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## Heavy Metals Content in *Paraphilometroides nemipteri* from South China Sea may Influence Level of Glutathione and P38 Protein Expression

(Kandungan Logam Berat dalam *Paraphilometroides nemipteri* dari Laut China Selatan Boleh Mempengaruhi Tahap Glutathione dan Ekspresi Protein P38)

NURUL HUDA ABD KADIR\*, MOHD FAUZI MAHMUD, MOHAMAD KHAIRI MOHD ZAINOL, MASTURA ABD MALEK & FAIZAH SHAHAROM HARRISON

### ABSTRACT

Numerous studies have shown that parasites potentially become bio-accumulators for heavy metals. The heavy metals content in parasite-infected fish was reported to be lower compared to the parasite non-infected fish. Evaluation of heavy metal content in *Nemipterus peronii* and *Paraphilometroides nemipteri* was performed using ICP-OES. Our result has shown that arsenic was the most abundance heavy metal content in muscle *N. peronii* and *P. nemipteri*, suggesting that the parasite has the ability to accumulate heavy metals. Heavy metals were reported to induce oxidative stress where glutathione and p38 protein may be involved. Thus, expression of the p38 protein was determined using western blot technique and glutathione content was measured fluorometrically. The p38 expression in *P. nemipteri* of Pulau Kambing was higher compared to *P. nemipteri* of Besut has shown that the parasite may exposed to stress. Glutathione content showed no significant changes due to detoxification mechanism occurred in the parasite. In this study, we could conclude that *P. nemipteri* could be a bio-accumulator, whereas p38 protein and glutathione as indicator of stress level in the parasite that exposed to the heavy metals.

**Keywords:** Glutathione; heavy metals, *Nemipterus peronii*; oxidative stress; *Paraphilometroides nemipteri*

### ABSTRAK

Banyak kajian telah menunjukkan bahawa parasit berpotensi menjadi bio-akumulator bagi logam berat. Kandungan logam berat dalam ikan yang dijangkiti parasit dilaporkan lebih rendah berbanding ikan parasit yang tidak dijangkiti parasit. Penilaian kandungan logam berat dalam *Nemipterus peronii* dan *Paraphilometroides nemipteri* dilakukan menggunakan ICP-OES. Keputusan yang diperoleh menunjukkan bahawa arsenik merupakan kandungan logam berat yang paling banyak di dalam otot *N. peronii* dan *P. nemipteri*, ini menunjukkan bahawa parasit mempunyai keupayaan untuk mengumpul logam berat. Logam berat dilaporkan menyebabkan tekanan oksidatif dengan protein glutathione dan p38 mungkin terlibat. Oleh itu, ekspresi protein p38 ditentukan dengan menggunakan teknik Western blot dan kandungan glutathione diukur secara fluorometri. Ekspresi protein p38 oleh *P. nemipteri* dari Pulau Kambing adalah lebih tinggi berbanding *P. nemipteri* di Besut, menunjukkan bahawa parasit mungkin terdedah kepada tekanan. Kandungan glutathione tidak menunjukkan perubahan ketara disebabkan oleh mekanisme detoksifikasi yang berlaku pada parasit. Dalam kajian ini, kita dapat menyimpulkan bahawa *P. nemipteri* boleh menjadi bio-akumulator melalui ekspresi protein p38 dan glutathione sebagai penunjuk aras tekanan dalam parasit yang terdedah kepada logam berat.

**Kata kunci:** Glutathione; logam berat; *Nemipterus peronii*; *Paraphilometroides nemipteri*; tekanan oksidatif

### INTRODUCTION

Heavy metals are known as one of the main contributors to aquatic pollution (Palaniappan & Karthikeyan 2009) and are considered as critical contaminants of aquatic ecosystems (Jarić et al. 2011). Industrial wastes, geochemical structure and mining of metals create a potential source of heavy metal pollution in the aquatic environment (Gümgüm et al. 1994; Karadede & Ünlü 2000; Lee & Stuebing 1990). Fish and other aquatic organisms were reported accumulating heavy metals from polluted sea water (Kumar et al. 2012). A study conducted by Saliu and Bawa-Allah (2012) has shown that high concentration of Manganese (Mn), Cadmium (Cd), Lead (Pb), Mercury (Hg) and Copper (Cu) were found accumulated in the liver, intestine, heart and

gills of the fish. Furthermore, Kamaruzzaman et al. (2011) and Rejomon et al. (2010) have reported that the heavy metals can be found in *Nemipterus japonicus* from South China Sea. In this study, we have chosen Pulau Kambing and Besut, which are situated along South China Sea as our sampling sites. Pulau Kambing is located at the adjunction of Sungai Nerus and Sungai Terengganu, which highly contaminated due to human activities in this populated area (Chee et al. 2008). On the other hand, Sungai Besut is considered to be low contaminant site, due to less populated area and has been reported as clean (Suratman et al. 2006).

P38 mitogen-active protein kinase (MAPKs) is known as stress indicator and usually involved in stress-response cell signalling that will affected by natural

and unnatural stimuli, for example redox change, uv light, hyperosmolarity, growth, death, migratory signal (Ressurreição et al. 2011). In the past decade, parasites in fish has been discovered to possess an ability in accumulating heavy metals from the fish (host) (Sures et al. 1999) and environment (Zimmermann et al. 1999). Numerous researches have reported that parasites in fish could be bio-accumulators of heavy metals (Brázová et al. 2012; Hassan et al. 2016; Mazhar et al. 2014) due to high heavy metals content in the parasites compared to host (fish). The findings lead to a speculation that the parasites have an ability to accumulate the heavy metals in their body. However, toxicological mechanisms of heavy metals accumulation in the parasites are not well studied.

The metals may induce oxidative stress that results in imbalance of reactive oxygen species (ROS) production when antioxidant enzymes activity changed (Nishida 2011). The antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione S-transferases (GSTs) are involved in defense mechanism against xenobiotics and heavy metals (Stohs & Bagchi 1995). There are two types of heavy metals which are redox active metals and metals without redox (Sevcikova et al. 2011). Redox active metals induced oxidative stress by altering redox cycling in the cells, which leads to glutathione reduction and elevation of antioxidant enzymes such as SOD, CAT and GPX. On the other hand, metals without redox potential such as arsenic, cadmium, mercury, lead and selenium, may inhibit antioxidant defenses that caused glutathione fluctuation against time of exposure and affecting SOD and CAT activities (Valko et al. 2005).

Glutathione (GSH) is a non-thiol protein that present in most living cells and has been shown to play numerous roles, such as protection against oxidative stress, detoxification, transport and enzymatic catalysis that involve GSTs and GPX (Kim et al. 2005). Heavy metals also could affect the glutathione level in fish as demonstrated by Canesi et al. (1999), which the level of GSH were decreased in contaminated fish compared to non-heavy metals fish when heavy metal was accumulated in the fish tissues. Furthermore, GSH was found to be responsible in inducing toxicity by mutating glutamylcysteine synthetase, which involve in GSH synthesis that leads to oxidative stress in worms (Liao & Yu 2005). Helmcke and Aschner (2010) reported that GSH content elevated in acute exposure of methylmercury-treated worms, whilst the GSH level of chronic exposure worms was shown to be depleted. According to Wilhelm et al. (1997), intracellular glutathione is responsible as a regulator in inducing cellular stress response, including Jun N-terminal protein kinases (JNK) and p38 kinase.

On the other hand, p38 MAPK acts as a signal transduction component of heavy metals stress that mediates redox stress component of signal induction pathway when exposed to heavy metals (Rios-Barrera et al. 2008). A review by Ventura-Lima et al. (2011), reported that arsenic in aquaporins could affect signalling

pathways by activating proteins such as ERK2, p38 and JNK as demonstrated in mammals. In this study, we believed that parasites might undergo stress when they accumulate metals in their body. Thus, a comparison study was conducted using a molecular approach to determine glutathione and p38 protein expression as stress biomarkers *P. nemipteri*, a bio-accumulator of heavy metals in the *N. peronii*.

## MATERIALS AND METHODS

### SAMPLING SITES

Fish (*Nemipterus peronii*) were collected from Pulau Kambing Kuala Terengganu, Terengganu (coordinate 05°12'60.00" N, 4°21'7.00" E) and Kuala Besut, Terengganu (Besut) (coordinate 5°49'52.032" N, 0°0'0" E) as shown in Figure 1. The fish samples were transported to the laboratory in an icebox and stored at -80°C prior further analysis. Their standard length and weight were documented during dissection. The fish were identified morphologically according to Ambak et al. (2012) and were sorted through labeling or coding.

### CHEMICALS

Arsenic, Manganese, Cadmium, Chromium and Lead were purchased from Merck, Germany and all other chemicals unless stated in the text were purchased from Sigma Aldrich, USA.

### HEAVY METAL ANALYSIS

Each sample left was taken out and thawed in an icebox. The thawed samples (6 g) were then transferred in a veil tube for freeze-drying process. The lyophilized samples were ground and kept in -80°C. Hydrogen peroxide (1 mL) and nitric acid (7 mL) were added in the ground samples (0.2 g). The mixtures in veil tubes were vortexed vigorously and then transferred to Teflon vessel before digested in microwave digester for 15 min at 200°C. The digested samples were diluted with deionized water (15 mL) for heavy metals analysis using ICP-OES. Arsenic (As), Manganese (Mn), Cadmium (Cd), Chromium (Cr) and Lead (Pb) were prepared by adding 1% nitric acid to each of the heavy metals standards to reach final volume of 100 mL at concentrations of 0.8 ppm, 1.6 ppm, 2.4 ppm, 3.2 ppm and 4.8 ppm.

### GLUTATHIONE ANALYSIS

The parasites (100 mg) in triplicates were lysed using a RNA/protein purification kit (Macharey-Nagel, Germany). The supernatant from lysed samples were mixed with 6.5% 5-sulfosalicylic acid (160 µL) on ice for 20 min and centrifuged at 14,000 rpm for 5 min at 4°C. The supernatants were kept at -80°C until required. The supernatant fraction was diluted 10-fold with sodium phosphate buffer (100 mM, pH7.5) and 100 µL used

for the assay in 96 well plates. In each well, 33.4  $\mu\text{L}$  of *O*-phthalaldehyde (OPA) was added and wells were covered to avoid light exposure, then incubated for 30 min at room temperature before fluorescence.

#### PROTEIN EXTRACTION AND QUANTIFICATION

Proteins from the samples (100 mg) were extracted by using NucleoSpin<sup>®</sup> RNA/Protein (Macherey-Nagel) protocol. Protein concentrations of the samples were obtained from Bradford assay, based on the protocol given by Bio-Rad Protein Assay (Bio-Rad, USA).

#### WESTERN BLOTTING

The extracted protein samples (0.06  $\mu\text{g}$ ) were separated using NuPAGE 4-12% bis-tris gels (Novex, Life Technologies, USA) according to the manufacturer protocol (Pub Part No. IM-8042) and transferred onto PVDF membrane (Novex, Life Technologies, USA). PVDF membranes were incubated overnight with primary antibodies at 4°C followed by incubation with HRP-conjugated secondary antibodies for an hour at room temperature. The following antibodies were used for western blotting: p38 MAPK (D13E1) XP<sup>®</sup>Rabbit mAb (Cell Signaling) (1:1000 dilution, 0.5  $\mu\text{g}/\text{mL}$ ) and HRP-conjugated Goat anti-Rabbit IgG (H+L) Secondary Antibody (G-21234, 1:2000 dilution, 2  $\mu\text{g}/\text{mL}$ ) for p38 protein expression. Mouse monoclonal anti- $\beta$ -actin (AM4302, 1: 5,000 dilution, 0.02  $\mu\text{g}/\text{mL}$ ) (Ambion, USA) and HRP-conjugated anti-mouse secondary antibodies (A16072, 1:2,200 dilution, 0.4545  $\mu\text{g}/\text{mL}$ ) (Life Technologies, USA) were prepared for  $\beta$ -actin protein expression as loading controls. The image p38 protein bands were analysed by using imageJ software.

#### STATISTICAL ANALYSIS

GraphPad Prism 5.0 was used to determine  $\text{IC}_{50}$  value. One-way analysis of variance followed by Dunnett's multiple comparison was performed using GraphPad Prism V 5.0 to determine significant differences between groups.

#### RESULTS

Heavy metal may not be decomposed by bacteria or other living organisms and it can be found everywhere (Tekaya et al. 2013). Some heavy metals such as iron, zinc, selenium is not dangerous and essential for living thing in small amount, however it may be hazardous if it is present in high concentration and some heavy metal was highly toxic in low concentration, such as cadmium, lead, arsenic, mercury and chromium (Bagal-Kestwal et al. 2008). In this study, we measured heavy metals content in the muscle of *N. peronii* from two different areas (Pulau Kambing and Besut as illustrates in Figure 1) in order to determine the level of heavy metal contamination. Analysis of arsenic (As), manganese (Mn), cadmium (Cd), chromium (Cr) and lead (Pb) content in muscle tissues of *N. peronii* from Pulau Kambing and Besut is shown in Figure 2. Our data has shown that arsenic ( $23.07 \pm 2.78 \text{ mg/g}$ ) was the most abundance, followed by chromium ( $4.19 \pm 1.08 \text{ mg/g}$ ), manganese ( $3.50 \pm 0.77 \text{ mg/g}$ ), cadmium ( $0.124 \pm 0.009 \text{ mg/g}$ ) and lead ( $-1.35 \pm 0.316 \text{ mg/g}$ ), respectively, in the fish muscle from Pulau Kambing compared to negligible content of all the heavy metals in the fish muscle from Besut. The results suggesting that Pulau Kambing is highly contaminated area compare to Besut.



FIGURE 1. Sampling sites (1) Pulau Kambing, Kuala Terengganu (2) Besut, Kuala Terengganu



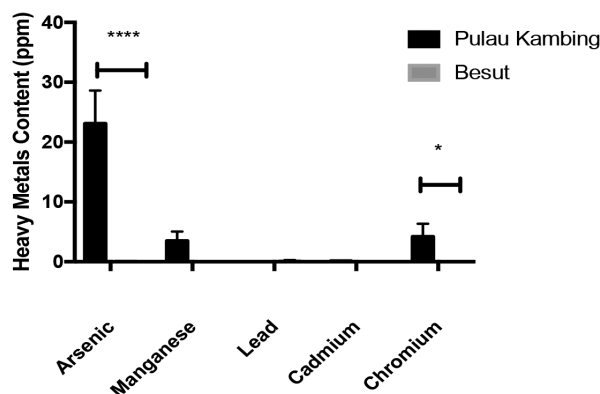


FIGURE 2. Arsenic, manganese, lead, cadmium, chromium content in muscle of *N. peronii* from Pulau Kambing and Besut, The heavy metal content was measured using ICP-OES. Statistical t test analysis was performed and \* indicate  $p < 0.05$ , \*\* indicate  $p < 0.01$ , \*\*\* indicate  $p < 0.001$  and \*\*\*\* indicate  $p < 0.0001$  significant different of the metal concentration in fish muscle of Besut and Pulau Kambing areas. Data is presented as the mean ( $n=3$ )  $\pm$  SEM

Table 1 shows the content of heavy metals in the muscle of *N. peronii* and its parasite, *P. nemipteri* from Pulau Kambing (high contaminated) and Kuala Besut (low contaminated). This comparison study of heavy metals in *N. peronii* muscle and *P. nemipteri*, has shown that all the heavy metals except chromium in the parasite was approximately 1.5 fold higher than the host (*N. peronii*) from Pulau Kambing, Similar trend could be seen for all other heavy metals content in *P. nemipteri* from Besut, which is approximately 1.5 fold higher than *N. peronii* muscle. The heavy metals level finding showed that the parasite (*P. nemipteri*) has the ability to accumulate heavy metals in their bodies.

Then, we carried out p38 protein and glutathione expression to determine physiological changes especially oxidative biomarkers of *P. nemipteri*, when they exposed to high and low contaminant areas. Based on our result (Figure 3), p38 protein bands were clearly expressed and the expression of the band was found to be higher in *P. nemipteri* from Pulau Kambing compared to Besut. The high p38 protein expression of *P. nemipteri* from Pulau Kambing compare to Besut was due to the higher level of

heavy metals especially arsenic in Pulau Kambing than Besut, suggesting that the parasites might be exposed to oxidative stress.

GSH involve in detoxification and redox in organisms to encounter oxidative stress, thus we measured GSH content of the *P. nemipteri* from Pulau Kambing (high contaminant area) and Besut (low contaminant area). Based on our finding (Figure 4), negligible difference of the GSH content in the parasites from both Pulau Kambing and Besut.

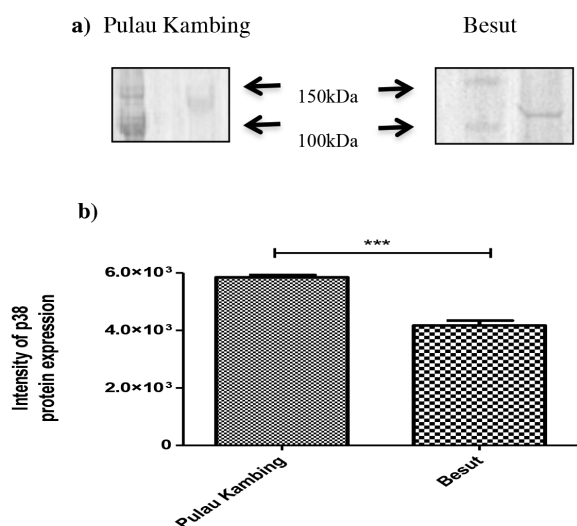
## DISCUSSION

High level of arsenic in the *N. peronii* muscle tissue and the fish muscle from Pulau Kambing has shown significance high level ( $p < 0.01$ ) of arsenic content compared to Besut. Arsenic level which was found to be predominant in *N. peronii* muscle from Pulau Kambing suggesting that, the area is considered to be highly contaminated compare to Besut. According to Mazhar et al. (2014), arsenic was among the most concentrated heavy metals in muscles of *N. peronii* which was collected from Marang, Terengganu that is located along the South China Sea.

TABLE 1. Heavy metals content of *N. peronii* (fish/host) and *P. nemipteri* (parasite) from Pulau Kambing and Besut

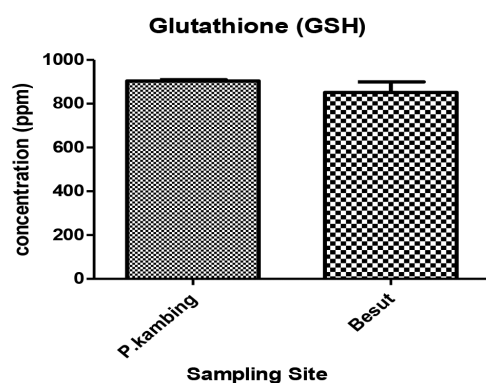
	Pulau Kambing		Besut	
	Fish muscles (N=4 $\pm$ SEM)	Parasites (N=4 $\pm$ SEM)	Fish muscles (N=4 $\pm$ SEM)	Parasites (N=4 $\pm$ SEM)
Arsenic	23.07 $\pm$ 2.78*	34.72 $\pm$ 3.28*	0.037 $\pm$ 0.0034*	0.0714 $\pm$ 0.013*
Manganese	3.50 $\pm$ 0.77*	4.99 $\pm$ 0.476*	0.0093 $\pm$ 0.0006	0.0577 $\pm$ 0.0298*
Lead	BDL	0.28 $\pm$ 1.142	BDL	BDL
Cadmium	0.124 $\pm$ 0.009*	0.24 $\pm$ 0.024	0.0055 $\pm$ 0.003	0.00071 $\pm$ 0.0004*
Chromium	4.19 $\pm$ 1.08*	3.35 $\pm$ 0.462	0.096 $\pm$ 0.005*	0.026 $\pm$ 0.0078*

The heavy metal content was measured using ICP-OES. Data is presented as the mean ( $n=4$ )  $\pm$  SEM and one sample t-test was performed. \*Indicates data significant at  $p < 0.05$  and BDL is 'Below Detection Level'



Data is presented as the mean ( $n=3$ )  $\pm$  SEM. Statistical analysis was performed using t-test and the significant values are indicated as follows \* $p<0.05$ , \*\* $p<0.01$  and \*\*\* $p<0.001$

FIGURE 3. a) Western blot band of p38 protein expression of *Paraphilometroides nemipteri* in Pulau Kambing and Besut, respectively and b) Intensity of P38 protein expression of *Paraphilometroides nemipteri* from Besut and Pulau Kambing was analysed using ImageJ software



Statistical analysis (one way ANOVA, Dunnet post-test) was performed and \* indicate  $p<0.05$ , \*\* indicate  $p<0.01$  and \*\*\* indicate  $p<0.001$  significant different of the glutathione concentration in *Paraphilometroides nemipteri* of Besut and Pulau Kambing areas. Data is presented as the mean ( $n=3$ )  $\pm$  SEM

FIGURE 4. Glutathione content in *Paraphilometroides nemipteri* from Pulau Kambing and Besut. The glutathione content was measured fluorometrically

*P. nemipteri* is an ectoparasite that adheres and consumes blood that contains heavy metals, from the muscle of dorsal fins, ventral fins and operculum of *N. peronii*. According to Mazhar et al. (2014), *P. nemipteri* contain high content of arsenic compared to the host (*N. peronii*) due to their ability in absorbing heavy metals such as cadmium, lead, aluminium directly from the blood of the host. In addition, *P. nemipteri* also was found to accumulate toxic elements from the water as an additional route of heavy metals uptake in the nematode

body (Zimmermann et al. 1999). Furthermore, a genomic study by Sahu et al. (2013) showed that genomic response of nematodes, *Caenorhabditis elegans* after the exposure with sodium arsenite exhibited oxidative stress response. Sahu et al. (2013) added that oxidative stress from arsenic as a result in production of ROS or the release of iron through induction of heme oxygenase. Oxidative stress, a pathological process relates to over-production of reactive oxygen species (ROS) in tissues and could be one of important general toxicity mechanism for many xenobiotics. Oxidative stress has shown to be induced after heavy metals exposure in fish (Farombi et al. 2007). Previous study by Farombi et al. (2007) also stated that alteration in the antioxidant enzymes, glutathione system and induction of lipid peroxidation reflects the presence of heavy metals. In addition, many organisms including fish have involved in xenobiotics metabolism mechanisms to counteract the impact of ROS and these include various antioxidant defense enzymes such as SOD, CAT and GSTs (Farombi et al. 2007).

Since heavy metals may causes oxidative stress to organisms when exposed to the chemicals and may cause a detrimental problem to the aquatic livings (Ercal et al. 2001), we conducted a molecular study using p38 protein expression to detect stress level of *P. nemipteri* after the exposure of heavy metals from the sea. Our results have shown that p38 protein and GSH content were elevated in *P. nemipteri* from Pulau Kambing due to high level of arsenic in host and the parasite itself.

A high amount of arsenic could lead to the oxidative stress (Labuda et al. 2005). According to Rios-Barrera et al. (2008), heavy metals such as cadmium and mercury exhibited p38 MAPK activity that mediates redox stress of the MAPKK signal pathways in *Euglena gracilis*. P38 MAPK pathways was discovered to be associated with apoptosis, stress responses and immunity (Johnson & Lapadat 2002). Blackwell et al. (2015) showed that sodium arsenite or other oxidative stressors activated p38 kinase through phosphorylation process in *C. elegans*. On the other hand, a study by Wang et al. (2008) reported that cadmium-induced germline apoptosis of *C. elegans* via JNK and p38 MAPK signalling pathways. Hence, we believed that arsenic and cadmium which are classified as metals without redox potential induced stress through p38 MAPK signalling pathways, but underlying stress pathways of *P. nemipteri* after the heavy metals exposure is not fully understood. To address this problem, we measured glutathione (GSH) level in order to investigate the biochemical changes in *P. nemipteri* when it exposed to heavy metals.

Espinoza et al. (2012) stated that there are changes in fish GSTs activity and glutathione (GSH) level may loss in accordance with the presence of high level of heavy metals. This condition will lead to oxidative stress that arise through normal metabolic processes (Hayes & Strange 2000). Thus, we speculated that GSH of *P. nemipteri* could be affected when they consume the blood that has been contaminated heavy metals from the

host. However, our result has shown that no significant changes of GSH level in *P. nemipteri* from both high and low contaminant areas. The unchanged GSH content in the parasite from both high contaminant and low contaminant areas was due to redox homeostasis of the parasite that may protect the parasite against oxidative stress. Elia et al. (2003) and Rana et al. (1996) found that GSH content was increased in liver and gill of fish after the exposure of mercury due to amino acid substrates such as glutamine, cysteine and glycine uptake to form GSH as protection against oxidative stress.

Furthermore, Bhattacharya and Bhattacharya (2007) reported that ratio of GSSG/GSH was found to be increased after 10 days of arsenic exposure in Indian catfish, *Clarias batrachus*. Whilst, Allen et al. (2004) mentioned that GSH levels in fish liver and kidney were fluctuated depending on days of the arsenic exposure. Based on previous finding, the induction of p38 protein expression explained the elevated concentration of GSH level of *P. nemipteri* from Pulau Kambing due to arsenic exposure. We also believed that the protection event might be occurred and some anti-oxidant enzymes such as GSTs, CAT and SOD could be affected. Further research should be carried out to investigate the expression of these proteins in order to enhance understanding mechanisms of action of the parasites after the exposure of heavy metals from the host.

Our results have shown that p38 protein and GSH content were elevated in *P. nemipteri* from Pulau Kambing due to high level of arsenic in host and the parasite itself. The biochemical toxicity changes might lead to a speculation that the parasite might be having an ability to detoxify the heavy metals in their body. A future study needs to be performed to explore this detoxification mechanism.

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- Nurul Huda Abd Kadir & Mohd Fauzi Mahmud  
School of Fundamental Science  
Universiti Malaysia Terengganu  
21030 Kuala Nerus, Terengganu Darul Iman  
Malaysia
- Mohamad Khairi Mohd Zainol  
School of Science and Food Technology  
Universiti Malaysia Terengganu  
21030 Kuala Nerus, Terengganu Darul Iman  
Malaysia
- Nurul Huda Abd Kadir, Mastura Abd Malek & Faizah Shaharom Harrison  
Institute of Tropical Aquaculture (AQUATROP)  
Universiti Malaysia Terengganu  
21030 Kuala Nerus, Terengganu Darul Iman  
Malaysia
- Faizah Shaharom Harrison  
School of Fisheries and Aquaculture  
Universiti Malaysia Terengganu  
21030 Kuala Nerus, Terengganu Darul Iman  
Malaysia

Mastura Abd Malek  
Institute of Medical Molecular Biotechnology (IMMB)  
Faculty of Medicine, Universiti Teknologi MARA  
Selangor Branch, Sungai Buloh Campus  
47000 Sungai Buloh, Selangor Darul Ehsan  
Malaysia

\*Corresponding author; email: nurulhuda@umt.edu.my

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