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Hormonal and ion regulatory response in three freshwater fish species following waterborne copper exposure

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ABSTRACT

We evaluated effects of sublethal copper exposure in 3 different freshwater fish: rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*) and gibel carp (*Carassius auratus gibelio*). In a first experiment we exposed these fishes to an equally toxic Cu dose, a Cu level 10 times lower than their 96 h LC_{50} value: 20, 65, and 150 μg/L Cu. In a second series we exposed them to the same Cu concentration (50 μg/L). Na^+/K^+-ATPase activity in gill tissue was disturbed differently in rainbow trout then in common and gibel carp. Rainbow trout showed a thorough disruption of plasma ion levels at the beginning of both exposures, whereas common carp and gibel carp displayed effects only after 3 days. Rainbow trout and common carp thyroid hormones experienced adverse effects in the beginning of the exposure. The involvement of prolactin in handling metal stress was reflected in changes of mRNA prolactin receptor concentrations in gill tissue, with an up regulation of this mRNA in rainbow trout and a down regulation in gibel carp, which was more pronounced in the latter. Overall, rainbow trout appeared more sensitive in the beginning of the exposure, however, when it overcame this first challenge, it handled copper exposure in a better manner then common and gibel carp as they showed more long term impacts of Cu exposure.

1. Introduction

Long and frequent use of metals by mankind, have led to a widespread release of these toxicants in aquatic systems. As the exposed organisms try to cope with this pollution, they develop species-specific defense mechanisms, which are rarely fully apprehended. For instance, fish are able to develop a copper (Cu) tolerance after sublethal Cu exposure in laboratory settings (Grosell et al., 2001, 2002; Hashemi et al., 2008) as well as in their natural habitat (Gale et al., 2003). However, not all fish species handle elevated Cu concentrations equally adequately. For example, rainbow trout appeared to be three times more sensitive to Cu exposure than common carp, and almost seven times more sensitive than gibel carp (De Boeck et al., 2004).

Considering Cu is an essential element, organisms build up homeostatic mechanisms and strictly regulate free Cu concentrations. When Cu concentration augments to a toxic range, oxidative stress, DNA damage and a disrupted ion osmo homeostasis will occur (Arabi, 2004; Arabi and Alaeddini, 2005; Bury et al., 1998; Bopp et al., 2008; Gravato et al., 2006; Grosell et al., 2002; Handy, 2003; Wood, 2001). Previous research revealed that Cu possibly targets sodium and chloride transport systems in the gills of freshwater animals. A reduction in branchial sodium uptake occurs by inhibiting the Na^+/K^+-ATPase by nonspecific binding to thiol groups on the subunits of the transporter (Lauren and McDonald, 1987; Li et al., 1998), and by binding to the Mg^{2+} binding site, as well as competitive inhibition at the apical Na^+-channel (Grosell and Wood, 2002; Pyle and Wood, 2008). Increases of sodium loss caused by a displacement of calcium by copper in the tight junctions, leading to changes in permeability, is another effect caused by Cu exposure in freshwater fish. These changes result in a net loss of Na^+, an increase in blood viscosity and blood pressure, a compensatory tachycardia and, when exposed to acute toxic concentrations, cardiac failure (Evans et al., 1999; Handy et al., 2002; Jorgensen, 2008; Lauren and McDonald, 1985; Lingwood et al., 2005; Lingwood et al., 2006; Niyogi et al., 2006; Pyle et al., 2003; Wilson and Taylor, 1993). Within these processes, it seems that the rate of Na^+ loss is more important in determining the sensitivity of a species than the amount of Na^+ lost (Grosell et al., 2002; Pyle and Wood, 2008).

Controlling ion osmoregulation, hormones such as cortisol, prolactin (PRL), growth hormone and thyroid hormones, are of outmost significance in dealing with the effects of these toxicants. PRL, produced in the pituitary gland, is active in a broad spectrum. This hormone is most characterized by its function in water and electrolyte balance (Sangiao-Alvarellos et al., 2006), yet it is equally essential for metabolism, growth, development, reproduction, behavior and immunoregulation (Power, 2005). In a freshwater environment, fish are forced to prevent loss of ions to the external hypo

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osmotic conditions and inhibit the influx of water. PRL's ability to increase plasma ion concentrations and decrease the permeability of osmoregulatory surfaces to water, plays an important role in adapting to these circumstances (Manzon, 2002). PRL initiates its actions through binding to a specific cell surface PRL receptor (PRLR). Generally, PRLR mRNA is found to be heavily expressed in osmoregulatory organs such as gills, kidney and intestine of different fish species (Lee et al., 2006; Le Rouzic et al., 2001; Sandra et al., 1995; Tse et al., 2000).

In teleosts, thyroid hormones are synthesized, stored, and released from thyroid follicles, which are distributed in a diffuse pattern on the surface of the ventral aorta and other sub- and parapharyngeal areas (Swapna and Senthilkumaran, 2007). The major hormone synthesized in these follicles is L-thyroxin (T4), which is metabolized to the more biologically potent 3,5,3′-triiodo-L-thyronine (T3) by outer ring deiodination via deiodinases, mostly in peripheral tissues. Extensive evidence indicates that most thyroid hormone effects are mediated at the genomic level via binding to the nuclear thyroid hormone receptors (TRs), of which there are two forms (TRα and TRβ). The expression patterns share some common features (high expression in pituitary and brain), however, TRβ shows higher expression in the muscle and gill compared to TRα (Filby and Tyler, 2007; Power et al., 2001; Swapna and Senthilkumaran, 2007). Thyroid hormones are involved in development and metabolism (Hontela et al., 1995; Nelson and Habibi, 2008; Zoeller et al., 2007).

Since the gill is one of the major sites for control of ion and water exchange and the first organ to accumulate metals under metal exposure due to its extensive surface area and direct contact with the external environment (Manzon, 2002), it is the best suited tissue to investigate species-specific differences in handling Cu exposure. The present study aimed at unraveling relationships between plasma ion concentrations, plasma T3/T4 concentrations, gill Na^+/K^+-ATPase activity and gill mRNA concentrations of PRL receptors and thyroid hormone receptor β in rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*) and gibel carp (*Carassius auratus gibelio)* after exposure to either the Flemish standard of surface water (50 µg/L Cu) or an equally toxic dose of 10% of the LC_{50} 96 h value, previously determined (De Boeck et al., 2004). These diverse exposures will give us an insight in how these fish species manipulate their hormones and ion osmoregulatory processes when introduced to a similar concentration of a toxicant or a concentration corresponding with a similar toxic load.

2. Materials and methods

2.1. Animal maintenance and copper exposure

Rainbow trout (*O. mykiss*), common carp (*C. carpio*) and gibel carp (*C. auratus gibelio*) were obtained from a fish farm at Luc and Patrick Bijnens, Zonhoven, Belgium. Average mass of rainbow trout was 296 ± 46 g, of common carp 231 ± 85 g, and of gibel carp 224 ± 96 g.

Fish were kept at the University of Antwerp in aquaria (200l) for at least a month before the exposure started. Tap water was filtered with an open trickling filter, consisting of filter wadding, activated charcoal and lava stones. Those lava stones provide a place for nitrifying bacteria that remove ammonia and nitrites from water. Experiments were conducted at 17 ± 1 °C in a flow through system that renewed total water volume of the aquaria each 7 h. Fish were fed *ad libitum* once a day. Water quality was checked every day: oxygen concentration stayed well above 90% saturation, hardness was 250 mg $CaCO_3$/L and pH 7.6 ± 0.2. Ammonia, nitrate and nitrite levels were kept below toxic concentrations (<0.1 mg/L). Cu was added manually at the start of the experiment as a copper nitrate solution ($Cu(NO_3)_2 \cdot 2H_2O$, Merck, Darmstadt, Germany) to the exposure aquaria. The desired concentration was maintained by using a peristaltic pomp (Watson Marlow 505 S) connected to a stock solution of Cu, for the duration of the entire experiment. Each fish species was exposed to the Flemish standard of surface water (50 µg/L) and to 10% of the 96 h LC_{50} value (20 µg/L for rainbow trout, 65 µg/L for common carp and 150 µg/L for gibel carp). Cu concentrations were measured using atomic absorption spectroscopy.

2.2. Sampling procedures

Eight fish of each fish species and each condition were sampled after 1 h, 12 h, 24 h, 3 days, 1 week and 1 month. Fish were quickly netted, anaesthetized in a buffered MS222 solution (100 mg/L Sigma-Aldrich Chemical, St. Louis, MO, USA) weighed and measured. A blood sample was collected from the caudal blood vessel using Li-heparinised 1 mL syringes. Blood was immediately centrifuged for 3 min in Li-heparinised 1.5 mL microcentrifuge tubes. Plasma was transferred to a fresh cryovial, flash frozen in liquid nitrogen and stored at −80 °C until $[Na^+]$, $[Cl^-]$, $[Ca^{2+}]$, [T3/T4] analysis. Gill tissue was dissected on ice, rinsed and flash frozen in liquid nitrogen followed by storage in −80 °C for determination of Na^+/K^+-ATPase activity and mRNA concentration of PRL and TH receptors.

2.3. Na^+/K^+-ATPase activity in gill tissue

Frozen gill filament samples were thawed and homogenized for determination of Na^+/K^+ ATPase activity using the method of McCormick (1993). Briefly, homogenization was carried out in a SEID buffer (10 mM EDTA, 150 mM sucrose, and 50 mM imidazole, with 0.1% w/v sodium deoxycholate). The samples were centrifuged for 1 min at 4 °C and 5000 g. The supernatant was used for further determination. Using a 96-well microplate, 10 µL of the resultant supernatant was run in duplicate with assay A mixture (400 U Lactate dehydrogenase, 500 U pyruvate kinase, 2.8 mM phosphoenolpyruvate, 0.7 mM ATP, 0.22 mM NADH, 50 mM imidazole) and in parallel, 10 µL of the supernatant in duplicate with assay B mixture (contains assay A with 0.4 mM ouabain). Using a spectrophotometer (Ultra microplate reader $ELX808_{IU}$ Bio-Tek Instruments Inc., VT, USA) at 340 nm, absorbance was measured. By subtracting the oxidation rate of NADH to NAD in the presence of ouabain from that in the absence of ouabain, the Na^+/K^+-ATPase activity was determined. The associated protein content was assayed using the technique of Bradford (1976).

2.4. Ion concentration in plasma

Plasma $[Na^+]$, $[Cl^-]$ and $[Ca^{2+}]$ were analyzed using an AVL 9180 Electrolyte Analyser (AVL, Roche Diagnostics, Belgium).

2.5. Radioimmunoassay for thyroid hormones in plasma

Radioimmunoassays were performed to determine plasma $[T_3]$ and $[T_4]$ as previously described by (Van der Geyten et al., 2001). All plasma samples were measured within a single assay and thyroid hormone levels were expressed as pmol/mL.

2.6. Real Time Reverse Transcriptase PCR analysis

2.6.1. Homogenization and mRNA extraction

For disruption, frozen gill tissue of all fish species and exposure conditions was grinded to a fine powder in liquid nitrogen, using mortar and pestle. The tissue was kept in frozen state using liquid nitrogen. The suspension was transferred into a cooled tube, allowing the liquid nitrogen to evaporate without allowing the sample to thaw. Lysis buffer was added and homogenization was performed using the homogenization kit of Qiagen (Qiashredder Cat no. 79654). Subsequently, Rneasy Mini kit from Qiagen (Cat. no 74104) was used to extract mRNA. Concentration was determined by measuring absorbance at 260 nm