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Developmental oestrogen exposure differentially modulates IGF-I and TNF-α expression levels in immune organs of *Yersinia ruckeri*-challenged young adult rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Intensified aquaculture has strong impact on fish health by stress and infectious diseases and has stimulated the interest in the orchestration of cytokines and growth factors, particularly their influence by environmental factors, however, only scarce data are available on the GH/IGF-system, central physiological system for development and tissue shaping. Most recently, the capability of the host to cope with tissue damage has been postulated as critical for survival. Thus, the present study assessed the combined impacts of estrogens and bacterial infection on the insulin-like growth factors (IGF) and tumor-necrosis factor (TNF)-α. Juvenile rainbow trout were exposed to 2 different concentrations of 17β-estradiol (E2) and infected with *Yersinia ruckeri*. Gene expressions of IGF-I, IGF-II and TNF-α were measured in liver, head kidney and spleen and all 4 estrogen receptors (ERα1, ERα2, ERβ1 and ERβ2) known in rainbow trout were measured in liver. After 5 weeks of E2 treatment, hepatic up-regulation of ERα1 and ERα2, but down-regulation of ERß1 and ERß2 were observed in those groups receiving E2-enriched food. In liver, the results further indicate a suppressive effect of *Yersinia*-infection regardless of E2-treatment on day 3, but not of E2-treatment on IGF-I whilst TNF-α gene expression was not influenced by *Yersinia*-infection but was reduced after 5 weeks of E2-treatment. In spleen, the results show a stimulatory effect of *Yersinia*-infection, but not of E2-treatment on both, IGF-I and TNF-α gene expressions. In head kidney, E2 strongly suppressed both, IGF-I and TNF-α. To summarise, the treatment effects were tissue- and treatment-specific and point to a relevant role of IGF-I in infection.

1. Introduction

A role for the growth hormone (GH)-insulin-like growth factor (IGF) system in immune competence and functionality has been debated for decades in mammals (for review see: Clark, 1997; Weigent, 2013). Both, GH and IGF-I, have been attributed beneficial and restoring effects in immune organ and cell development, differentiation and recovery, e.g., for murine B cell lineage differentiation (Landreth et al., 1992), for shaping and sustaining the human lymph node architecture (Oberlin et al., 2009), and for macrophage functionality in rat, mice and humans (Hansson et al., 1988; Arkins et al., 1993; Kelley and Arkins, 1994). A particular role for the GH/IGF-I axis was found in the recovery of CD4+ T helper cells in HIV-infected individuals treated with GH where beneficial effects coincided with increased IGF-I serum levels (Napolitano et al., 2008). Vice versa, lowered IGF-I serum levels were measured in children during sepsis and septic shock (Onenli-Mungan et al., 2004) and in non-survivors from septic shock as compared to the survivors (De Groof et al., 2002). Nevertheless, data are very fragmentary still (for further reading see: Oberlin et al., 2009; Weigent, 2013).

Intensified aquaculture has strong impact on fish growth and health by stress and infectious diseases and has tremendously stimulated the interest in the complex orchestration of cytokines and growth factors in fish, particularly their influences by environmental factors (Bowden, 2008; Secombes, 2008; Verburg

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van-Kemenade et al., 2009; Workenhe et al., 2010; Wang and Secombes, 2013; Cabas et al., 2012, 2013). However, only very scarce data are available on the potential involvement of the GH/IGF-system in the immune defense in fish. For instance, lower IGF-I serum levels were found in channel catfish infected with *Edwardsiella ictaluri* (Peterson et al., 2007) and in confinement stress-exposed gilthead sea bream (*Sparus aurata*) (Saera-Vila et al., 2009). IGF-I gene expression was dropped in liver (main source of endocrine IGF-I) and kidney of sea bream during vibriosis (Deane and Woo, 2005), and in head kidney of *Enteromyxum leei*-infected gilthead sea bream. Vice versa, in the non-infected individuals IGF-I was elevated, so that an increase in IGF-I expression was proposed as advantage of the immune cells to better combat the parasite (Sitjà-Bobadilla et al., 2008). Thus, data point to a stimulatory role of IGF-I in immune response to bacteria, but are derived from rare observations in patients and fish, only.

Furthermore, there exist some sparse reports through the literature on mutual interactions between IGF-I and TNF-α, e.g., in mammals, where human and murine myeloid cells, particularly macrophages, produced IGF-I under TNF-α stimulation (Fournier et al., 1995) or, vice versa, TNF-α induced a state of resistance against IGF-I (O'Connor et al., 2008). IGF-I (and IGF-II) counteracted some pro-inflammatory effects of cytokines (including TNF-α) in mice (Park et al., 2011). GH, which is used for therapy in growth-deficient children and kachectic patients, has been reported to enhance cytokine production and thus is suggested to be involved in adverse affects including septic shock, uncontrolled infections and depression (Uronen-Hansson et al., 2003). In fish, flounder TNF-α mRNA was induced in a GH-releasing hormone-treated embryonic cell line but in primary pituitary cell culture decreased (Nam et al., 2011) and in GH-injected tilapia elevated in liver (Shved et al., 2011). Thus, data on alterations of the levels of the IGFs and TNF-α expression are scarce in vertebrate species in general, and even less is known on both in the state of illness despite their central physiological roles.

Also estrogens, both natural and artificial, which are ubiquitous in the aquatic environment and may act as "endocrine disruptors" (e.g. Jobling et al., 1998; Segner et al., 2006), are known to interact with both, the immune system (Bowden, 2008; Nakayama et al., 2008; Shved et al., 2009; Casanova-Nakayama et al., 2011; Segner et al., 2013) and the GH/IGF axis, respectively (Shved et al., 2007, 2008, 2009) whereby only few studies have addressed the potential of estrogen-active substances to modulate the immunocompetence of fish (e.g., Thilagam et al., 2009; Jin et al., 2010; Liarte et al., 2011; Raida et al., 2011; Cabas et al., 2012, for further reading see: Wenger et al., 2012) and none with particular view on the GH/IGF-axis.

Strong evidence for a reduced capability of estrogen-exposed fish to combat a bacterial pathogen has recently been provided (Wenger et al., 2011, 2012) in juvenile rainbow trout (*Oncorhynchus mykiss*) after pre-treatment with E2 which showed significantly reduced survival after infection with *Yersinia ruckeri*, compared to the non-E2-exposed infected control group (Wenger et al., 2012). Thus, further exploration with respect to the IGFs and TNF-α – sometimes proposed as counter-players as described above – in light of estrogen treatment and infection, will help to gain further insights into this complex network of growth factors and cytokines in fish.

Thus, the aim of the present study was to investigate the regulation of local IGF-I and IGF-II and TNF-α in the immune-related organs liver, spleen and head kidney in juvenile rainbow trout. For that purpose, specimens were selected from individuals exposed to different doses of 17ß-estradiol (no E2, low E2, high E2) and then infected with the bacteria *Y. ruckeri*, aetiological agent of red mouth disease at that dose (10^6 cfu) where the most pronounced effects on fish survival had been observed (Wenger et al., 2011, 2012). Efficiency of the E2-treatment was assessed in the present study by measuring hepatic mRNA levels of all 4 estrogen receptors (ER) known in rainbow trout (ERα1, ERα2, ERß1 and ERß2) in order to complement previous data using hepatic vitellogenin *vtg* (Wenger et al., 2011, 2012) and to serve as an internal positive control (ERß2) in head kidney and spleen. Alterations of the IGFs and TNF-α were measured as compared to the untreated (no E2, no *Yersinia*) group by qPCR.

2. Materials and methods

2.1. Fish culture and hormone treatment

Juvenile rainbow trout were challenged with a combined treatment of 17ß-estradiol (E2) and infection with bacteria as previously described (Wenger et al., 2011, 2012). Shortly, fish obtained from a local trout farm (Pisciculture de la Gruyère, Neirivue, Switzerland) were kept in aerated glass tanks in normal tap water (non-chlorinated, UV-treated municipal water, temperature: 13.8 °C ± 0.7 °C) with a flow-through of 1 L/s until 3 days before bacteria challenge. The fish were divided into 3 different treatments groups (2 replicates/group): No E2 – control feed = NE2, low dose E2 = 2 mg E2/kg food = LE2, high dose E2 = 20 mg E2/kg food = HE2. Feeds were prepared as described (Wenger et al., 2011) from commercially available dry trout food (Biomar, Brande, Denmark) which was spiked with E2 (Sigma, Buchs Switzerland) and processed by the alcohol evaporation method (Guerrero, 1975). The feeding protocol started with 3% of body weight (BW)/day and reduced to 1.5% of BW/day until end of the experiment as described (Wenger et al., 2012). The work described in this article has been carried out in accordance with the EC Directive 86/609/EEC for animal experiments.

2.2. Infection with Y. ruckeri

Three days before the bacteria challenge experiment, the 3 groups were sub-divided into 12 challenge groups (2 replicates/group, 75 fish/replicate). Each replicate was kept in a separate aerated glass tank for the duration of the experiment. Infection with *Y. ruckeri* strain JF3685 was performed as previously described (Wenger et al., 2011). In brief, the fish (n = 75/treatment group) were transferred and kept in aerated 30 L tanks/replicate filled with 5 L of normal tap water for 1 h with 4 different bacterial concentrations: No *Yersinia* = negative control, 10^2 colony forming units/ml (cfu) = Y2, 10^4 cfu = Y4, 10^6 cfu = Y6) and then re-transferred to their original tanks. The experiment was terminated at day 15 p.i., when no more mortality occurred. To confirm *Yersinia*-infection as the cause of mortality, *Y. ruckeri* were re-isolated from infected fish after each sampling as described by Wenger et al. (2011). In brief, samples were taken freshly from liver, spleen and head kidney of 5 sampled fish, plated on blood agar and incubated for 48 h at 22 °C. Re-isolated bacteria were identified phenotypically using the API20E® system (BioMérieux (Suisse) SA, Geneva, Switzerland). Identity was then confirmed by sequencing of the 16S rRNA gene according to Kuhnert et al. (2002). The survival rates were determined in control and E2-treated rainbow trout exposed to *Y. ruckeri*. No significant differences in mortalities were detected between the replicates ($p > 0.05$) and therefore results of the replicates were combined. Also, no mortalities occurred in the negative control group (No bacteria, no E2), which was further evidence for *Yersinia*-infection-related mortalities in bacteria-infected groups Y4 and Y6 (Wenger et al., 2011, 2012). Since treatment effects were most pronounced with 10^6 cfu of *Y. ruckeri* (Y6) as described (Wenger et al., 2012) with markedly reduced survival rates of 61.1% in Y6-NE2 and a further reduction