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17β-Estradiol induces changes in cytokine levels in head kidney and blood of juvenile sea bass (*Dicentrarchus labrax*, L., 1758)

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ABSTRACT

The cytokine network is involved in the immune system communication. As estrogens influence the cytokine expression in mammals, this study investigated the impact of exogenous estrogenic pollutants on selected cytokines in *Dicentrarchus labrax*. The gene expression of Interleukin 6, Tumour Necrosis Factor α, Transforming Growth Factor β1 and Interleukin 1β was assessed and accomplished with protein measurements in the blood for the last two. Impacts through 17β-estradiol mainly occurred at the beginning of organ regionalisation, thus falling together with a developmentally induced increase of Interleukin 1β and Tumour Necrosis Factor α gene expression in 102 dph fish. 17β-estradiol depressed this modification after 35 days of exposure and the cytokine gene expression tended to be generally down-regulated independently of the 17β-estradiol concentrations after 56 days of exposure. This impact was confirmed at the protein level, showing that 17β-estradiol affects the fine control of the cytokine network in sea bass.

1. Introduction

To date, studies of the fish immune system have been primarily directed (I) to aquaculture applications to strengthen the immunocompetence in reared fish and (II) to phylogenetic aspects of fish immunology (Alvarez-Pellitero, 2008; Bird et al., 2006; Bricknell and Dalmo, 2005; Flajnik and Du Pasquier, 2004; Pancer and Cooper, 2006; Van Muiswinkel, 1991). But, since the immune system is a very sensitive indicator of sub-lethal insults, environmental pollutants may evoke direct or indirect effects on the pathogen defence capacity as well. Especially during larval and juvenile periods, which generally characterise very sensitive stages (Hutchinson et al., 1998), a weakening of immune defence and impairment of the development of immune relevant organs are likely to impact organism's health persistently. This underlines the importance of investigating possible modifications of the immune response at early stages.

Far from being fully investigated and understood, immune responses in fish, as in mammals, consist of two main axes: the innate and the adaptive immunity. Both are linked through bilateral interactions and a complex signalling system (Tort et al., 2003). Within these two fundamental lines of host defence, cytokines act as pleiotropic, multifunctional mediators (Jin et al., 2010). Being produced by different leukocyte types, they are the principal effectors of signal transduction between the different components of the immune system and orchestrate elements of innate and adaptive immunity, like the inflammation cascade, phagocytosis as well as lymphocyte development and proliferation. In addition, they regulate their synthesis in a reciprocal manner (Secombes et al., 1996). Therefore, cytokines constitute a favourable biological surrogate for immune system integrity. Already small changes in their fine-tuning can provoke changes in the immune response, as has been pointed out for humans (Straub, 2007). In fact, recent research in fish confirms that 17β-estradiol (E2) is involved in cytokine regulation during development (Jin et al., 2010).

E2 is commonly present between 1 and 50 ng/L in rivers and might reach concentrations up to 200 ng/L in sewage effluents (Desbrow et al., 1998; Pojana et al., 2007). It is introduced in the aquatic environment predominantly by livestock breeding and sewage effluents (Ahmad et al., 2009; Teles et al., 2006). Thus, lakes, rivers and estuaries represent most affected environments. For European sea bass (*Dicentrarchus labrax* L. 1758) estuaries serve as nursery grounds, and consequently, juvenile stages most likely experience higher levels of aquatic pollution than adults. A comprehensive body of knowledge on the development of the sea bass immune system has been provided by the research of Abelli et al. (1996a, b), Breuil et al. (1997) and dos Santos et al. (2000). In

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sea bass, the head kidney is the first immune organ to be developed. It appears around 10 days post hatching (dph) and provides immunoreactive cells from 35 dph on. The regionalisation of the organ into hematopoeitic, neuroendocrine and reticuloendothelial compartments starts about 98 dph and is completed around 238 dph. The literature mostly agrees upon the fact that *D. labrax* is immunologically mature at least 145 days post hatching (dph) when cultured at 16 °C (dos Santos et al., 2000). Thus, in sea bass, the head kidney appears to be the first primary immune organ to become mature. Additionally, it is possible to make use of known gene sequences of several cytokines, cytokine receptors and lymphocyte receptors (Nascimento et al., 2007; Picchietti et al., 2009, 2008; Pinto et al., 2007; Scapigliati et al., 2000) to investigate gene expression.

In this study, juvenile sea bass (60 dph) were exposed for 7, 35 and 56 days to different waterborne concentrations of E2 (0; 2; 20; 200 ng/L). Dose- and time-dependent effects of E2 were examined to determine critical E2 concentrations and possible minimal exposure periods. The experiment aimed at identifying particularly sensitive stages in the development of the head kidney by assessing possible disruptions of the cytokine mediation during immune system maturation of juvenile sea bass. The gene expression of four cytokines and the blood protein levels for two of them were investigated. The cytokines of particular interest were Interleukin 1β (IL-1β), Interleukin 6 (IL-6), Transforming Growth Factor β1 (TGF-β) and Tumour Necrosis Factor α (TNF-α). They are amongst the cytokines best investigated in fish and possess a high functional homology to their human counterparts (Harms et al., 2000). Furthermore, they are implicated in the organism's cell and organ development (Wride and Sanders, 1998, 1995). Briefly, IL-1β is released by macrophages (Pelegrín et al., 2001; Scapigliati et al., 2001). It interacts with TNF-α and induces the inflammation cascade. Moreover, IL-1β influences lymphocyte maturation and proliferation. IL-6 is essentially produced by stimulated monocytes/macrophages, fibroblasts and vascular endothelial cells in mammals. It enhances the monocyte recruitment and their differentiation into macrophages (Bird et al., 2005). Furthermore, it seems to influence the B-cell and T-cell differentiation (Savan and Sakai, 2006). IL-6 plays as well a role in the inflammation reaction through decreasing neutrophil infiltration. TNF-α, the third pro-inflammatory cytokine, regulates the lymphoid organogenesis and the induction of apoptosis (Zou et al., 2003). TGF-β, on the other hand, activates or deactivates, according to its concentration level, macrophages and monocytes in an anti-inflammatory manner (Letterio and Roberts, 1998). This leads to a down-regulation of the expression of monocyte/macrophage released cytokines, e.g. IL-1β and IL-6. In addition, TGF-β also affects the development of the organism by modulating cell proliferation and differentiation in various tissues (Gitelman et al., 1994). The selected cytokines belong to the main modulators of fish immune response. Hence, an impairment of expression and secretion of these cytokines could be crucial for head kidney development, a functional immune reaction as needed in pathogen control and to a lesser extend for individual survival. A reduced individual survival and impaired immunocompetence may eventually even imply negative effects on the population level. To the best of our knowledge, the present study is the first investigating the effect of environmentally relevant concentrations of E2 on cytokine gene expression and protein levels in fish in a chronic exposure scenario.

2. Material and methods

2.1. Animals

Sea bass (*D. labrax*, 60 dph, total length 4.03 ± 0.67 cm, weight 0.62 ± 0.36 g) were obtained from the aquaculture hatchery "L'écloserie marine de Gravelines" (Gravelines, France) and acclimated to experimental conditions for one week. 320 individuals were randomly distributed across eight aquaria (120 L), comprising two tank replicates per exposure concentration and control. The animals were treated in accordance to the European Convention for the protection of vertebrate animals used for experimental and other scientific proposes (Strasbourg: Council of Europe, 1986). The glass tanks were filled with 100 L artificial seawater and kept at 18 °C ± 0.5 in a semi-static condition with daily renewal of 70% of the water body. The system was equipped with an oxygen supply and a filtration system (eco pro, Eheim, Germany) to maintain water quality. General water quality parameters were measured daily (temperature, salinity, pH, oxygen) or every second day (nitrite, nitrate, ammonium). Fish were fed once daily on formulated feed (1% body weight; by courtesy of "L'écloserie marine de Gravelines"). The light cycle was set at 12 h:12 h.

2.2. Exposure and estrogenicity measurement

At the start of the experiment, the aquaria were charged with an appropriate amount of 17β-estradiol (Sigma, France), diluted in methanol (Distillery Hauguel, France), to meet the nominal exposure concentrations 0, 2, 20 and 200 ng/L. The concentrations were chosen in consideration of the values measured by Desbrow et al. (1998) in sewage effluents. The dosing was carried out every day after the water exchange. Water estrogenic activity, 24 h after dosing E2, was determined once a week with the yeast estrogen screen (YES) established by Routledge and Sumpter (1996) as modified after Denier et al. (2009). Briefly, the estrogenic compounds were extracted using Oasis HLB 6cc extraction cartridges (200 mg, Waters, France) from a 500 mL sample of each aquarium, resolved in 6 mL methanol (Sigma, France) and evaporated to 1 mL. The samples were diluted in ethanol in a serial dilution with a final concentration of $0.48*10^{-3}$. 10 mL of each sample dilution were transferred to a microplate and the ethanol was evaporated. Subsequently, 200 μL of growth medium, supplemented with genetically modified yeast and chlorophenol-red β-D galactopyranoside, were added in each well. An E2 standard dilution series and a negative control, ethanol and growth medium only, were preformed on each plate. The samples were dosed in 2 replicates. After 72 h of incubation at 29 °C, the glucose production of the yeast was measured photometrically at 540 and 630 nm.

2.3. Sampling

Five individuals were sampled from each tank after 7, 35 and 56 days of exposure to obtain a sample size of ten fish per concentration and time point ($n = 10$). The fish were anaesthetised with tricaine methane sulfonate (MS 222, Sigma, France), weighed and measured. Blood from 10 fish per treatment was sampled with a heparinised syringe from the caudal vein and stored on ice for less than half an hour before centrifugation at 1000 *g*, 4 °C, for 10 min followed by a second centrifugation at 3000 *g* for 10 min at 4 °C. The plasma was stored at −80 °C until further processing. The head kidney was dissected, immediately frozen in liquid nitrogen and stored at −80 °C until further processing.

2.4. Gene expression of IL-1β, IL-6, TNF-α and TGF-β

Each head kidney was homogenised in 500 μL of TRI-Reagent® (Sigma, France) employing extraction tubes supplemented with ceramic beads by centrifugation for 2*10 s at 5000 rpm in a Precellys 24 (Bertin technologies, France). The supernatant was transferred into new tubes and mixed with chloroform, incubated for 15 min and centrifuged (12 000 *g*, 15 min, 4 °C). The aqueous