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Physiological and proteomic responses to corticosteroid treatments in Eurasian perch, *Perca fluviatilis*: Investigation of immune-related parameters

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ABSTRACT

The comparative effects of cortisol and 11-deoxycorticosterone (DOC), two major corticosteroids in fish, have yet received little attention in teleosts. We evaluated the proteomic and immune responses of Eurasian perch to chronic corticosteroid treatments. We implanted immature perch with cortisol (80 mg/kg) or DOC (4 mg/kg) and measured the proportions of blood leucocytes, immune indices in the plasma, spleen and liver (complement and lysozyme activity, total immunoglobulin and immune gene expression in the tissues) and differential proteome expression (corticosteroid versus control) in the liver and the spleen on days 2, 4 and 14 post-treatment. Implantation of cortisol decreased the ratio of blood leucocytes and depressed Ig levels in both organs while DOC modulated the proportion of leucocyte sub-populations (increase in lymphocytes and decrease in granulocytes). In contrast, the innate humoral immunity was not strongly influenced by any of corticosteroid implants. The only immune parameter that was significantly affected was lysozyme, after DOC treatment. A number of proteins were differentially regulated by these hormones and some were identified in the liver (21 for cortisol and 8 for DOC) and in the spleen (10 for cortisol and 10 for DOC). None of the proteins was directly linked to immunity, except the natural killer enhancing factor, which was repressed by cortisol in the spleen. Our results also confirm that the proteins involved in energetic and glucose metabolism are affected by corticosteroids. Furthermore, these corticosteroids differently regulate immune status in Eurasian perch and they primarily impact leucocytes, as opposed to innate immune function.

1. Introduction

Corticosteroids are a vital component of the teleost endocrine system and are involved in the regulation of a range of physiological functions. Corticosteroids can be divided into two groups of hormones: the glucocorticoids, of which cortisol is the primary hormone, and mineralocorticoids such as 11-deoxycorticosterone (DOC), a strong agonist of the mineralocorticoid receptor in teleost fish (Sturm et al., 2005). The effects of these corticosteroid hormones are mediated by intracellular receptors that act as ligand-dependent transcription factors. Under the current paradigm, cortisol binds to either glucocorticoid receptors (GR) or mineralocorticoid receptors (MR) whereas DOC appears to act only via the MR (Sturm et al., 2005; Arterbery et al., 2011). Whereas cortisol has been shown for a long time to exert pleiotropic actions, the physiological roles of DOC in teleosts are still poorly understood. Still, DOC is often measured at substantial levels in fish notably in Eurasian perch, in the range of some ng/ml (Milla et al., 2009; Mathieu et al., 2013). In particular, the dynamics of plasma DOC during the reproductive cycle suggest its involvement in the final stages of reproduction in a range of fish species (Milla et al., 2009). In trout

Abbreviations: ACH, alternative pathway haemolytic complement; CHAPS, (3-[(3-Cholamidopropyl)dimethylammonio]-1 propanesulfonate); $DiOC_6(3)$, 3,3 dihexyloxacarbocyanine; DOC, 11-deoxycorticosterone; DTT, dithiothreitol; GRs, glucocorticoid receptors; HSP, Heat Shock Proteins; LPS, Lipolysaccharides; MR, mineralocorticoid receptor; MS, mass spectrometry; RBC, red blood cells; WBC, white blood cells

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males, the plasma DOC increase during the spermiation period together with the effects of DOC on spermatocrit values, led to the hypothesis of a role for this hormone in the endocrine control of spermiation in rainbow trout (Milla et al., 2008). But the only confirmed DOC action came out from several studies conducted in the maturing females showing that DOC participates in the triggering of the final oocyte meiotic maturation in some fish species (Nagahama and Yamashita, 2008) while negative effects on sex-steroid secretion have also displayed in both sexes (Milla et al., 2008; Mandiki et al., 2017). Aside from these implications in reproductive functions, other studies suggest its implication in teleost behavior (Takahashi and Sakamoto, 2013) though its role in osmoregulation is still under debate as its capacity to regulate the ionic transporters is species-dependent and generally lower than the cortisol one (McCormick et al., 2008; Kiilerich et al., 2011a, 2011b, 2011c). Finally, a recent study showed up-regulation of plasma DOC level in confined trouts suggesting its implication in fish stress endocrinology (Kiilerich et al., in press). However, as the blood concentration of plasma DOC is 10–1000 fold lower than cortisol depending on the species, the reproductive stage and the stress status (Milla et al., 2009; Kiilerich et al., 2011a, in press), the role of DOC as mineralocorticoid-like hormone acting via the MR is still cryptic. Thus, the research of this hormone in fish raises interesting challenges for fish physiologists. Given the wide ranging effects of cortisol, the identification of specific DOC actions deserves attention.

In vertebrates, including fish, the immune system is regulated, in part, by corticosteroids notably in case of chronic stressor exposure. Indeed, chronic stress, which is often accompanied by elevation of blood cortisol in fish, is generally associated with modulation of hematological and immune parameters (Tort, 2011). For instance, confinement stress induces enhancement of serum cortisol and lysozyme levels, and changes of Reactive Oxygen Species (ROS) production in tilapia *Oreochromis mossambicus* (Binuramesh et al., 2005) and in Eurasian perch *Perca fluviatilis* (Douxfils et al., 2011). The immunomodulatory effects of cortisol suggest that these stress effects are partly evoked by this sustained rise of cortisol in the blood. Cortisol is believed to induce a decrease of circulating lymphocyte number, total Ig production and phagocytosis although it may increase the number of phagocytes by limiting neutrophil apoptosis (Harris and Bird, 2000; Esteban et al., 2004). This hormonal immunoregulation appears to be mediated by glucocorticoid receptors (GRs) which are located in the leucocytes and immune organs (Maule and Schreck, 1991; Di Bella et al., 2008; Stolte et al., 2009). Reciprocally, LPS treatment or infection with blood parasites induces variation in GR mRNA expression in the head kidney phagocytes or in the spleen of gilthead seabream, *Sparus aurata*, common carp, *Cyprinus carpio* and Eurasian perch *Perca fluviatilis* (Acerete et al., 2007; Stolte et al., 2008; Stolte et al., 2009; Mathieu et al., 2014). While there is a large body of evidence suggesting that cortisol is a potent endocrine regulator of fish immunity, little is known about the influence of other corticosteroids.

Indeed, the role of mineralocorticoids in vertebrate immunity has received very little attention. In mammals, there are scattered reports supporting the participation of aldosterone in immunoregulation. For example, the exposure of human cells to aldosterone induces their adhesion to leucocytes, the synthesis of complement C3, or the activation of CD8(+) T cells (Zach et al., 1993; Krug et al., 2007; Herrada et al., 2010). In rodents, DOC exerts some roles in the inflammatory response in relation with activation of interleukin pathways (Krishnan et al., 2016). In fish, the MR is also expressed in the immune organs, but at lower levels than GRs (Stolte et al., 2009). Aldosterone exerts a suppressive effect on leucocyte phagocytosis in vitro, though the effect is less marked than with cortisol (Law et al., 2001). Recently, we showed that DOC regulates some immune gene expression at short term following a hormonal injection (Mathieu et al., 2013). Thus, we hypothesize that other corticosteroids, such as DOC, participate in hormonal immunoregulation.

In addition to investigation of mRNA and physiological parameters, characterizing the proteome is a useful tool to study the endocrine regulation. Compared to transcriptomics, proteomics is closer to the cellular phenotype and gives a more functional knowledge of the product of gene expression (Silvestre et al., 2012). Notably 2D gel electrophoresis has been used to point out some markers of steroid regulation (Ibarz et al., 2013), stress response (Naderi et al., 2017) or immune response (Hang et al., 2013) in fish. All together, we hypothesize that the immunoregulation of corticosteroids, notably DOC, may be detected at the proteome level using 2D gel electrophoresis.

Our objective was to evaluate the effect of chronic exposure to cortisol and DOC on the immune status of Eurasian perch, an aquacultural species known to be quite sensitive to stressors. We injected fish with cocoa butter containing cortisol or DOC and measured blood leucocyte proportions, immune parameters, immune gene expression and the proteomic profile in the liver and spleen. The spleen was selected as it is one of the major lymphoid organ in teleost fishes (Zapata et al., 2006). The liver was selected as this well-known glucocorticoid target contains melanomacrophage centers, antibacterial peptides and high level of immunoglobulins in Eurasian perch (Vijayan et al., 2003; Rossi et al., 2007; Dezfuli et al., 2015).

2. Materials and methods

2.1. Fish and in vivo corticosteroid implantation

Investigations and animal care were conducted according to the guidelines for the use and care of laboratory animals and in compliance with Belgian and European regulations on animal welfare. One-year-old immature Eurasian perch, *Perca fluviatilis* (105 ± 15 g) were provided by the CEFRA (Centre de Formation et de Recherches en Aquaculture, University of Liège, Belgium). Fish were then maintained at the University of Namur (Belgium) experimental fish facilities at 23 °C under constant photoperiod (12 L:12D) at a density of 15 kg/m^3 in recirculated water systems. Fish were fed once daily at apparent satiation with a commercial diet and were allowed acclimatising for 3 weeks before the experiment.

Perch were subjected to corticosteroid administration through the implantation of cortisol (80 mg/kg of fish, Sigma, Steinheim, Germany) or DOC (4 mg/kg of fish, Sigma) or only the cocoa butter vehicle (control) in the body cavity. The DOC concentration was chosen to increase mildly the hormonal level in the plasma to reach supraphysiological concentrations and to avoid pharmacological considerations. Indeed the average plasma DOC level obtained after DOC implantation (4 mg/kg) corresponds to the highest level measured in one specimen of immature perch (Mathieu et al., 2013; around 10–15 ng/ml). The cortisol implant dose (80 mg/kg) was selected to conduct to cortisol increase in the blood corresponding to the maximum of the plasma cortisol level found in perch specimens after exposure to a sharp stressor (Milla et al., 2010; 650 ng/ml). For both steroids, the choice of these doses allowed to generate a 3–20 fold-increase of the hormone level in the blood relatively to the average level measured in control fish. The difference in the induction level between the 2 steroids, higher for cortisol than DOC, reflects the difference in the stability of their physiological concentrations (high lability and inter-individual variability for cortisol; low variability for DOC).

Implants were prepared by suspending a known concentration of corticosteroids in liquid cocoa butter at 40 °C and by injecting the warm liquid into the peritoneal cavity of the fish (1 ml/100 g. of fish). The cocoa butter solidified rapidly within the fish and acted as a solid implant. Fish sampled before implantation served as initial control. Twelve fish for each treatment (cortisol-treated, DOC-treated and controls) were sampled 2, 4, 14 days after implantation (3 fish per tank at each sampling time, 4 tanks per treatment randomly distributed). The 2 first sampling times were selected as over such middle-term period, some regulations of immune gene expression associated with increase of plasma cortisol and MR gene expression were observed after