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Waterborne copper disrupts circadian rhythm in red seabream (*Pagrus major*)

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ABSTRACT

We investigated the effect of copper (Cu) on circadian rhythms in red seabream, *Pagrus major*, under various concentrations of Cu (10, 20, 30 and 40 µg/L). To examine variability in circadian rhythms, we measured changes in the period 2 (Per2), cryptochrome 1 (Cry1), serotonin and arylalkylamine N-acetyltransferase (AANAT2) proteins. We found that circadian rhythm-related plasma proteins were significantly lower in a high-Cu environment (30–40 µg/L) than in low-Cu concentration (0, 10, or 20 µg/L). Our results indicate that environmental Cu at concentrations greater than 30 µg/L can have deleterious effects on fish circadian rhythms.

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Copper; circadian rhythms; clock proteins; *Pagrus major*

Introduction

The concentrations of heavy metals in seawater have risen in recent years due to outflow of industrial and domestic wastewaters (Karadede & Ünlü 2000; Chen et al. 2012), and concentrations of essential trace elements required for biological metabolism such as copper (Cu), zinc (Zn) and iron (Fe) are increasing accordingly (Canli & Atli 2003; Tuzen 2009). When Cu is present in small amounts, it acts as a cofactor for essential enzymes such as phosphofructokinase, pyruvate kinase, lactate dehydrogenase and glycose-6-phosphate dehydrogenase, and acts as an essential trace element *in vivo* (Watanabe et al. 1997; Dos Santos & Fernandes 2008). In high concentrations, copper can be toxic in marine environments, accumulating mainly in the liver and gill in fish and negatively affecting physiological functions such as tissue repair, osmotic pressure control, antioxidant ability and growth regulation (Tellis et al. 2012; Luzio et al. 2013). When fish are exposed to such environmental stressors, physiological functions such as antioxidant ability and immunity can be adversely affected (Grim et al. 2010; Choi et al. 2016), and circadian rhythms are among these (Choi et al. 2014; Rhee et al. 2014).

Many organisms have circadian rhythms regulating physiological, biochemical and behavioural functions over a 24-h cycle (King & Takahashi 2000). Period 2 (Per2) and cryptochrome 1 (Cry1) are representative clock protein regulating these circadian rhythms which are expressed in response to light exposure (Klein 2007; Takemura et al. 2008; Shin et al. 2014). Their corresponding proteins bind to one another to form protein complexes (Besharse

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et al. 2004; Choi et al. 2014). The arylalkylamine N-acetyltransferase (AANAT) enzyme is also a factor in controlling circadian rhythms. AANAT2 is a rate-limiting enzyme regulating melatonin synthesis from 5-hydroxytryptamine (serotonin), a neurotransmitter secreted in the pineal gland and retina (Arendt 1998; Klein et al. 2002; Iuvone et al. 2005). Depolarisation occurs in light receptors during night time, allowing the entry of Ca^{2+}, which activates adenylyl cyclase to result in an increase in cyclic AMP (cAMP). In turn, this increases intracellular activity through increasing phosphorylation of AANAT2 by cAMP. A second function of AANAT2 regulates the hypothalamus–pituitary–interregnal gland axis (HPI axis) to stimulate cortisol secretion, as a response to stress (Lim et al. 2013).

Therefore, this study has been carried out to determine the impact on the circadian rhythm of aquatic organisms according to the water pollution due to water within the copper, which gradually increases. In order to do this, we measured the plasma concentrations of Per2, Cry1, serotonin and AANAT2 at various Cu concentrations in the red seabream over intervals of 4 h for 36 h.

Materials and methods

Experimental fish

Red seabream, *Pagrus major*, was supplied from a fish farm in Tong-yeong, Korea, at a size of 18.5 ± 1.5 cm in length and 127.2 ± 0.18 g in mass. The fish were allowed to acclimate for 1 week in eight 300 L circulation filter tanks in the laboratory. A constant temperature was maintained at 20 °C using an automated regulatory system (JS-WBP-170RP; Johnsam Co., Seoul, Korea). Salinity was maintained at 35, and photoperiod followed a 12 h light/12-h dark cycle under a white fluorescent 27 W bulb.

Cu treatment and sampling

Four tanks containing 30 individuals apiece were used for to test circadian rhythm response to Cu, and a fifth tank was kept Cu-free as a control. Experimental groups were treated with waterborne copper (II) sulphate pentahydrate (Cu, $CuSO_4 \cdot 5H_2O$, 7758-99-8; Sigma-Aldrich, St. Louis, MO, USA) at concentrations of 10, 20, 30 and 40 µg/L. Blood was drawn from three fish from each tank at the beginning of the experimental period, and at 4, 8, 12, 16, 20, 24, 32 and 36 h. Prior to blood sample collection, fish were anesthetized using 200 µg/L 2-phenoxyethanol (Daejung Chemicals & Metals Corporation Ltd., Siheung, Gyeonggi, Korea) to minimise stress prior to blood collection. Blood was collected from the caudal vein using a 3 mL heparin-coated syringe. Plasma was separated by centrifugation at 1000 *g* for 10 min at 4 °C, and stored at −80 °C for further analysis.

Plasma parameter analysis

The enzyme-linked immunosorbent assay (ELISA) is a technique for quantitatively detecting proteins using an antigen-antibody reaction. The centrifuged plasma sample from red seabream was examined using ELISA kits for Per2, Cry1, serotonin, and AANAT2 levels (Per2, MBS9368536; Cry1, MBS041774; serotonin, MBS012786; AANAT2, MBS021281; Mybiosource Inc., San Diego, CA, USA). Absorbance was read at 450 nm, and the concentration was interpolated from a standard curve.

Statistical analysis

All data were analysed using SPSS version 10.0 (SPSS Inc., USA). A one-way ANOVA followed by Tukey's *post hoc* test was used to compare differences in the data, with differences considered significant at $p < 0.05$. Values are expressed as means ± standard error (SE).

Results

Changes in plasma levels of Per2

Plasma Per2 level was significantly lower under treatment with 30 and 40 μg/L Cu than under 0, 10 and 20 μg/L Cu ($p < 0.05$) (Figure 1). In addition, Per2 levels tended to be higher during photophase than during scotophase.

Changes in plasma levels of Cry1

Plasma Cry1 level was significantly lower under treatment with 30 and 40 μg/L Cu than under 0, 10 and 20 μg/L Cu ($p < 0.05$) (Figure 2). Cry1 levels tended to be higher during photophase and decreased in scotophase.

Changes in plasma levels of serotonin

Plasma serotonin level was significantly lower under treatment with 30 and 40 μg/L Cu than under 0, 10 and 20 μg/L Cu ($p < 0.05$) (Figure 3). In addition, serotonin levels tended to be higher during photophase compared to scotophase.

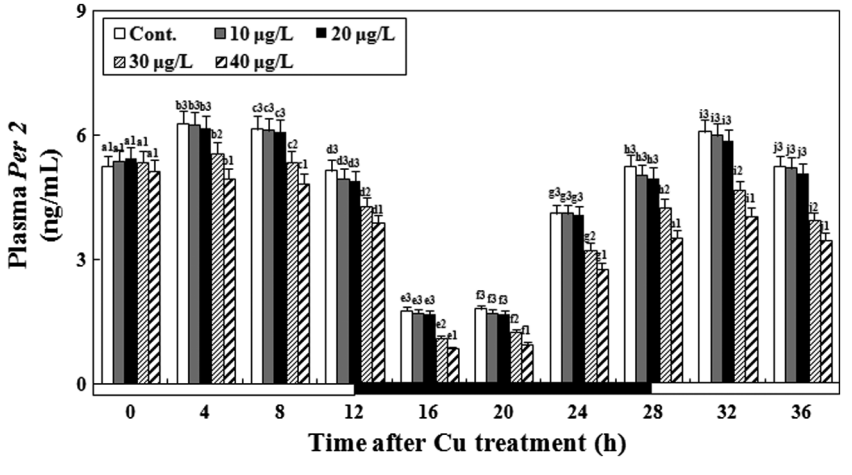

Figure 1. Change in plasma Per2 levels in red seabream during exposure to Cu at 0, 10, 20, 30, and 40 μg/L in red seabream, as measured using a microplate reader.

Notes: The white bar represents photophase and the black bar represents scotophase. Lowercase letters indicate significant differences among the different exposure periods at the Cu concentrations ($p < 0.05$). Numbers following the letters indicate significant differences among the different parameter values at the same Cu concentration and exposure period ($p < 0.05$). All values are means ± SE ($n = 5$). The white bar represents photophase and the black bar represents scotophase.

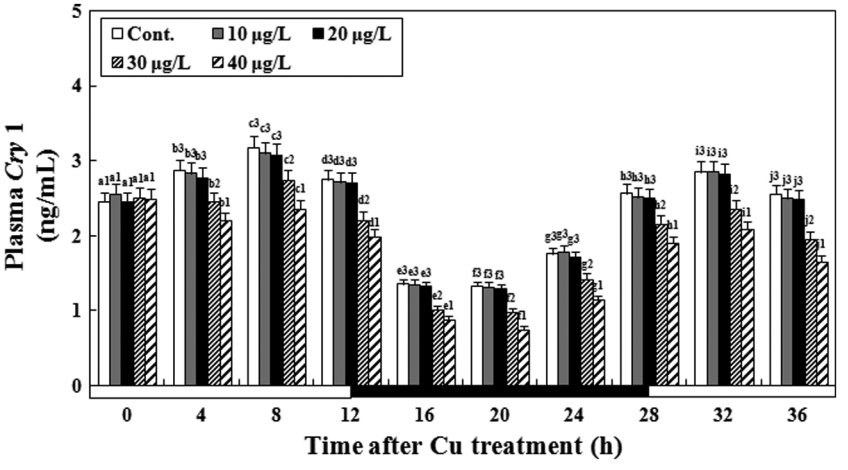

Figure 2. Change in plasma Cry1 levels in red seabream during exposure to Cu at 0, 10, 20, 30, and 40 μg/L in red seabream, as measured using a microplate reader.
Notes: The white bar represents photophase and the black bar represents scotophase. Lowercase letters indicate significant differences among the different exposure periods at the Cu concentrations ($p < 0.05$). Numbers following the letters indicate significant differences among the different parameter values at the same Cu concentration and exposure period ($p < 0.05$). All values are means ± SE ($n = 5$).

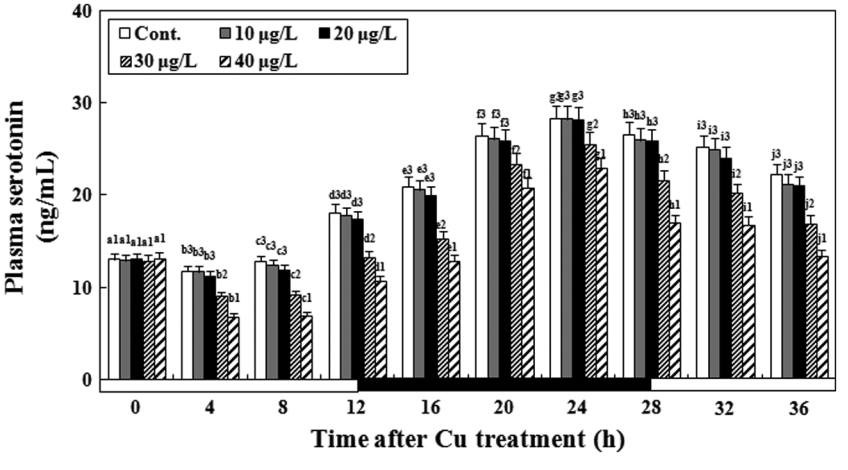

Figure 3. Change in plasma serotonin levels in red seabream during exposure to Cu at 0, 10, 20, 30, and 40 μg/L in red seabream, as measured using a microplate reader.
Notes: The white bar represents photophase and the black bar represents scotophase. Lowercase letters indicate significant differences among the different exposure periods at the Cu concentrations ($p < 0.05$). Numbers following the letters indicate significant differences among the different parameter values at the same Cu concentration and exposure period ($p < 0.05$). All values are means ± SE ($n = 5$).

Changes in plasma levels of AANAT2

Plasma AANAT2 was significantly lower under treatment with 30 and 40 μg/L Cu than under 0, 10 and 20 μg/L Cu ($p < 0.05$) (Figure 4). AANAT2 levels were higher during photophase compared to scotophase.

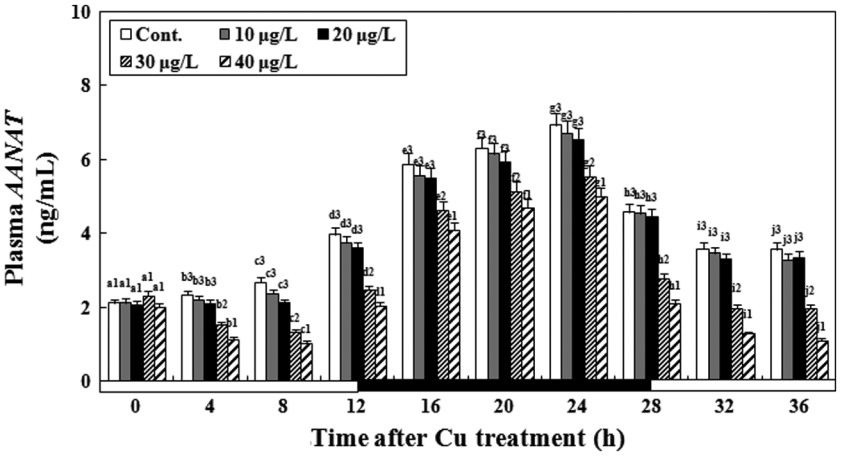

Figure 4. Change in plasma AANAT2 levels in red seabream during exposure to Cu at 0, 10, 20, 30, and 40 μg/L, as measured using a microplate reader.

Notes: The white bar represents photophase and the black bar represents scotophase. Lowercase letters indicate significance differences among the different exposure periods at the Cu concentrations ($p < 0.05$). Numbers following the letters indicate significant differences among the different parameter values at the same Cu concentration and exposure period ($p < 0.05$). All values are means ± SE ($n = 5$).

Discussion

To investigate changes in the circadian rhythm of red seabream with Cu exposure, we analysed changes in two circadian rhythm-regulating proteins, Per2 and Cry1 in blood plasma. We found that the concentration of Per2 and Cry1 in plasma was significantly higher during scotophase, and significantly lower when Cu concentration was above 30 μg/L. A similar study was conducted by Jung et al. (2016), measuring plasma Per2 concentration after exposure of goldfish (*Carassius auratus*) to ammonia at 0.25 and 0.5 mg/L. Per2 concentration in plasma was significantly lower in all experimental groups exposed to ammonia, and lowest under exposure to the higher concentration. Similarly, Rhee et al. (2014) exposed mangrove killifish (*Kryptolebias marmoratus*) to the environmental hormone bisphenol A at 600 μg/L and the surfactant 4-tert-octyphenol at 300 μg/L, which act in a high-concentration which is toxic, such as copper. Here, the mRNA transcription of Per2 and Cry1 genes was significantly lower than in the control group.

Therefore, comparing the results of this study with previous studies, Cu concentrations above 30 μg/L are suggested to be a concentration that disturbs the secretion of clock protein in the red sea bream.

We also examined Cu effects on serotonin and AANAT2. Serotonin is produced during scotophase in the retina and pineal glands and converted to N-acetylserotonin by AANAT; in turn, this is converted to melatonin, which known as circadian rhythm regulator at scotophase. Our results demonstrated higher plasma serotonin levels during scotophase than during photophase, and significantly lower serotonin levels in fish exposed to 30–40 μg/L Cu.

In a similar study, Tsai et al. (1995) showed that plasma serotonin levels were significantly reduced when tilapia (*Oreochromis mossambicus*) were exposed to mercury concentrations (15 and 30 μg/L), which acts as toxic and disturbed in fish body such as Cu, with serotonin

decreasing under high concentration of mercury. Mercury and other heavy metals are toxic to the brain and interfere with the normal function of the Na^+ and Ca^{2+} channels in the serotogenic system and cell membrane. We suggest that Cu acts similarly, with levels above 30 µg/L becoming toxic to red seabream. We suggest that this may occur due to the cause of functional disturbance in the central nervous system and cell membrane ion-channels to lead to a decrease in plasma serotonin levels.

Furthermore, we found that plasma AANAT2 was significantly higher during scotophase than in photophase, but significantly lower when the Cu concentration was above 30 µg/L. A similar study was conducted by Preud'homme et al. (2015), when African clawed frogs (*Xenopus laevis*) were exposed to the insecticides endosulfan (0.1 and 1 µg/L), which act toxic in a high concentration such as copper. AANAT mRNA expression level was found to be significantly lower under endosulfan exposure compared with the control group, with the greater level of exposure yielding lower AANAT transcription. Similarly, the results of our study suggest that exposure to copper at levels of over 30 µg/L can have a negative effect on seabream circadian rhythm, causing a reduction in plasma AANAT2.

We performed this study to determine the concentration of copper acting as toxic (= internal disturbance). In conclusion, Cu concentration above 30 µg/L disturbs the secretion of circadian rhythm-related hormones such as Per2, Cry1, serotonin and AANAT2, potentially adversely affecting physiological functions such as growth and reproduction of red seabream. The results of this study may be used as the basis for future studies on the effects of Cu water pollution gradually increasing on marine life. In addition, research on various heavy metals and essential trace elements as well as copper is needed.

Disclosure statement

No potential conflict of interest was reported by the authors.

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