F: CTGTCAAGGAAATCCGTCGT	60	Guardiola et al. (2014)
	R: TGACCTGAGCGTTGAAGTTG		

F forward; *R* reverse; *Tm* annealing temperature; *MT* metallothionein; *HSP70* heat shock protein 70 kilodalton; *EF1α* elongation factor 1α as reference gene

Table 4 Metal concentrations (mean ± SD μg/g dry weight) in liver and muscle of nonparasitized fish and parasitized fish at three sampling sites along the East Coast of Peninsular Malaysia

Tissue	Metal	Sampling site		
		Batu Rakit	Kemaman	Sedili
Liver	Nonparasitized fish			
	Fe	855.7 ± 180.0^{a}	1550.7 ± 179.5^{b}	1523.5 ± 392.3^{b}
	Cu	14.94 ± 2.32	16.95 ± 2.44	17.12 ± 5.08^{NS}
	Zn	80.7 ± 10.3^{a}	101.3 ± 15.3^{b}	104.2 ± 15.7^{b}
	Cd	3.01 ± 1.94^{a}	4.22 ± 1.48^{a}	11.46 ± 3.93^{b}
	Parasitized fish			
	Fe	845.4 ± 228.2^{a}	1348.6 ± 452.6^{b}	1456.4 ± 596.9^{b}
	Cu	13.59 ± 1.68^{a}	15.16 ± 4.22^{a}	19.57 ± 3.63^{b}
	Zn	84.8 ± 13.4^{a}	98.0 ± 14.1^{a,b}	114.6 ± 12.2^{b}
	Cd	3.80 ± 0.98^{a}	4.80 ± 2.02^{a}	9.40 ± 4.35^{b}
Muscle	Nonparasitized fish			
	Fe	21.91 ± 3.62^{a}	30.79 ± 4.27^{b}	27.13 ± 7.10^{a,b}
	Cu	1.74 ± 0.24	1.64 ± 0.15	1.63 ± 0.39^{NS}
	Zn	16.42 ± 1.97	17.26 ± 1.16	16.49 ± 2.34^{NS}
	Cd	0.0045 ± 0.0028	0.0043 ± 0.0020	0.0038 ± 0.0013^{NS}
	Parasitized fish			
	Fe	22.69 ± 3.84	26.94 ± 5.13	26.17 ± 2.82^{NS}
	Cu	1.57 ± 0.28	1.48 ± 0.22	1.55 ± 0.21^{NS}
	Zn	15.84 ± 1.56	16.73 ± 1.54	16.37 ± 2.20^{NS}
	Cd	0.0037 ± 0.0016	0.0037 ± 0.0026	0.0044 ± 0.0022^{NS}

Values with different letters denote significant difference ($P < 0.05$) among sampling sites (one-way ANOVA followed by post hoc Tukey's test). NS indicates no significant difference for that analyzed element. No significant differences in metal bioaccumulation were found between nonparasitized fish and parasitized fish at each sampling site

Table 5 Two-way ANOVA analysis showing interactive effects of sampling sites (Batu Rakit, Kemaman and Sedili) and parasite infection (nonparasitized fish and parasitized fish) on heavy metal concentrations in fish tissues

Organ	Metal	*F*	*P* value
Liver	Fe		
	Site × nonparasitized fish	0.377	0.688
	Site × parasitized fish		
	Cu		
	Site × nonparasitized fish	3.025	0.057
	Site × parasitized fish		
	Zn		
	Site × nonparasitized fish	1.212	0.306
	Site × parasitized fish		
	Cd		
	Site × nonparasitized fish	2.399	0.100
	Site × parasitized fish		
Muscle	Fe		
	Site × nonparasitized fish	1.572	0.217
	Site × parasitized fish		
	Cu		
	Site × nonparasitized fish	0.346	0.709
	Site × parasitized fish		
	Zn		
	Site × nonparasitized fish	0.082	0.921
	Site × parasitized fish		
	Cd		
	Site × nonparasitized fish	0.588	0.599
	Site × parasitized fish		

$$\text{Ef} = 10^{-1/\text{slope}}$$

$$\text{Efficiency} = (\text{Ef} - 1) \times 100$$

Statistics and Data Analysis

Heavy metal data analysis was performed using SPSS version 20 (IBM, USA). Heavy metal concentrations in fish tissues were presented as means ± SD. The data were log transformed to satisfy the assumptions of parametric statistical methods. Student *t* test was used to compare heavy metal concentration between parasitized fish and nonparasitized fish at each sampling site. One-way ANOVA analysis was performed to determine the differences of heavy metal levels among sampling sites, followed by Tukey's test when there was significant differences among sites ($P < 0.05$). Two-way ANOVA was analyzed to investigate interactive effects of sampling sites (Batu Rakit, Kemaman, and Sedili) and parasite infection on heavy metal accumulation in *N. furcosus*.

Relative quantification of each gene expression level was normalized according to the expression of the EF1α (elongation factor 1α) reference gene for each biological and technical samples using Relative Expression Software Tool 384 v. 1 (REST). The transcription levels between nonparasitized fish and parasitized fish, as well as transcription levels in fish among sampling sites for each gene, were compared and converted to relative fold changes by the relative quantification method (Pfaffl et al. 2002). Relative fold changes for each gene between nonparasitized and parasitized groups as well as between sampling sites were assessed for statistical significance ($P < 0.05$) using a randomization test in the REST software. To compare gene expression among sampling sites, Batu Rakit was chosen as the reference site.

Results

Heavy Metal Bioaccumulation

To discover the role of parasitic isopods on heavy metal accumulation in *N. furcosus*, heavy metal concentrations in nonparasitized fish and parasitized fish were compared. Our result indicated that no significant difference was shown in metal bioaccumulation between nonparasitized fish and parasitized fish at each sampling site for both liver and muscle tissues ($P > 0.05$).

Concentrations of Fe, Cu, Zn, and Cd in liver and muscle of nonparasitized and parasitized *N. furcosus* were compared among three sampling sites (Table 4). For fish liver, heavy metal levels in both nonparasitized (except Cu) and parasitized fish were significantly different between all sampling sites ($P < 0.05$). While Fe and Zn were elevated in both nonparasitized and parasitized fish from Kemaman and Sedili, Cu in liver of parasitized fish from Sedili was significantly higher than those from Batu Rakit and Kemaman. Noticeably, Cd was highest in fish from Sedili, whereas there was no significant difference in Cd level between fish from Batu Rakit and Kemaman.

For fish muscle (Table 4), all heavy metals in nonparasitized fish (except Fe) and parasitized fish indicated no significant differences among sites ($P > 0.05$). Although Fe in muscle of fish from Kemaman was not significantly different compared with that in fish from Sedili ($P > 0.05$), it was significantly higher compared to that in fish from Batu Rakit ($P < 0.05$).

Two-way analyses of variance (ANOVA) assessed interactions between sampling sites and parasitic cymothoid on heavy metal accumulation in fish tissues (Table 5). However, we found no significant interaction between sites and parasitic cymothoid ($P > 0.05$) for heavy metal concentrations in the collected fish.

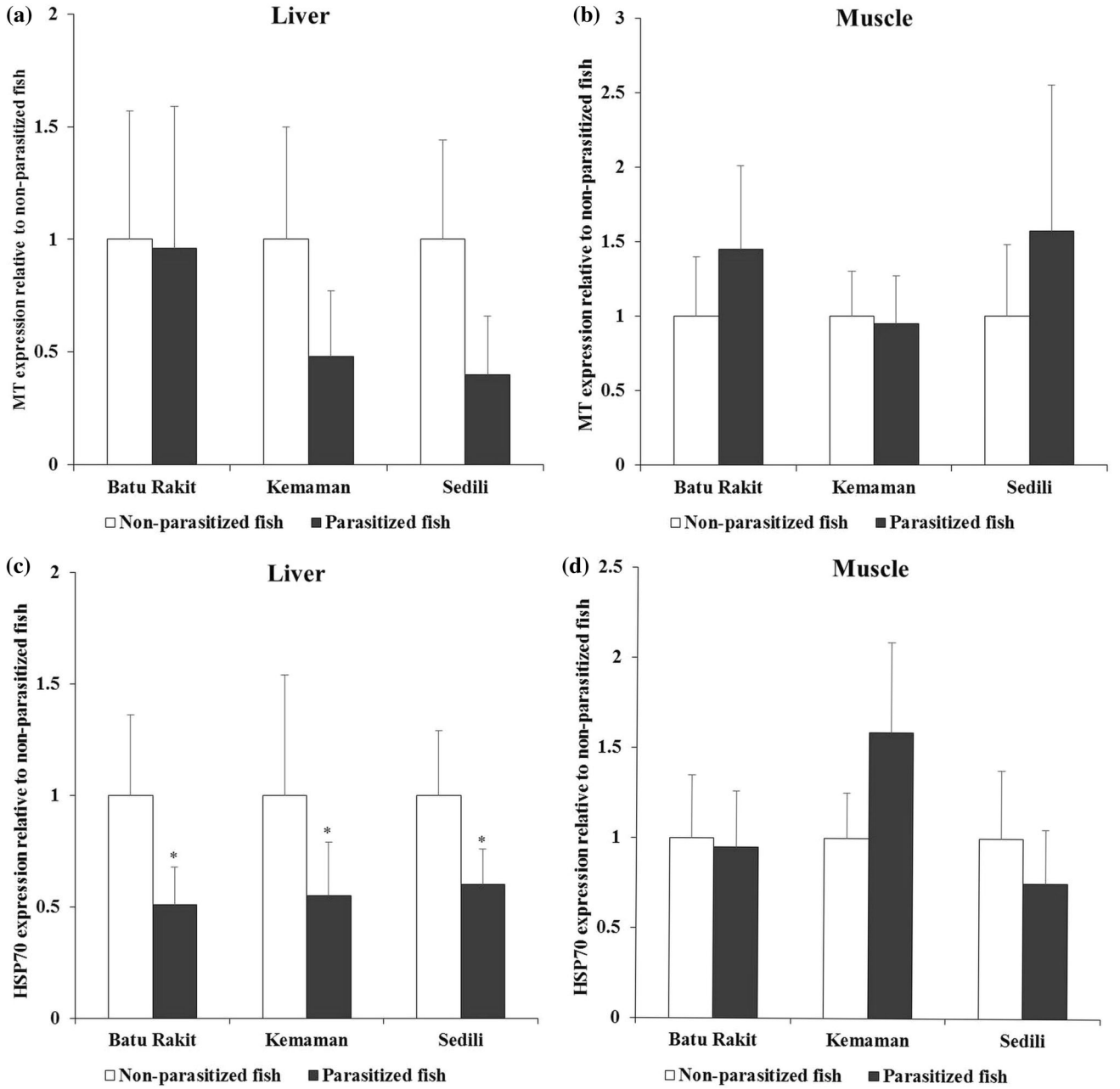

Fig. 2 Relative expression of metallothionein gene (**a**) and (**b**) and heat shock protein 70 (HSP70) gene (**c**) and (**d**) in liver and muscle of parasitized fish were expressed as fold change over nonparasitized fish at each sampling site. *Error bars* represent standard error. *Asterisks* indicate statistical significance ($P < 0.05$)

Expression of MT and HSP70 Genes

Similar to the heavy metal data, the MT gene was not significantly differentially expressed ($P > 0.05$) between parasitized and nonparasitized fish at all sampling sites for both muscle and liver tissue (Fig. 2a, b). However, gene expression pattern of the HSP70 gene was inherently different from that of the MT gene (Fig. 2c). Although no significant difference gene expression occurred in fish muscle ($P > 0.05$) between the two fish groups (Fig. 2d), HSP70 gene expression was significantly down-regulated in liver of parasitized fish at all studied sites. The expression level of HSP70 gene in parasitized fish was reduced by 0.51-fold, 0.55-fold, and 0.6-fold for individuals from Batu Rakit, Kemaman, and Sedili, respectively.

Figure 3 depicted the comparison of MT gene expression among all sampling sites, in which Batu Rakit was selected as the reference site. Liver of fish from Sedili had the highest expression level of MT gene across all sampling sites. In fact, the expression of MT gene in liver of

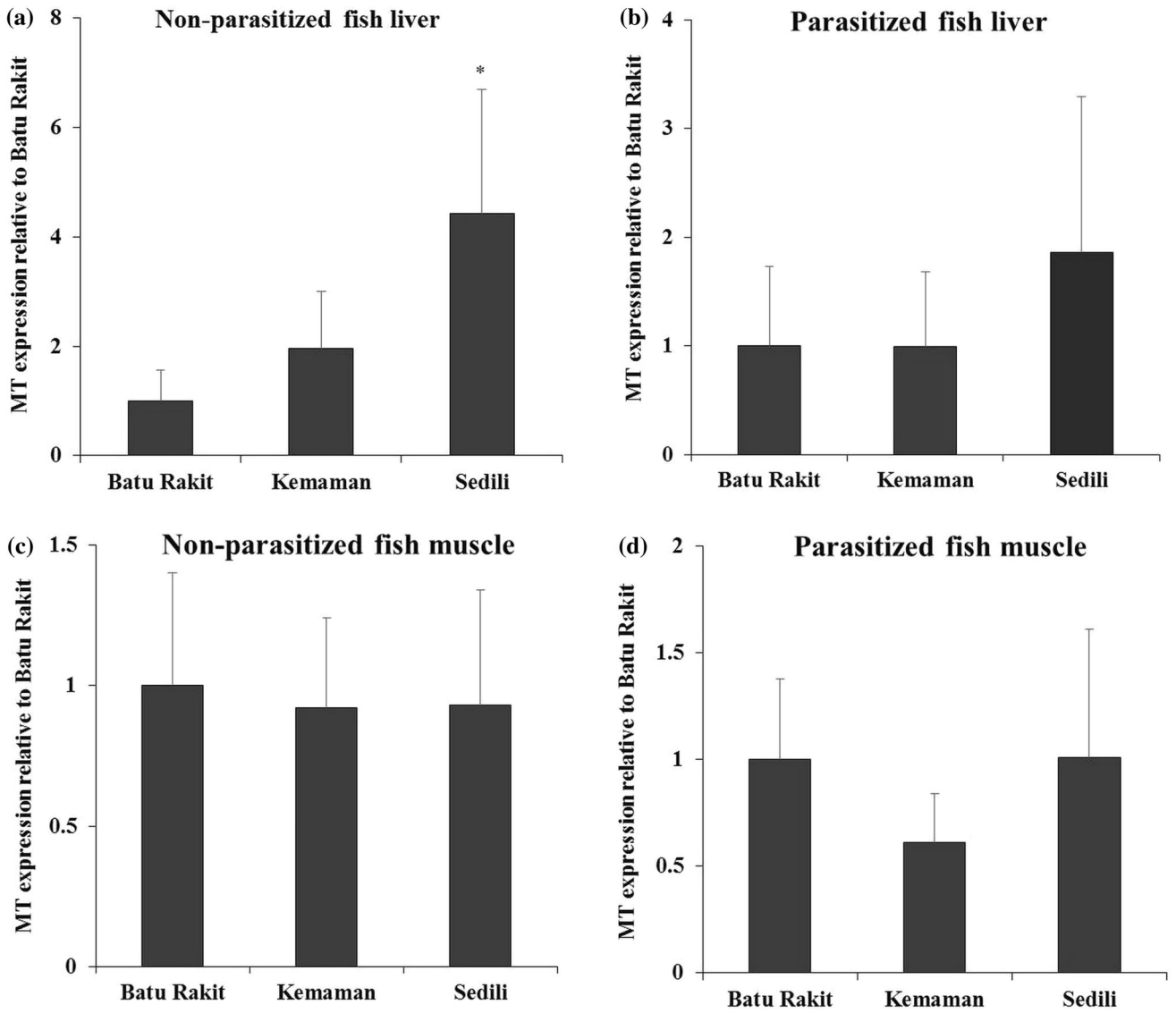

Fig. 3 Relative expression of metallothionein gene in different fish tissues of nonparasitized fish and parasitized fish. RNA expression values are presented relative to that of Batu Rakit: **a** and **b** fish liver; **c** and **d** fish muscle. *Error bars* represent standard error. *Asterisks* indicate statistical significance ($P < 0.05$)

nonparasitized fish from Sedili showed a 4.43-fold up-regulation compared with individuals from Batu Rakit. However, no significant difference was detected for MT gene expression in fish muscle from all sampling sites for both groups.

The expression of HSP70 mRNA expression in fish liver was not significantly different among sampling sites (Fig. 4a, b). In contrast, HSP70 was down-regulated in muscle tissue of both nonparasitized and parasitized fish from Kemaman and Sedili (Fig. 4c, d). The expression of HSP70 gene in muscle of non-parasitized fish from Kemaman and Sedili differed by 0.33-fold and 0.46-fold relative to that in Batu Rakit, respectively. In muscle tissues of parasitized fish, HSP70 expression was significantly lower by 0.55-fold (Kemaman) and 0.38-fold (Sedili) relative to Batu Rakit.

Discussion

A remarkable array of metal regulatory capacities of fish parasites have been revealed in recent years, in which parasites reduce metal levels in their fish host by competing with their host for essential metal elements (Sures 2002; Oyoo-Okoth et al. 2010, 2012). These parasites are capable of absorbing various substances from the host gut, including metal binding proteins (Jawale et al. 2011; Oyoo-Okoth et al. 2012). The present study represents the first

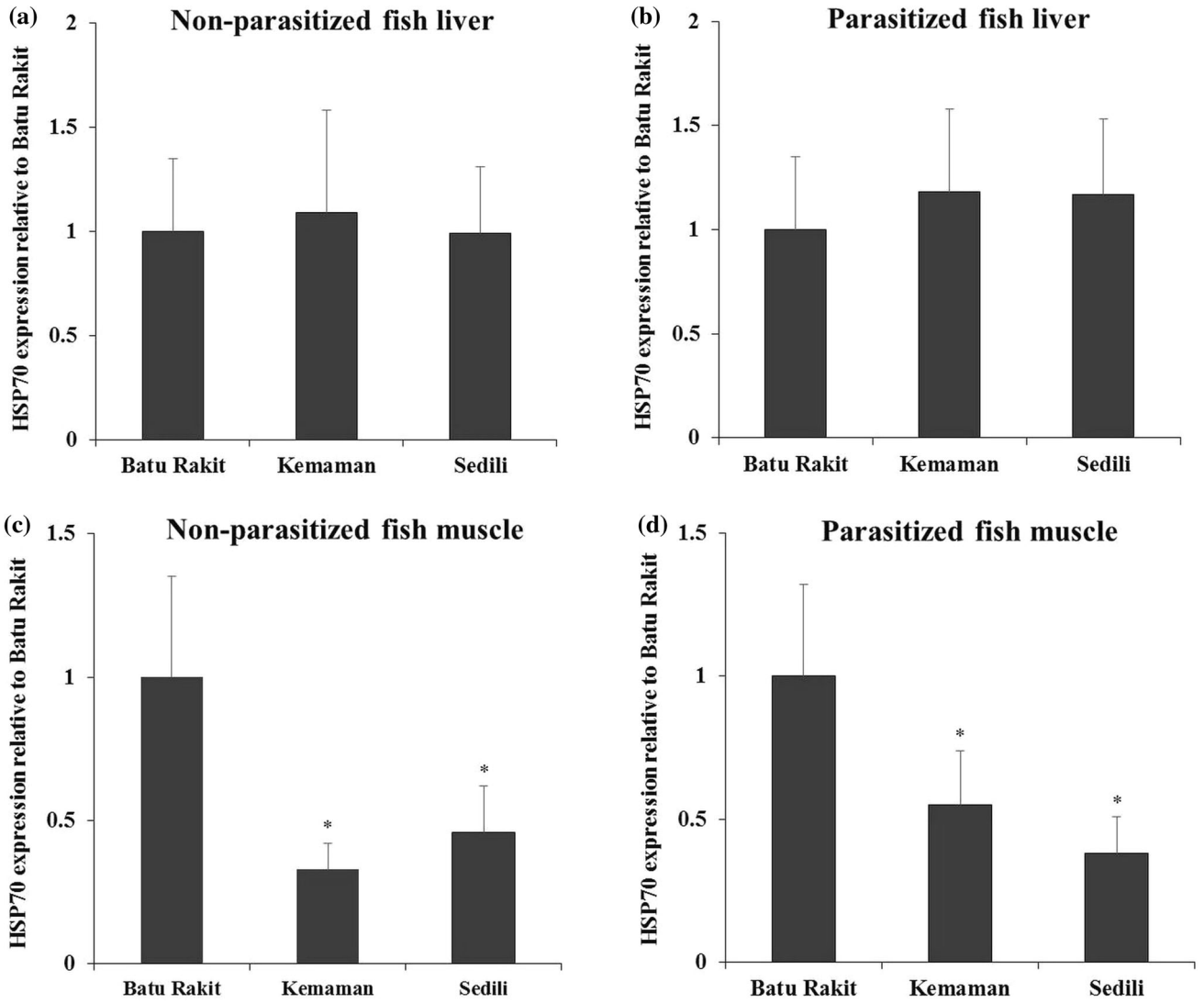

Fig. 4 Relative expression of Heat shock protein 70 (HSP70) gene in different fish tissues of nonparasitized fish and parasitized fish. RNA expression values are presented relative to that of Batu Rakit: **a** and **b** fish liver; **c** and **d** fish muscle. *Error bars* represent standard error. *Asterisks* indicate statistical significance ($P < 0.05$)

investigation of interaction between ectoparasites (a parasitic isopod) and heavy metal accumulations in fish tissues. Unlike other fish parasites, such as acanthocephalans and cestodes, our result demonstrated that these cymothoid individuals are not directly involved in metal regulations in their fish host, *N. furcosus*.

Overall, we observed a consistent trend in the concentration for all heavy metals (Fe, Cu, Zn, and Cd) among studied sites. Fish sampled from Sedili accumulated the highest level of heavy metal, followed by individuals of Kemaman and Batu Rakit. Concordant with other studies (Kalay et al. 1999; Yilmaz 2003; Javed 2005; Ashraf et al. 2012; Squadrone et al. 2013), heavy metal bioaccumulation in fish correlates positively with the level of aquatic pollution. Following exposure to heavy metal, these toxicants will be accumulated through their gill, skin, or gut via food consumption (Weber et al. 2013). As expected, the sites with highest level of anthropogenic activities along the East Coast of Peninsular Malaysia, Sedili, and Kemaman displayed a higher heavy metal concentration in the fish collected. Heavy metals discharged from industrial and shipping activities in these areas may be the main sources of pollution. Similarly, bigeye scad (*Selar crumenophthalmus*) and oyster (*Saccostrea cucullata*) was found to have consistent pattern of metal bioaccumulation with our results at the respective sampling sites (Agusa et al. 2005; Fuad et al. 2013).

In recent years, a wide array of biomarkers has been established to study potential ecological impacts of various anthropogenic contaminants including metal elements on aquatic organisms (Pina et al. 2007; Yudkovski et al. 2008). The expression profiles of stress-related transcripts may represent a powerful ecotoxicology tool to measure the direct

response of aquatic organism to external stressors. In this context, the level of expression of the gene coding for metallothionein (MT), a metal-detoxicating protein in fish would be measured to estimate the metal contamination level of polluted sites. The main functions of MTs include antioxidant defence and detoxification of both essential and nonessential metals (Monserrat et al. 2007). In aquatic animals, MTs are suggested as suitable biomarkers for heavy metal exposure (Kim et al. 2012). However, very limited studies have examined the roles of MT as bioindicator in wild fish populations (Knapen et al. 2007). Our findings revealed that the expression profiles of the MT gene in liver tissue were congruent with the heavy metal analysis, whereas MT gene expression was observed highest in fish collected from Sedili. These results have been anticipated, because Sedili is a heavily industrialized area located near a river mouth, which is contaminated with metal discharge from anthropogenic activities. In fact, higher levels of MT transcript and protein have been vastly documented in several teleost species, such as striped seabream (*Lithognathus mormyrus*), rainbow trout (*Oncorhynchus mykiss*), and gudgeons (*Gobio gobio*), all collected from sites differing in pollution gradients (Tom et al. 1998; Farag et al. 2003; Knapen et al. 2007). Collectively, MT expression demonstrated marked increase in expression level, specifically at heavily polluted area compared with least exploited control sites. Taken together, it appears that MT gene is a highly sensitive biomarker to assess heavy metal pollution in both fish and its habitat.

In contrast to transcript expression of MT gene in fish individuals among sampling site, expression of HSP70 gene was not correlated with heavy metal levels. Interestingly, HSP70 gene expression in fish muscle from Kemaman and Sedili were significantly lower than was observed in samples from Batu Rakit (the least exploited site). Unlike the MT gene, the HSP70 gene is a universal biomarker, which could be induced by other environmental stressors, such as heat, organic chemicals, and pathogenic infection (Ming et al. 2010). Our contrasting results, which show an inverse correlation of HSP70 with metal pollutants, are particularly noteworthy as this may reflect the presence of others stressors on fish collected from Batu Rakit. Concomitant with this assumption, we observed significantly lower HSP70 expression in fish infected with parasites across all sampling sites. Notably, parasite infection incurs negative interference with the fish host immune system by producing a variety of molecules and enzymes damaging to host immune function and protection mechanisms (Dzik 2006; Sures 2008). Indeed, down-regulation of the HSP70 gene following parasitic infection was reported extensively, indicating the likely specific response of HSP70 as molecular chaperone in immunity defense against pathogenic invasion (Basu et al. 2003; Fazio et al. 2008; Frank et al. 2013). However, as described previously, the parasitic cymothoid in our study has no significant effect on the heavy metal accumulation in *N. furcosus*. Predictably, the MT expression was not significantly altered, which is consistent with its main role as heavy metal chaperone. Clearly, our results emphasize that parasitic cymothoids are not directly involved in heavy metal accumulation in their fish hosts unlike many other endoparasites, such as helminths (Sures and Siddall 1999; Sures et al. 2003; Marijić et al. 2013).

Conclusions

Our comparison between nonparasitized and parasitized fish among sampling sites revealed that the cymothoid parasite had no significant impact on heavy metal concentration in their host, *N. furcosus*. In this study, metal concentration displayed significant variation among sampling sites with the highest bioaccumulation level in fish liver from Sedili. While there was no significant difference in MT gene expression of nonparasitized fish and parasitized fish, HSP70 gene expression was lower in the liver of parasitized fish. The down-regulation of HSP70 transcription level showed that parasites caused a decrease in fish's HSP70 gene expression. The present study also demonstrated that while HSP70 gene expression in fish liver was not related to the pollution gradient, MT levels increased in parallel with the pollution level of sampling sites. The highest level of MT expression gene was observed in the liver of fish collected from Sedili. Based on these findings, MT gene expression is suggested as a potential biomarker for heavy metal pollution. Our present study advances our knowledge of the role of ectoparasites on heavy metal accumulation and biomarker expression in fish hosts, which represent another step forward towards a more comprehensive aquatic environment assessment and management. Further research is recommended to determine the role of cymothoid parasites on metal regulation functions and expression of stress and immunity genes in different fish species.

Acknowledgments This study was funded by the Ministry of Higher Education, Malaysia under the Fundamental Research Grant Scheme (FRGS Vote No. 59291). The experiments and analyses were conducted at Institute of Tropical Aquaculture, Institute of Marine Biotechnology, Institute of Oceanography, and School of Fisheries and Aquaculture Sciences (University of Malaysia, Terengganu). The authors thank science officers in these institutes and school for their technical assistance.

References

Agusa T, Kunito T, Yasunaga G, Iwata H, Subramanian A, Ismail A, Tanabe S (2005) Concentration of trace elements in marine fish and its risk assessment in Malaysia. Mar Pollut Bull 51:896–911

Ameur WB, de Lapuente J, El Megdiche Y, Barhoumi B, Trabelsi S, Camps L, Serret J, Ramos-López D, Gonzalez-Linares J, Driss MR, Borràs M (2012) Oxidative stress, genotoxicity and histopathology biomarker responses in mullet (*Mugil cephalus*) and sea bass (*Dicentrarchus labrax*) liver from Bizerte Lagoon (Tunisia). Mar Pollut Bull 64:241–251

Ashraf MA, Maah MJ, Yusoff I (2012) Bioaccumulation of heavy metals in fish species collected from former tin mining catchment. Int J Environ Res 6:209–218

Atli G, Canli M (2008) Enzymatic responses to metal exposures in a freshwater fish *Oreochromis niloticus*. Comp Biochem Physiol C 145:282–287

Basu N, Kennedy CJ, Iwama GK (2003) The effects of stress on the association between HSP70 and the glucocorticoid receptor in rainbow trout. Comp Biochem Physiol A 134:655–663

Bio-Rad Laboratories Inc. (2006) Real-time PCR applications guide. Bulletin 5279

Deane EE, Woo NY (2006) Impact of heavy metals and organochlorines on HSP70 and HSC70 gene expression in black sea bream fibroblasts. Aquat Toxicol 79:9–15

Dhaneesh KV, Gopi M, Ganeshamurthy R, Ajith Kumar TT, Balasubramanian T (2012) Bioaccumulation of metals on reef associated organisms of Lakshadweep Archipelago. Food Chem 131:985–991

Dzik JM (2006) Molecules released by helminth parasites involved in host colonization. Acta Biochim Pol 53:33–64

Farag AM, Skaar D, Nimick DA, MacConnell E, Hogstrand C (2003) Characterizing aquatic health using salmonid mortality, physiology, and biomass estimates in streams with elevated concentrations of arsenic, cadmium, copper, lead, and zinc in the Boulder River watershed, Montana. Trans Am Fish Soc 132:450–467

Fazio G, Moné H, Lecomte-Finiger R, Sasal P (2008) Differential gene expression analysis in European eels (*Anguilla anguilla*, L 1758) naturally infected by macroparasites. J Parasitol 94:571–577

Feder ME, Hofmann GE (1999) Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu Rev Physiol 61:243–282

Frank SN, Godehardt S, Nachev M, Trubiroha A, Kloas W, Sures B (2013) Influence of the cestode *Ligula intestinalis* and the acanthocephalan *Polymorphous minutus* on levels of heat shock proteins (HSP70) and metallothioneins in their fish and crustacean intermediate hosts. Environ Pollut 180:173–179

Fuad MM, Shazili NAM, Faridah M (2013) Trace metals and rare earth elements in *Saccostrea cucullata* along the east coast of Peninsular Malaysia. Aquat Ecosyst Health Manag 16:78–87

Giguère A, Couillard Y, Campbell PGC, Perceval O, Hare L, Pinel-Alloul B, Pellerin J (2003) Steady-state distribution of metals among metallothionein and other cytosolic ligands and links to cytotoxicity in bivalves living along a polymetallic gradient. Aquat Toxicol 64:185–200

Guardiola FA, Gónzalez-Párraga P, Meseguer J, Cuesta A, Esteban MA (2014) Modulatory effects of deltamethrin-exposure on the immune status, metabolism and oxidative stress in gilthead seabream (*Sparus aurata* L.). Fish Shellfish Immunol 36:120–129

Gupta SC, Sharma A, Mishra M, Mishra R, Chowdhuri DK (2010) Heat shock proteins in toxicology: how close and how far? Life Sci 86:377–384

Javed M (2005) Heavy metal contamination of freshwater fish and bed sediments in the river Ravi stretch and related tributaries. Pak J Biol Sci 8:1337–1341

Jawale S, Fartade A, Borde S (2011) Biochemical studies of Caryophyllidean tapeworms in fresh water fish *Clarias batrachus*. Recent Res Sci Tech 3:35–36

Kalay M, Ay O, Canli M (1999) Heavy metal concentrations in fish tissues from the Northeast Mediterrenean Sea. Bull Environ Contam Toxicol 63:673–681

Kim JH, Rhee JS, Dahms HU, Lee YM, Han KN, Lee JS (2012) The yellow catfish, *Pelteobagrus fulvidraco* (Siluriformes) metallothionein cDNA: molecular cloning and transcript expression level in response to exposure to the heavy metals Cd, Cu, and Zn. Fish Physiol Biochem 38:1331–1342

Knapen D, Reynders H, Bervoets L, Verheyen E, Blust R (2007) Metallothionein gene and protein expression as a biomarker for metal pollution in natural gudgeon populations. Aquat Toxicol 82:163–172

Man AKY, Woo NYS (2008) Upregulation of metallothionein and glucose-6-phosphate dehydrogenase expression in silver sea bream, *Sparus sarba*, exposed to sublethal levels of cadmium. Aquat Toxicol 89:214–221

Marijić VF, Smrzlić IV, Raspor B (2013) Effect of acanthocephalan infection on metal, total protein, and metallothionein concentrations in European chub from a Sava River section with low metal contamination. Sci Total Environ 463–464:772–780

Ming J, Xie J, Xu P, Liu W, Ge X, Liu B, He Y, Cheng Y, Zhou Q, Pan L (2010) Molecular cloning and expression of two HSP70 genes in the Wuchang bream (*Megalobrama amblycephala*). Fish Shellfish Immunol 28:407–418

Monserrat JM, Martinez PE, Geracitano LA, Amado LL, Martins CM, Pinho GL, Chaves IS, Ferreira-Cravo M, Ventura-Lima J, Bianchini A (2007) Pollution biomarkers in estuarine animals: critical review and new perspectives. Comp Biochem Physiol C 146:221–234

Oyoo-Okoth E, Admiraal W, Osano O, Hoitinga L, Kraak MHS (2010) Metal specific partitioning in a parasite-host assemblage of the cestode *Ligula intestinalis* and the cyprinid fish *Rastrineobola argentea*. Sci Total Environ 408:1557–1562

Oyoo-Okoth E, Admiraal W, Osano O, Kraak MHS, Gichuki J, Ogwai C (2012) Parasites modify sub-cellular partitioning of metals in the gut of fish. Aquat Toxicol 106–107:76–84

Padmini E, Geetha BV, Usha Rani M (2009) Pollution-induced nitrative stress and heat shock 70 proteins over expression in fish liver mitochondria. Sci Total Environ 407:1307–1317

Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30:e36

Pina B, Casado M, Quiros L (2007) Analysis of gene expression as a new tool in ecotoxicology and environmental monitoring. Trends Anal Chem 26:1145–1154

Scheil V, Zurn A, Kohler HR, Triebskorn R (2009) Embryo development, stress protein (HSP70) responses, and histopathology in zebrafish (*Danio rerio*) following exposure to nickel chloride, chlorpyrifos, and binary mixtures of them. Environ Toxicol 25:83–93

Squadrone S, Prearo M, Brizio P, Gavinelli S, Pellegrino M, Scanzio T, Guarise S, Benedetto A, Abete MC (2013) Heavy metals distribution in muscle, liver, kidney and gill of European catfish (*Silurus glanis*) from Italian rivers. Chemosphere 90:358–365

Sures B (2002) Competition for minerals between *Acanthocephalus lucii* and its definitive host perch (*Perca fluviatilis*). Int J Parasitol 32:1117–1122

Sures B (2008) Host-parasite interactions in polluted environments. J Fish Biol 73:2133–2142

Sures B, Siddall R (1999) *Pomphorhynchus laevis*: the intestinal acanthocephalan as a lead sink for its fish host, chub (*Leuciscus cephalus*). Exp Parasitol 93:66–72

Sures B, Dezfuli B, Krug H (2003) The intestinal parasite *Pomphorhynchus laevis* (Acanthocephala) interferes with the uptake

and accumulation of lead (210Pb) in its fish host chub (*Leuciscus cephalus*). Int J Parasitol 33:1617–1622

Tom M, Moran O, Jabukov E, Cavari B, Rinkevitch B (1998) Molecular characterization of metallothionein-cDNA of *Sparus aurata* used for detecting heavy metal pollution along the Mediterranean coast of Israel. Mar Pollut Bull 36:131–137

Wang WX, Rainbow PS (2010) Significance of Metallothioneins in metal accumulation kinetics in marine animals. Comp Biochem Physiol C 152:1–8

Weber P, Behr ER, Knorr CL, Vendruscolo DS, Flores EMM, Dressler VL, Baldisserotto B (2013) Metals in the water, sediment, and tissues of two fish species from different trophic levels in a subtropical Brazilian river. Microchem J 106:61–66

Yılmaz AB (2003) Levels of heavy metals (Fe, Cu, Ni, Cr, Pb, and Zn) in tissue of *Mugil cephalus* and *Trachurus mediterraneus* from Iskenderun Bay, Turkey. Environ Res 92:277–281

Yudkovski Y, Rogowska-Wrzesinska A, Yankelevich I, Shefer E, Herut B, Tom M (2008) Quantitative immunochemical evaluation of fish metallothionein upon exposure to cadmium. Mar Environ Res 65:427–436