The disruptive effect of mercury chloride (HgCl) on gene expression of gonadotrophin hormones and testosterone level in male silver sharkminnow (*Osteochilus hasseltii* C.V.) (Teleostei: Cyprinidae)

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Abstract
Endocrine-disrupting compounds in water affect reproductive activities in fish. Mercury is a toxic and persistent pollutant, which bioaccumulates in the food chain. To investigate the effect of mercury chloride (HgCl) on fish reproduction, animals were kept in four aquaria containing increasing levels of HgCl (0 mg/L [control]; 0.025 mg/L [low]; 0.05 mg/L [medium]; 0.1 mg/L [high]) for 60 days. The effects of HgCl on reproduction performance of male silver sharkminnow were evaluated by GtH-Iα, GtH-IIα and GtH-IIβ gene expression, testosterone levels, and GSI levels. A significant decrease in Gonadothropin Hormone type I sub unit alpha (GtH-Iα), Gonadothropin Hormone type II sub unit alpha (GtH-IIα) and Gonadothropin Hormone type II sub unit beta (GtH-IIβ) gene expression, testosterone levels, and Gonado Somatic Index (GSI) levels was detected in fish receiving the high mercury dose compared to controls after 2, 4, 6 and 8 weeks (P < 0.05). After 4, 6 and 8 weeks, all treatment groups had significantly lower GtH-Iα, GtH-IIα and GtH-IIβ gene expression, testosterone levels and GSI levels compared to the control group (P < 0.05). These findings demonstrate a disruptive role of mercury on reproductive performance in male silver sharkminnow.

Keywords: HgCl, GtH-Iα, GtH-IIα and GtH-IIβ testosterone levels, GSI

Introduction
Reproductive activities in fish are regulated by several environmental and physiological factors (Bromage et al. 2001; Rodriguez et al. 2004). The external environment exerts an effect on reproduction through the hypothalamus, regulating the synthesis and secretion of gonadotropin-releasing hormones (GnRHs) (Vickers et al. 2004; Sherwood & Adams 2005; Prayogo et al. 2011; Piccione et al. 2015). GnRHs regulate the synthesis and secretion of gonadotropins (GtHs) (Li et al. 2004; Mortehavizavizadeh et al. 2010; Yaron & Levavi-Sivan 2011). The GtHs regulate the two main activities of the gonads, i.e. hormone and gamete production (Kamler 1992; Rodriguez et al. 2004). Ovarian hormones, especially estradiol and progesterone, play an important role in maintaining and promoting gamete production (Miranda et al. 2009). In salmonids, plasma levels of GTH-I are elevated during spermatogenesis; they decline during final spermiation in males (Saligaut et al. 1998). GtH-II levels are low during the early stages of the reproductive cycle; they increase during spermiation in males (Breton et al. 1998). Both GtHs are equipotent in stimulating testosterone (T2) production (Sulistyo et al. 1998), but GtH-II is more potent than GtH-I in stimulating maturation-inducing steroid, DHP (17, 20-dihydroxy-pregnen-3-one) (Saligaut et al. 1998; Utoh et al. 2003).

Heavy metals are one of the pollutants that are very persistent in nature. One of the most dangerous heavy metals is mercury (Hg). Mercury is becoming one of the most concerning heavy metals because it can spread through the atmosphere. Mercury is a rare element in nature, but it also comes from anthropogenic sources, such as power plants that...
still use coal as fuel. Atmospheric mercury can pollute aquatic systems from its deposition, as can stationary pollution sources such as mining and industry (Eisler 2004). In fish, mercury bioaccumulation and poisoning can lead to a high death rate, weight loss, larval deformation, depressed protein synthesis, hormonal imbalance, abnormal behavior, reduction in spawning, sexual dysfunction, and color changes (Friedmann et al. 1996; Oliviera Robeiro et al. 2002; Dervnick & Sandheinrich 2003; Houck & Cech 2004; Devline 2006; Gharaei et al. 2010).

Recent studies show that HgCl has a toxic effect on the human brain, having a destructive effect on human gene integrity (i.e. it is genotoxic). HgCl acts as an inhibitor on proliferation, increasing metastasis and micronucleation indexes, and the nucleoplasmic bridge index, as well decreasing the binucleation index (Zalups 2000; Crespo-López et al. 2007). Recent studies have identified the mechanism responsible for toxicity of MeHg, such as its reactivity to bioactive protein, thiols (Yasutake et al. 1997), arachidonic acid (AA), production (Shanker et al. 2002), occurrence of reactive oxygen species (ROS) (Limke & Atchison 2002), and intracellular calcium increase (Castoldi et al. 2001).

However, despite extensive studies, the effect of HgCl on male fish reproduction has received little attention. From the first study in our laboratory we found that in female silver sharkminnow, HgCl treatment decreased the gene expression of cGnRH-II and sGnRH, and the level of estradiol (Prayogo et al. 2016a). In the present study we investigated the specific effects of HgCl concentration, on gene expression of GtH-Ia, GtH-IIa and GtH-IIb, testosterone, GSI and spermatid proportion in male silver sharkminnow Ostecichilus hasseltii (Valenciennes, 1842). Silver sharkminnow are economically fished in Indonesia and have a large market (Prayogo et al. 2012, 2016b). The biology, physiology, and nutrition of this species have been studied in detail, and there have been several global efforts to conserve this species. Thus, silver sharkminnow is a suitable freshwater model species to provide insight into the toxic effect of HgCl in other cyprinid species. Silver sharkminnow is a synchronous batch spawner capable of spawning several times during the peak of the spawning period.

Material and methods

Experimental fish

One hundred and forty-four male hard-lipped barbs weighing 100 g on average were purchased from local farmer in Banyumas regency and maintained at the Laboratory of Fisheries and Marine, Jenderal Soedirman University. The experiments were divided into four groups. Each group consisted of four aquaria with nine fish/50 L water. In this study, different treatments of HgCl concentration (Prayogo et al. 2016b) – 0.025 mg/L (T1), low concentration; 0.05 mg/L (T2), medium concentration; and 0.1 mg/L (T3), high concentration – and a control were tested for concentration of GtH genes, testosterone and GSI, for 8 weeks. The aquaria were designed with recirculation for oxygen supply. Fish were kept for 8 weeks at the laboratory of the Aquaculture Department of Marine and Fisheries, University of Jenderal Soedirman. During the research, fish were fed on commercial pellets (37% protein and 10% fat) as much as 3% of total body weight daily. The water was siphoned regularly to maintain water quality. The water temperature, dissolved oxygen, pH and carbon dioxide were monitored every 2 weeks. Every sampling time, nine fish were captured for the samples. All fish were anesthetized with tricaine methane sulfonate (Sigma) and decapitated prior to tissue collection. Gonad samples from the fish were removed, and after being dissected and weighed, the gonads were fixed in Bouin’s solution and subjected to histological observation. The serum samples were separated by centrifugation (4°C, 10,000 g, 5 min) and stored at −80°C until analysis. The GSI was calculated for each fish. The calculation used to determine GSI was Gonad Weight/Total Tissue Weight × 100 (Prayogo et al. 2012).

RNA isolation and Reverse Transcriptase-Polymerase Chain Reaction

Total mRNA was extracted from the whole brain using an RNA extraction mini kit (geneaids). The samples were treated with DNase-free RNase (Takara). The quality and concentrations of total RNA were assayed by agarose gel electrophoresis and optical density reading at 260 and 280 nm; the RNA were loaded in batches and frozen at −70°C.

Reverse Transcriptase-Polymerase Chain Reaction

Total mRNA samples (1 μL) were reverse transcribed using a cDNA synthesis kit (PrimeScript™ Reverse Transcriptase) from Takara using Random 6 mers (50 μM) primers and Prime script R-tase according to the manufacturer’s instructions.

Quantitative real-time analysis

The primers were designed based on GtH-Ia (KT947119), GtH-IIa (KT947120) and GtH-IIb (KT762151) using the Primer 3.0 software. Silver sharkminnow beta actin gene, used as endogenous
control (housekeeping gene), was amplified by the following primers – actin forward 5’-GAGCTATGAGCTCCCTGACGG-3’, and actin reverse 5’-AAACGCTCATTGCCAATGGT-3’. They were used to normalize variations in RNA (Table I).

After optimization, PCR reactions were performed in a 10-μL volume containing 2 μL cDNA, 5 μL SYBR mix (Applied Biosystems), 0.3 μL forward primer, 0.3 μL reverse primer, and 2.4 μL Double Destilled Water (DDW) using the following conditions: 95°C for 45 s, (45 cycles of 95°C for 15 s and 60°C for 1 min), then 95°C for 15 s, 60°C for 15 s, and 95°C for 15s. Relative fold change of gene expression was calculated using the ΔΔCt method. Elongation actin gene, a stable reference gene in silver sharkminnow (12), was used to normalize the Ct values of the target genes. Normalized qPCR data were log transformed prior to statistical testing.

**Measurement of testosterone levels by enzyme-linked immunoasorbent assays**

At the sampling points, 1 mL blood was taken from the caudal vein of each male. The blood was allowed to clot at room temperature for 15 min. The serum was aspirated by centrifugation at 3000 g for 10 min, then was kept at −20°C until further analysis. Serum testosterone (T2) levels were determined using DRG Testosterone ELISA (EIA-1559) according to the manufacturer’s instructions. Duplicates were provided for each measurement.

**Data analysis**

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., Chicago, IL, USA). A one-way analysis of variance followed by Tukey’s post-hoc test was used to test for significant differences in the data (p ≤ 0.05). The values are expressed as means ± standard error (SE).

**Results**

*GtH-Ia, GtH-IIa and GTH-IIb mRNA expression*

Expression levels of GtH-Ia mRNA in 8-week-old silver sharkminnow males were 0.52 to 55.61 (Figure 1). The peak level of GtH-Ia mRNA expression (55.61) was recorded after 8 weeks’ treatment in the control group (PK; P < 0.05). Expression of mRNA in the control group was increased based on treatment period (P < 0.05). The expression of GtH-Ia mRNA in HgCl-treated groups and the control group at the

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**Table I. The primer used to amplify the GtH genes and their PCR products.**

| No. | Name/Primer Codes          | DNA Sequence (primer)             | Tm  | PCR Product (bp) |
|-----|-----------------------------|-----------------------------------|-----|-----------------|
| 1.  | Forward G1a real time (F2)  | GCGCTTCGTGTGTATGTTGA              | 62.81| 175             |
| 2.  | Reverse G1a real time (R2)  | CATTGACCGAGGAGTCTCG               | 62.83|                 |
| 3.  | Forward GIIa real time (F3) | TGGATGAGGAGGTGCAAC               | 59.89| 200             |
| 4.  | Reverse GIIa real time (R3) | CATCATTGAGAAGACCCGT              | 59.97|                 |
| 5.  | Forward GIIb real time (F3) | AGCTCTATTCTTCCACCTGT              | 61.23| 150             |
| 6.  | Reverse GIIb real time (R3) | AAGACTGTGAGTCGAGCAGG              | 62.34|                 |
| 7.  | Forward Actin (FA)          | GAGCTGAGCTTCCCTGACGG              | 58.3 | 53              |
| 8.  | Reverse Actin (RA)          | AAAAGCTTGTGAGTTGCAATGTTGA         | 55.6 |                 |

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![Figure 1. mRNA levels of GtH-Ia of male silver sharkminnow kept under HgCl treatment for 8 weeks. (C = control, T1 = 0.025 mg/L, T2 = 0.05 mg/L, T3 = 0.1 mg/L, W = weeks, * = significantly different).](image-url)
second week and fourth week were not significantly different (P > 0.05). The GtH-Ia mRNA expression of the control group was found to be higher than in the HgCl-treated group at the sixth week and eighth week (P < 0.05).

The expression level of GtH-IIa mRNA was recorded at 0.39–54.20 (Figure 2). The peak level of GtH-IIa mRNA expression (54.20) was recorded after 8 weeks’ treatment in the control group (PK; P < 0.05). The expression of mRNA in the control group increased based on treatment period (P < 0.05). Expression levels of GtH-IIa mRNA in HgCl-treated groups and the control group at the second week and fourth week were not significantly different (P > 0.05). The GtH-IIa mRNA expression of the control group was found to be higher than that of the HgCl-treated group at the sixth week and eighth week (P < 0.05). This result of our study on GtH alpha gene expression level in male silver sharkminnow shows a correlation between bioaccumulation of mercury and gene expression level.

The expression level of GtH-IIb mRNA was recorded at 9.89–45.20 (Figure 3). The peak level of GtH-IIb mRNA expression (45.20) was recorded after 6 weeks’ treatment in the control group (PK; P < 0.05). The GtH-IIb mRNA of male sharkminnow had the same trend as the GtH alpha expression pattern: at the second and fourth weeks there were no significant differences between the control group and the HgCl-treated groups (P > 0.05). The expression of GtH-IIb showed a significant difference between control and treated groups at the sixth and eighth weeks. This result of our study on GtH beta gene expression level in male silver sharkminnow shows a correlation between bioaccumulation of mercury and gene expression level.

**Testosterone serum levels**

The testosterone serum concentrations in male sharkminnow treated with HgCl after 2, 4, 6 and 8 weeks are illustrated in Figure 4. Testosterone serum level in male sharkminnow showed no significant difference

Figure 2. mRNA levels of GtH-IIa of male silver sharkminnow kept under HgCl treatment for 8 weeks. (C = control, T1 = 0.025 mg/L, T2 = 0.05 mg/L, T3 = 0.1 mg/L, W = weeks, * = significantly different).

Figure 3. mRNA levels of GtH-IIb of male silver sharkminnow kept under HgCl treatment for 8 weeks. (C = control, T1 = 0.025 mg/L, T2 = 0.05 mg/L, T3 = 0.1 mg/L, W = weeks, * = significantly different).
between control and HgCl-treated groups in the second week, but showed more significant differences after 4–8 weeks (P < 0.05). This result of our study of testosterone serum level in male silver sharkminnow shows a correlation between bioaccumulation of mercury and testosterone serum level.

**Gonadosomatic index (GSI)**

The percentage of GSI in male sharkminnow treated with HgCl after 2, 4, 6 and 8 weeks is illustrated in Figure 5. The GSI levels in control and HgCl-treated groups after the fourth and eight weeks were statistically different (P < 0.05). The HgCl-treated groups showed a significantly lower GSI level compared to the control group (P < 0.05). This result of our study on GSI level on male silver sharkminnow showed a correlation between bioaccumulation of mercury and GSI level. The GSI level of male silver sharkminnow decreases as mercury bioaccumulation level increases.

**Discussion**

This study analyzed the effect of endocrine disruption-based mercury treatment on GtH-Ia, GtH-IIa and GtH-IIb gene expression, testosterone level and GSI in male silver sharkminnow. This study confirms previous studies showing effects of mercury treatment on male silver sharkminnow, that found a decrease in Grh alpha and Grh beta gene expression.
expression, testosterone serum, and GSI levels after eight weeks of treatment. This study shows that GrH alpha and GrH beta gene expression, testosterone serum, and GSI levels decreased as the mercury concentration increased. Mercury was proven to inhibit synthesis of GrH protein and secretion of testosterone in male silver sharkminnow. Mercury poisoning effects in fish include elevated death rate, weight loss, larval deformation, depressed protein synthesis, hormonal imbalance, abnormal behavior, spawning reduction, sexual disfunction and color changes (Friedmann et al. 1996; Oliviera Robeiro et al. 2002; Dervnick & Sandheinrich 2003; Houck & Cech 2004; Devline 2006). Mercury acts to inhibit transcription factors for gene expression, resulting in depressed protein synthesis process. Mercury also mimics the effects of endogenous hormones, such as estrogen and androgens, antagonizing the effects of endogenous hormones, altering the pattern of synthesis and metabolism of normal hormones, and modifying hormone receptor levels (Duffaud et al. 1985; Dervnick & Sandheinrich 2003). The results of this study show that HgCl can have an effect on the hypothalamic–pituitary–reproductive axis by altering the transcription of key genes involved in reproductive physiology even before a fish reach its sexual maturity. GrH-I and GrH-II are genes that control gametogenesis (Litwack & Schmidt 2002). GrH-I plays a role in the early stages of gametogenesis (oogenesis and spermatogenesis), whereas GTH plays a role in maturation and ovulation (Kawauchi et al. 1989). Gonadotrophic hormone plays a role in the regulation of hypothalamic and pituitary activity for reproductive cycles (Yaron 1995; Volkoff & Peter 1999; Matsuwaki et al. 2006). Testosterone production in fishes was decreased after 4–8 weeks as the level of bioaccumulation of mercury in fishes increased. The Leydig cells are a major source of testosterone (T2) in male fishes. The GrH receptor level in follicular cells can also be altered by mercury, causing a decrease in testosterone production (Friedmann et al. 1996). Testosterone leads maturation in testes. The result of testosterone activity is an increase in size and weight of the testes as well as an increase in reproductive hormone produced by the testes (Figure 5). High levels of mercury alter the regulation of testosterone, testosterone receptors, and heat shock proteins 50, 70, and 90 by binding testosterone reception in the cell. The cause of mercury interference might be a genomic action of testosterone in the transcription response for protein production.

Testosterone is known to be a factor in the progression of the spermatogenesis process. The testosterone level gradually increases as spermatogenesis progresses, exceptionally 11α testosterone (Houck & Cech 2004; Schulz & Nobrega 2012). The level of testosterone circulating inside fish increases in parallel with maturation of fish, as shown by the level of GSI and spermatogenesis progression (Thomas & Stacia 2005). Testosterone inhibition results in decreased GSI level and maturation progress. Therefore, mercury acts as an endocrine disruptor of spermatocyte development in male silver sharkminnow.

**Conclusion**

In summary, the results of this study show that mercury acts as an endocrine disruptor and has a toxic effect on reproductive performance in male silver sharkminnow. Fishes that were treated and exposed to the HgCl (mercury) showed a depressed expression of Gth-Ia, GtH-IIa and GtH-IIb genes, decreased secretion of testosterone, and disrupted gonad activity and final maturation of the testes.

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