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Contents of hepatic and renal metallothioneins in *Hyposarcus pardalis*: For construction of biomarker for heavy metal contamination in environments

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Abstract The pollution of water by heavy metals is one of the most serious problems in the developing countries, where watercourses play important roles in transport and economic activities. The aim of this study was to examine whether *Hyposarcus pardalis*, a fish species widespread in the freshwater environment in Indonesia could be used as a biomarker for pollution of the environment by metals. To this effect, the concentrations of metallothioneins and metals in the livers and kidneys of *Hyposarcus pardalis* were measured. In addition, to clarify the relationship between metallothionein concentrations and metal exposure, the concentrations of metallothioneins and metals were determined in the liver and the kidney of fish exposed to 50 and 500 ppb Cu and 500 ppb Mn, compared with those kept in clean water. Sufficient concentrations of metallothionein were detected in fish captured from Lake Rawakalong located in an industrial area in the suburbs of Jakarta. The results of exposure experiments suggested that *Hyposarcus pardalis* retained a history of pollution in its organs for a long duration, and the metals bound to metallothioneins in the liver and kidney could be replaced with Cu following exposure. In conclusion, the hepatic and renal metallothioneins in *Hyposarcus pardalis* are a useful candidate biomarker for monitoring heavy metal contamination.

Key word: biomarker; developing countries; heavy metals; *Hyposarcus pardalis*; metallothionein

Introduction

Heavy metals constitute some of the most hazardous substances that can be bioaccumulated in biota (Tarifeno-Silva et al. 1982). Aquatic ecosystems are major recipients of pollutants, which can have serious consequences for biota that may not become apparent until changes occur at the population or ecosystem level (Linde-Arias et al. 2008). The need to detect and assess the impact of pollutants, particularly when present at low, sublethal concentrations, on environmental quality has led to a range of biological responses being measured in number of different species (Clements 2000; Fent 2004). Biomarkers defined as biochemical or physiological indicators of polluted environments, can be applied to detect the impact of pollutants on fish. Biomarker responses can subsequently be used to give early warnings of worse effects and pathological destruction of tissues (Roosmini et al. 2006). Molecular biomarkers are considered to be the most sensitive and earliest responses to pollutants (Rodríguez-Ortega et al. 2009). Metallothioneins (MTs) are recognized within a suite of "core biomarker", because their induction represents a biochemical response to an increased bioavailability of metals in the environment (Amiard et al. 2006). MTs are a class of low molecular weight, cysteine-rich (>30%), metal-binding proteins (Olsson et al. 1990). Although their physiological importance remains debated, these proteins appear to be primarily involved in the homeostatic control of essential metals such as Zn and Cu, but they are also believed to play an important role in the defense of organisms against metal poisoning and other stressors (Roesijadi 1996). The biosynthesis of MTs as a result of heavy metal exposure has been confirmed in many wildlife groups including fish, thus it has been suggested that the MT is useful as a biomarker of heavy metal contamination, although it is well known that physiological

and oxidative stress can be induced MTs (Hidalgo et al. 1988; Kadota et al. 2010). For example, a significant correlation was observed between a MT level in the liver and Cd or inorganic Hg in carp, eels and seals (Sato and Tohyama 1999).

Fish populations exploited commercially often inhabit in freshwater environments that contain high levels of heavy metals, resulting from contamination by industrial or agricultural wastes. Therefore, the reasons for utilizing edible fish as biomarkers of heavy metal pollutions are the following two points; first, they are intended for human consumption and any possibility of risk for consumers ingesting heavy metals through the food chain should be assessed; second, bioaccumulation allows the comparison of metal concentrations among sites where water samples are close to or below the detection limits of the atomic absorption technique (Ramelow et al. 1989). The expression and roles of MTs in fish have mostly been studied in organs that play a central role in metal uptake and accumulation, i.e., the liver, kidney, and gills. It has also been shown that significant differences can occur in the expression and induction of MT among different fish species (De Boeck et al. 2003).

In Indonesia, the pollution of the environments by heavy metals has reached worry levels (Yasuda et al. 2011). In West Java of Indonesia, lake water plays important roles on the lives of people. Crucially, the inhabitants of this area, especially those who live beside the rivers, use the river and/or lake water on a daily basis for cooking, washing, bathing and even drinking. If these substances contaminating the water are able to accumulate in humans and wildlife, human and wildlife health may be affected in the long-term.

From these viewpoints, the present study was carried out with the aim of clarifying whether metallothioneins in *Hyposarcus pardalis*, a fish species widespread in the

Indonesian freshwater environment, can be used as a biomarker for metal pollution. This species was selected based on our first results (see Results, Fish species captured in Lake Rawakalong). To this effect, MTs were measured in the liver and kidney of this species alongside measurement of the concentrations of metals in the tissues and in the lake they inhabit. In addition, to confirm the results of the field work, the levels of MTs and metals in the kidney and liver of fish exposed to 50 and 500 ppb Cu and 500 ppb Mn, compared with fish maintained in clean water, were also determined in the laboratory.

Materials and Methods

Sampling sites

Water samples were collected at 4 sites in the suburbs of Jakarta, West Java, Indonesia (Lake Rawakalong, Lake Cibinong, Lake Cikalet and Lake Sunter) for determination water quality. Based on the results of these analyses, a single sampling site, Lake Rawakalong, was selected for further study. The locations of the sampling sites are shown in Fig. 1. Numerous industrial factories were located near Lake Rawakalong producing a range of products, for example, plastics, electrical lamps, electrical home appliances and cosmetics. Water, sediment, and fish were collected twice, on August 6^{th}-8^{th}, 2008, and October 12^{th}-15^{th}, 2009. The lengths and weights of all fish caught were recorded. Condition factor (CF), calculated using the formula; $CF=FW \times 100/l^3$ (where FW = fish weight and l = fish length), was chosen as an estimation of the body size of the fish.

Study species

At first, we have caught 3 species of fish, *H. pardalis*, Mujar and Gabus (each 2 fish in each species) at August 6^{th}-8^{th}, 2008 in Lake Rawakalong to decide the candidate of biomarker. In addition, at October 12^{th}-15^{th}, 2009 thirteen *H. pardalis* individuals were caught by net from whole area of Lake Rawakalong. All caught *H. pardalis* individuals were on the sediments in the lake. After capture, all fish were anesthetized according to low temperature and, liver and kidney in each fish were removed to measure the contents of metals and metallothioneins.

General water quality

Water samples were taken from the surface of the lakes. The conductivity, chemical oxygen demand (COD), and concentrations of NO_2, NO_3, and PO_4 in the samples were measured immediately at each sampling location using a conductivity meter (Iuchi model TDS-can3, Japan) and simple water quality testing packages for COD, NO_2^-, NO_3^- and PO_4^{3-} (Kyoritsu chemical-check, WAK [200], Japan) according to the manufacturer's instructions.

Determination of metals in river and lake waters, sediments, and fish

The water samples were filtered with a 0.45 µm Millipore filter (USA). Before determining the concentrations of metals in the water samples, 1 mL of ultrapure analytical grade concentrated HNO_3 for the measurement of toxic metals (Tama Kagaku

Japan, Tokyo) was added to each 50 mL sample. To extract the heavy metals from the sediments, 2.5 mL of concentrated HNO_3 and the same volume of water were added to 5 g of the dried sediment, and the mixture was agitated for 24 h. The supernatants were collected after centrifugation at 3,000 rpm for 15 min. To remove the insoluble materials, the supernatants were filtered with a 0.45 µm Millipore filter (USA). The weights and lengths of the fish caught from the lake and grown in water tanks were measured. The fish were stored on ice and sacrificed. The livers and kidneys were taken, weighed, and stored in ethanol until analysis. To digest the weighed livers and kidneys with acid, they were heated in an oven (Isuzu Seisakusho, Tokyo, Japan) at 95°C for 3 h using Teflon reactors with concentrated HNO_3. The digested samples were transferred into volumetric flasks and the volumes of the samples were adjusted to 50 mL with 1M HNO_3.

The concentrations of Mg, Mn, Al, Co, Cd, Pb, Cu, and Zn in the water and sediment samples were analyzed with an Inductively Coupled Plasma Mass Spectrometry (ICP-MS; Seiko SPQ-6500, Tokyo, Japan) according to the method of Yasuda et al. (2011). The results were presented as µg metal/L for the water samples, µg metal per kg for the sediment samples, and µg metal per g wet weight tissue for fish samples.

Laboratory exposure

H. pardalis was obtained from an aquarium shop (Nature Club, in Tokyo), and maintained in 40 L tanks filled with tap water, changed once a day, at a constant temperature (26±1^{o}C), and aerated using an air pump (Suisaku, Tokyo). The fish were fed once a day with fish food (Tetra Pleco, Tetra, Japan) at a ratio of 0.5% of the fish

biomass. In the Cu and Mn exposure experiment, a total of 9 *H. pardalis* divided into 3 groups, were exposed to 0 (n=3), 500 ppb Cu (n=3), and 500 ppb Mn (n=3) for 7 days at 25±3^{o}C. In the Cu exposure experiment, a total of 9 *H. pardalis* divided into 3 groups, were exposed to 0 (n=3), 50 (n=3) and 500 ppb Cu (n=3) for 8 days at 25±1^{o}C. After the exposure, the fish were sacrificed under a cold treatment at 0°C. The experimental scheme was shown in Fig. 2.

Determination of MT levels in *H. pardalis*

The obtained tissues were homogenized on ice with 3 mL of 50 mM Tris-25 mM HCl. The tissue homogenates were centrifuged at 12,000 rpm for 30 min at 4°C. After centrifugation, the obtained supernatant was applied to a Sephadex G-50 column (1.0 × 110 cm) equilibrated with 50 mM Tris-25 mM HCl. The metal concentrations in the fractions were measured by ICP-MS, as described above. The MT concentration in each tissue sample was presented as μg MT/g wet weight tissue. The MT concentration was calculated using the following equation:

$$MT_{Total} = Mor_{cu} \cdot [Cu] / K_{cu} + Mor_{Zn} \cdot [Zn] / K_{zn} + Mor_{Cd} [Cd] / K_{cd}$$
$$Mor_{metal} = 6100 + K_{metal}$$

where [Cu], [Zn], and [Cd] were the total amounts of each metal bound to MT in the total MT fraction (μg), K_{metal} was the total atomic weight of the metal bound to one molecule of MT (g) (e.g., 7 atoms for Cd and Zn, and 12 atoms for Cu, bind to one molecule of MT), and Mor_{metal} was the molecular weight of the MT including the bound metals.

Statistical analysis

All values are given as the mean ± standard error (S.E.). Statistical analyses were performed by one-way analysis of variance (ANOVA), followed by the Fisher's test using InStat 3 (GraphPad Software, USA).

Results and Discussion

General water quality and concentrations of metals in the surface water

To understand the environmental conditions in Indonesia, four lakes were selected in West Java, Indonesia, as water sampling locations, as shown in Fig. 1. As listed in Table 1, the sample with the highest conductivity was that from Lake Sunter (600 µS cm^{-1}; site 4 in Fig. 1). This conductivity value was higher than the standard conductivity value for Indonesian water of 225 µS cm^{-1}. The conductivity of the water in Lake Rawakalong (240 µS cm^{-1}; site 1 in Fig. 1) was also above the Indonesian standard value (225 µS cm^{-1} as above mentioned). The concentrations of NO_2 and NO_3 in the water samples were considered to be within the normal range, because Indonesian standard values for NO_2 and NO_3 are 1 and 10, respectively. The highest phosphate concentration was 0.35 mg/L measured in Lake Rawakalong. This concentration of phosphate was higher than those measured in Japan, described in Kido et al. (2009).

To examine whether metal pollution was present in the sampling area, the concentrations of 8 metals such as Al, Cd, Co, Cu, Mg, Mn, Pb, and Zn, were measured in samples of water from the lakes. Mg, Mn, and Al were detected in all analyzed samples from the lakes. As shown in Table 2, the levels of metals of water were much

lower than previously described (Kido et al. 2009; Kurasaki et al. 2000; Yasuda et al. 2011). Cu, Zn, Co, Pb and Cd were not detected or it was present at a concentration less than the standard value for Indonesian water.

Concentrations of metals in the sediments

The concentrations of metals in the sediments from the lakes in Indonesia are listed in Table 3. All of the metals analyzed were detected at each sampling point. The concentrations of Pb in the lake sediments studied were markedly high, especially in Lake Rawakalong and Lake Sunter. In addition, the concentrations of Cd at 2 sites (Lake Rawakalong and Lake Cikalet) exceeded the environmental standard value. In particular, the concentration of Cd in Lake Rawakalong was more than 10 times that of the standard value. It is suggested that this contamination may be caused by the discharge of waste water from the industrial factories near Lake Rawakalong. Because Cu, Zn, Cd and Pb were the highest in Lake Rawakalong, even if Mn was low, we chose Lake Rawakalong as the capture site for the fish.

Fish species captured in Lake Rawakalong

As the first step towards developing a monitoring system for metal contaminants in the water of the Indonesian lakes, the levels of MT were measured in liver of fish captured from Lake Rawakalong (each 2 fish in each species). As shown in Fig. 3, the mean level of MTs in the liver of *H. pardalis* (Sap Sap) was higher than that of the 2 other species (Mujar and Gabus). Based on these results, we considered *H. pardalis* to be the best

candidate for a biomarker of environmental contaminants. *H. pardalis* was chosen as the candidate biomarker of heavy metal contamination. This species originates in the Amazon River in Brazil but is cultivated across Southeast Asia for the aquarium hobbyist trade. *H. pardalis* eats most food offered, including processed foods such as algae wafers, flake, and sinking pellets, many kinds of fruits and vegetables, and small live or frozen foods such as bloodworm. In addition, *H. pardalis* is widespread in Indonesia. It will also scavenge on dead or dying fish if the opportunity arises. The mouth is located on the underside of the head and is shaped like a suction cup (Fig. 4).

Biological characteristics of *H. pardalis*

The biological characteristics of the fish captured from Lake Rawakalong and their CF values are shown in Table 4. There was no major difference in the weights, lengths, or CFs of the fish caught from Lake Rawakalong in August 2008 compared with October 2009. There were also no significant differences in the CF values between the fish collected from the field and those used in the laboratory exposures.

Concentrations of metals and MTs in *H. pardalis* collected from the field

The concentrations of metals (Cu, Zn, and Mn) in *H. pardalis* caught from the field are shown in Fig. 5A. The concentration of Cu in the livers was significantly higher than that in the kidneys ($P<0.05$). Although the concentrations of Mn in water and sediments of Lake Rawakalong were higher than those of Cu and Zn, Mn concentration in the cytosol of both tissues was lower than Cu and Zn concentration. There is no significant

difference of Zn or Mn concentration in between livers and kidneys. In addition, unexpectedly, the concentrations of Cd in the livers and kidneys of *H. pardalis* in the lake was less than 50 ng/g wet weight (data not shown), although the Cd concentration of the sediment of Lake Rawakalong was more than 1.0 mg/kg (Fig. 5A). It was hypothesized that, after Cd induced the synthesis of MTs in the liver and kidney of the fish, the Cd bound to MT would be replaced with Cu. It is well known that Cu can be replaced with other metals bound to MT, because the binding affinity of Cu to MT is higher than that of other metals (Nielson and Winge 1983).

To clarify whether metal concentrations and metal species in the cytosol had a close relationship with the bound metals in MT, MT amounts and bound metals in MT were measured using gel filtration technique. A typical elution profile of the cytosol from the liver of *H. pardalis* caught from the field is shown in Fig. 5B. Since MTs were detected in all samples, their concentrations were calculated for each fish, as detailed in the Materials and Methods. The concentrations of MTs in *H. pardalis* obtained from Lake Rawakalong are shown in Fig. 5C. There were no significant differences in the concentrations of MTs in the liver compared with the kidney, although the concentrations of Cu in the liver were higher than those in the kidney (Fig. 5A). In addition, Cu- and Zn-binding MTs were detected at levels appropriate for the amounts of metal contaminations in the sediment. About 60% of the total MTs in the liver and kidney were bound to Cu (other MTs were almost bound to Zn). Ratio of Cu and Zn-MTs in both tissues was similar. MT was scarcely observed to be bound Cd and Mn. After exposure to Mn, Mn-MT was hardly detected, despite the increase in the concentration of MTs.

Cu and Mn exposure laboratory first experiment

In the next step, to clarify whether *H. pardalis* could be used as a biomarker for pollution by metals, the relationship between the concentrations of MTs in the liver and kidney of *H. pardalis* and concentrations of metals in water were investigated in the laboratory. At first, to compare the induced ability of MTs by Cu and Mn in *H. pardalis*, the concentrations of metals and the total metal-binding, Cu-binding and Zn-binding MTs were measured in the livers and kidneys of the fish exposed to 500 ppb Cu or 500 ppb Mn for 7 days, compared with control fish (Fig. 6). Zn bound to MT was rarely observed in the liver and kidney of the control (0 ppb) fish. The concentrations of total, Cu-binding, and Zn-binding MTs in the liver and kidney of the fish exposed to Cu or Mn were increased compared with those in the control fish. However, the concentrations of MTs in the liver and kidney of the fish exposed to Cu were the higher than those in the fish exposed to Mn. In addition, although the concentrations of MTs in the liver were not different from those in the kidney following Cu exposure, the concentrations of MTs in the liver were significantly higher than those in the kidney following Mn exposure. As shown in Fig. 5, after exposure to Mn, Mn-MT was hardly detected, even if MTs was increased in the cytosol. This occurrence was also reported by Sato and Tohyama (1999). In this study, following Mn exposure, Cu-MT accumulated in the liver and kidney of the fish. These results were supported by the finding that the Cd bound to MT in *H. pardalis* of Lake Rawakalong might be able to be replaced by other contaminant metals such as Cu, as mentioned above.

Cu exposure laboratory second experiment

Basing on the results of previous experiment, we focus on relationship between exposure of Cu and induction of MT. Especially, to determine the dose-dependency of the responses to Cu exposure, the concentrations of total metal-binding, Cu-binding, and Zn-binding MTs in the liver and kidney of *H. pardalis* exposed to 0, 50, and 500 ppb Cu for 8 days were measured (Fig. 7). The concentrations of Cu and Zn in the liver and kidney of the fish exposed to 500 ppb Cu were increased compared with those in the control fish and the fish exposed to 50 ppb Cu. According to the results of the ANOVA analysis ($p=0.035$), significant differences were present between the concentrations of Cu-MT in the control compared with the 500 ppb exposed group, and between the concentrations of Cu-MT in the liver and kidney of the fish exposed to 50 ppb Cu (Fig. 7). The concentrations of total MTs and Cu-binding MTs in the liver and kidney of the fish exposed to 50 and 500 ppb Cu increased in a dose-dependent manner ($P<0.1$).

In addition, the Cd-binding MT was present in the liver and kidney of all groups, although there was no Cd in the fish tank water. In the case of the exposure to Cd, the levels of hepatic and renal MT in carp (*Cyprinus carpio*) increased with increasing exposure Cd concentration (Van Campenhout et al. 2004). However in this study, hepatic and renal Cd-MTs were detected in fish that were not exposed to Cu (the water the fish maintained in did not contain Cd). We proposed that the metals bound to MTs in the unexposed fish must reflect the environment they inhabited prior to the study. Previous study suggest that Cd is accumulated slowly in fish (Calamari et al. 1982; Haux and Larsson 1984), with an extremely slow elimination (Olsson and Hogstrand 1987). From the results presented here, it appears likely that the fish can retain a history

of the pollution in their organs for a long duration, and the metals bound to MTs in the liver and kidney could be replaced with Cu. As for the hepatic and renal MTs in the fish, the concentrations of Cu and Zn in the livers and kidneys were also increased when the fish were exposed to 500 ppb Cu (data not shown). The relationship between the concentrations of the metals in the environment and the concentrations of the MTs in animal tissues has led to the use of MTs for monitoring the biological effects of metal exposure (Hylland et al. 1992).

Conclusion

In developing countries, there is some metal pollution due to developing of industry. It will be expected to confirm the biomarker of the pollutant in surface water. In this study, Cu- and Zn-MTs were detected in *H. pardalis* obtained from Lake Rawakalong polluted with Cd, which is located in an industrial area near to Jakarta. To confirm whether this fish can be used as biomarker for heavy metal pollution, the laboratory experiments were carried out. As results, MTs in liver and kidney of *H. pardalis* were increased by increase of Cu exposure. When heavy metal which cannot bind to MT was exposure such as Mn, MTs in the liver and kidney of *H. pardalis* exposed to Mn were also increased compared with those in control fish. In addition, the results of laboratory and field experiments suggested that *H. pardalis* could retain a history of pollution in its organs for a long duration and the metals bound to MTs in the liver and kidney could be replaced with Cu. Based on these results, the MTs in *H. pardalis* appear to be a useful candidate for a monitoring system of heavy metal pollutiom.

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Figure legends

Fig. 1 The sampling sites in West Java, Indonesia

Fig. 2 Scheme of laboratory experiments

Fig. 3 Mean levels of MTs in the liver of *H. pardalis* (SapuSapu), Gabus, and Mujar collected from Lake Rawakalong on August 6^{th}-8^{th}, 2008 (each n=2). Data are shown mean values of each two fish.

Fig. 4 *H. pardalis* A: Overall, B: Mouth

Fig. 5 Concentrations of Cu, Zn, and Mn in the livers and kidneys (n=13) of *H. pardalis* caughted from Lake Rawakalong on October 12^{th}-15^{th}, 2009 (A). The bars indicate the mean ±S.E. Identical letters designate significant differences between two groups at $p<0.05$. A representative elution pattern from Sephadex G-50 chromatography using the hepatic cytosol from the fish from Lake Rawakalong (B). Levels of hepatic (n=13) and renal (n=9) MTs in *H. pardalis* from Lake Rawakalong (C). The bars indicate the mean ± S.E.

Fig. 6 Concentrations of hepatic and renal MTs of *H. pardalis* exposed to 0, 500 ppb Cu, and 500 ppb Mn (each n=3). The bars indicate the mean ± S.E. Identical letters designate significant differences between two groups at $p<0.05$ (a and a, or b and b).

Fig. 7 Concentrations of hepatic and renal MTs MT of *Hyposarcus pardalis* exposed to 0, 50, and 500 ppb Cu (each n=3) The bars indicate the mean ± S.E. Identical letters designate significant differences between two groups at $p<0.05$.