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High environmental ammonia elicits differential oxidative stress and antioxidant responses in five different organs of a model estuarine teleost (*Dicentrarchus labrax*)

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ABSTRACT

We investigated oxidative status and antioxidant profile in five tissues (brain, liver, gills, muscle and kidney) of European sea bass (*Dicentrarchus labrax*) when exposed to high environmental ammonia (HEA, 20 mg/L ~ 1.18 mM as NH_4HCO_3) for 12 h, 2 days, 3.5 days, 7.5 days and 10 days. Results show that HEA triggered ammonia accumulation and induced oxidative stress in all tissues. Unlike other organs, hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) accumulation in liver were restored to control levels. This recovery was associated with a concomitant augmentation in superoxide dismutase (SOD), catalase (CAT), components of glutathione redox cycle (glutathione peroxidase GPX, glutathione reductase, reduced glutathione), ascorbate peroxidase activity and reduced ascorbate content. On the contrary, in brain during prolonged exposure many of these anti-oxidant enzymes were either unaffected or inhibited, which resulted in persistent over-accumulation of H_2O_2 and MDA. Branchial and renal tissue both involved in osmo-regulation, revealed an entirely dissimilar compensatory response; the former rely mainly on the ascorbate dependent defensive system while the glutathione catalytic pathway was activated in the latter. In muscle, GPX activity first rose (3.5 days) followed by a subsequent drop, counterbalanced by simultaneous increment of CAT. HEA resulted in a relatively mild oxidative stress in the muscle and kidney, probably explaining the modest anti-oxidative responses. Our findings exemplify that oxidative stress as well as antioxidant potential are qualitatively diverse amongst different tissues, thereby demonstrating that for biomonitoring studies the screening of adaptive responses at organ level should be preferred over whole body response.

1. Introduction

In culture based systems and in confined natural ecosystems such as enclosed bays and estuaries, ammonia levels can rise to unsafe levels as a consequence of sewage effluents, industrial wastes, agricultural run-off and decomposition of biological wastes. Moreover, the majority of teleosts are ammonioteles, and excrete most of their nitrogenous wastes as ammonia across the gills to the external milieu. A possible accumulation of metabolic waste products of fish can also contribute to a high ammonia load in the water (Boeuf et al., 1999). Waterborne ammonia exist in two forms, the unionized ammonia (NH_3) and the ionized form (NH_4^+), and the sum of NH_3 and NH_4^+ comprises the total ammonia concentration. Throughout this paper, the term 'ammonia' is used to refer to total ammonia. High environmental ammonia (HEA) is a worldwide concern as it may affect the performance of fish in several ways. HEA induces a range of ecotoxicological effects in fish, including a decrease in growth rate (Dosdat et al., 2003; Sinha et al., 2012a), alteration in energy metabolism (Arillo et al., 1981; Sinha et al., 2012a, 2015), disruption of ionic balance (Wilkie, 1997; Sinha et al., 2012b, 2014a, 2015; Diricx et al., 2013), alterations in hormone regulation (Knoph and Olsen, 1994; Dosdat et al., 2003), increase vulnerability to diseases, and even mortality. To protect against ammonia toxicity, various defensive strategies have been reported in freshwater and marine teleosts (Ip et al., 2001, 2004a,b). These include—minimize the ammonia production by suppression of amino acid catabolism, conversion of accumulated ammonia into free amino acids particularly glutamine, detoxification of ammonia to the less toxic urea, and augmentation of ammonia excretion by up-regulation of 'Na^+/NH_4^+ exchange complex' involving the Rh glycoproteins (Wright and Wood, 2009). Furthermore, some studies suggest that ammonia exerts the (cyto)toxic effects in fish by the production of reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), peroxyl ($ROO^{\bullet}$), and hydroxyl radicals ($^{\bullet}OH$) (Ching et al., 2009; Hegazi et al.,

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2010; Hegazi, 2011; Sun et al., 2011, 2012; Sinha et al., 2014b). ROS are generated as by-products of oxidative metabolism, and high production and accumulation of ROS could result in build-up of oxidized and damaged lipids and proteins in the cellular compartments, eventually inducing oxidative damage (Droge, 2003).

Cellular oxidative stress is established when the pro-oxidant forces overwhelm the antioxidant defenses, and this situation can damage biomolecules, such as lipids, proteins and DNA (Winston and Di Giulio, 1991; Kelly et al., 1998). Some ROS can initiate lipid peroxidation and this is considered one of the most prevalent mechanisms of cell damage (Halliwell and Gutteridge, 1990; Lushchak, 2011). Consequently, estimation of lipid peroxidation (in terms of malondialdehyde, MDA) has been found to have promising importance as a biomarker for oxidative stress (Lackner, 1998; Lushchak, 2011). In order to convert ROS to harmless metabolites as well as to protect and restore normal cellular metabolism and functions, animals including fish possess enzymatic and non-enzymatic antioxidant defense systems (Guerriero et al., 2002; Basha and Rani, 2003; Erdoğan et al., 2005). The key enzymes for the detoxication of ROS includes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), glutathione-s-transferase (GST), dehydroascorbate reductase (DHAR) and ascorbate peroxidase (APX) (Livingstone, 2001; Valavanidis et al., 2006; Kelley et al., 2010; Lushchak, 2011).

Ammonia is a potent neurotoxin that predominantly affects the brain tissue. Compared to other organs, brain is also extremely prone to oxidative damage as it contains high levels of polyunsaturated fatty acids (Dringen, 2000). In general, the susceptibility of organs to oxidative damage is dependent on their metabolic function, structural characteristics, their antioxidative arsenal and the different routes of exposure to pollutants. It is reported that in response to environmental stressors (e.g. metal pollution, starvation, hypoxia, anoxia, salinity reduction, heat shock and pesticide exposure), the anti-oxidant defense systems in fish are modulated differentially among various organs (Lushchak et al., 2001; Oruc et al., 2004; Lushchak and Bagnyukova, 2006, 2007; Ballesteros et al., 2009; Furné et al., 2009; Li et al., 2010a; Yin et al., 2011; Hao and Chen, 2012; Stara et al., 2012). Although there has been some evidence on the potential hazards of acute and chronic exposure of HEA on the oxidative status of fish, there are no detailed studies on the effects of ammonia on oxidative toxicity and antioxidant defense systems at the level of different organ systems. Consequently, we hypothesized that in response to ammonia exposure oxidative stress and countervailing response of enzymatic and non-enzymatic antioxidants systems can vary considerably between different organs. Therefore, in this comparative study we aimed to investigate the differential oxidative and antioxidant defense responses among different organs (brain, liver, gills, muscle and kidney) to evaluate which one was more severely affected by HEA exposure to fish. This will provide a better understanding of the specific protective strategies employed by these organs to sustain their normal metabolism when threatened with oxidative damage induced by high ammonia. Furthermore, the antioxidant defense response has a potential applicability as biochemical biomarker for contaminant-mediated oxidative stress, and could also be used as a monitoring tool for assessing the ecotoxicological impact of environmental stressors (Ballesteros et al., 2009; Hao and Chen, 2012).

To accomplish our aims, a set of pro-oxidant status indicators were assessed by quantifying the contents of MDA, H_2O_2 and the activity of xanthine oxidase (XO) in various organs with a parallel investigation on ammonia accumulation. A comprehensive analysis of antioxidant defense system i.e., antioxidant molecules (reduced glutathione, GSH and ascorbate, ASC) and enzymes (SOD, CAT, APX, GPX, GR, DHAR and GST) was conducted to understand the tissue-specific dynamics of anti-oxidant defense system in response to ammonia pollution. We also aimed to examine an association between tissue ammonia accumulations and the response of oxidative stress markers.

Furthermore, since the implementation of the water framework directive in European Union countries, ammonia pollution in coastal ecosystem has increasingly been the focus of monitoring programs using fish as bioindicators. European sea bass is a marine teleost whose juveniles use both coastal and estuarine nurseries, and is one of the most preferred fish species for pollution studies associated with oxidative stress (Gwozdzinski et al., 1992; Roméo et al., 2000; Ahmad et al., 2008; Gravato and Guilhermino, 2009; Maria et al., 2009; Mieiro et al., 2011; Vinagre et al., 2012). This species is widely distributed throughout the Europe and is extensively used for aquaculture. It is therefore of great commercial and ecological importance. Consequently, in the present study we used juveniles of European sea bass (*Dicentrarchus labrax*) as a test organism. The concentration of ammonia-nitrogen (20 mg/L ~ 1.18 mM) used in the present study represents 50% of 96 h LC_{50} value for European sea bass (Person-Le Ruyet et al., 1995).

2. Materials and methods

2.1. Experimental system and animals

European sea bass (*D. labrax*) juveniles (14–18 g; 9–11 months old) were obtained from Ecloserie Marine (Gravelines, France). Fish were kept at the University of Antwerp in tanks (1000 L), filled with artificial seawater (Meersalz Professional Salt, 32 ppt salt) for at least a month. Thereafter, a total of 120 fish were distributed into four 200 L tanks ($n = 30$ per tank; 32 ppt) equipped with a recirculating water supply in a climate chamber where temperature was adjusted at 17 ± 1 °C and photoperiod was set at 12 h light and 12 h dark. Temperature can considerably influence the ammonia toxicity to aquatic animals; therefore, the temperature consistency (17 ± 1 °C) was monitored every day. Water quality was ensured through an additional bio-filter containing wadding, activated charcoal and lava stones. Fish were acclimated to the above mentioned constant salinity, temperature and photoperiod for 3 weeks prior to the experiment and were fed with commercial pellets (Skretting, Boxmeer, The Netherlands) at a rate of 2% of their wet body weight/day. All animal experiments were approved by the local ethics committee, University of Antwerp, and conducted according to the guidelines of the Federation of European Laboratory Animal Science Associations.

2.2. Exposure and sampling intervals

The experimental setup consisted of exposing the fish to 20 mg/L (~1.18 mM) ammonia-nitrogen for a period of 12 h and 2, 3.5, 7.5 and 10 days. The exposure was conducted in 8 L glass aquaria (water volume set to 6 L). Control groups (no HEA) were set up in parallel to each exposure group. The experimental aquaria were shielded with black plastic to minimize visual disturbance and fitted with individual air-stones.

Individual fish were placed in a glass aquarium the evening before an experiment and left overnight to settle with continuous aeration, and were subsequently fed daily at a rate of 2% of their wet body weight. The experimental protocols consisted of exposing 8 fish (in 8 separate aquaria) per experiment to HEA. Each exposure aquaria was spiked with the required amount of an NH_4HCO_3 stock solution (Sigma, Germany). A constant concentration of 20 ± 0.18 mg/L of ammonia-N was maintained throughout the experiment. Exposure ammonia concentrations were measured (using the salicylate-hypochlorite method, Verdouw et al., 1978) 6 h after the onset of treatment and the concentration of ammonia in the aquaria was maintained by adding calculated amount of the NH_4HCO_3 solution. Moreover, to avoid the microbial breakdown of test chemical and build-up of other waste products, 60–80% of the water was discarded after each 2–3 days and replaced with salt water (32 ppt) containing the respective amount of ammonia. Water pH was maintained at 8.0–8.1 throughout the experimental period using dilute HCl and/or KOH. At this experimental pH and temperature, the speciation of ammonia-N (the amount