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Spleen immune status is affected after acute handling stress but not regulated by cortisol in Eurasian perch, *Perca fluviatilis*

Sylvain Milla [a,*], Cédric Mathieu [a], Neil Wang [a], Sophie Lambert [a], Stéphanie Nadzialek [a], Sophie Massart [a], Emilie Henrotte [a], Jessica Douxfils [a], Charles Mélard [b], S.N.M. Mandiki [a], Patrick Kestemont [a]

[a] University of Namur, Unit of Research in Organismal Biology, rue de Bruxelles 61, B-5000 Namur, Belgium
[b] University of Liège, CEFRA, Chemin de la Justice 10, B-4500 Tihange, Belgium

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ABSTRACT

The effects of acute stress on immune status and its regulation by cortisol/corticosteroid receptors have received little attention in percids. To address that question, we investigated the physiological and immune responses of Eurasian perch, *Perca fluviatilis* to acute stress. We exposed immature perch to an 1-min exondation and measured at 1 h, 6 h, 24 h and 72 h post-stress: (1) stress-related parameters including plasma cortisol and glucose levels, (2) immune parameters in the plasma and in the spleen (complement, respiratory burst and lysozyme activity, total immunoglobulins; gene expression of lysozyme, complement unit 3, apolipoprotein A1 and 14 kDa, hepcidin and chemotaxin) (3) the corticosteroid receptors gene expression in the spleen after having cloned them. In addition, the *in vitro* effects of cortisol on the spleen immune parameters were also investigated.

Plasma cortisol and glucose levels increased markedly 1 h post-stress and returned at basal levels after 24 h. *P. fluviatilis* mineralocorticoid receptor, but not glucocorticoid receptors, was significantly up-regulated both *in vivo* after the stress and *in vitro* by cortisol at a physiological concentration (100 ng/ml). The plasma immune parameters were not significantly affected by the stress. In contrast, spleno-somatic index, spleen lysozyme activity, lysozyme and hepcidin gene expression were depleted and total immunoglobulins increased along the whole time-course (1–72 h). But, these immune parameters were not regulated *in vitro* by cortisol at physiological or supra-physiological doses.

Our results indicate that handling stress may affect spleen antibacterial defences without clear effects on circulating immune compounds and that the elevation of plasma cortisol after handling stress may not be related to the regulation of this splenic response.

1. Introduction

In aquaculture, fish are subjected to a wide range of stressors (handling, confinement, hypoxia), which in turns disrupt numerous biological mechanisms. Immunity is particularly affected after exposure to mechanical stressor and, chronically, these stresses have often been described to be detrimental to fish immunity [1,2]. However, the reports about the consequences of acute stress are much more rare and controversial. Acute stressors may be associated with enhancement or deprivation of immune defenses and modulation of hematological parameters [3]. For instance, handling stress promoted a progressive drop in plasma lysozyme activity in sheatfish, *Silurus glanis*, and an increase in rainbow trout, *Oncorhynchus mykiss* [4–6]. Although stress response in terms of immune system indicators has been extensively studied in salmonids, the case of percids remains poorly investigated. Still, the differential physiological response to stressors [7] and history in terms of adaptation to husbandry conditions lead us to hypothesize that the immune status of percids may be differently regulated than that of salmonids after stress.

To date, studies dedicated to understand the stress effects on innate immunity mainly focused on plasma immune defences with little attention paid to the tissular response. Among the lymphoid organs, fish spleen contains various lytic enzymes which protect fish against bacteria. These are hydrolases like lysozyme, the lytic pathways of the complement system and other bacteriolytic enzymes recently suspected to be important components of fish innate immunity. Hepcidin and apolipoprotein A1 are mammalian

* Corresponding author. Tel.: +32 (0)81724371; fax: +32 (0)81724362.
E-mail addresses: sylvain.milla@fundp.ac.be (S. Milla), cedric.mathieu@fundp.ac.be (C. Mathieu), neil.wang@fundp.ac.be (N. Wang), lsof2@hotmail.com (S. Lambert), stephanie.nadzialek@fundp.ac.be (S. Nadzialek), smassart@student.fundp.ac.be (S. Massart), emilie.henrotte@fundp.ac.be (J. Henrotte), jessica.douxfils@fundp.ac.be (J. Douxfils), c.melard@ulg.ac.be (C. Mélard), robert.mandiki@fundp.ac.be (S.N.M. Mandiki), patrick.kestemont@fundp.ac.be (P. Kestemont).

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and piscine antimicrobial peptides whose transcripts are regulated after lipopolysaccharides (LPS) treatments or bacterial infections in various fish species [8–10]. Leucocyte cell-derived chemotaxin-2 is a chemotactic factor released from inflammatory sites to activate neutrophils, which is also up-regulated in spleen from infected fish [11]. While all these immune genes are believed to participate in the fish immune defence, the modulation of their expression by stress remains unknown.

Some of the stress effects on immune parameters have been reported to be mediated by cortisol, the main corticosteroid in fish. In fish treated with cortisol, a modulation of phagocytosis activity [3,12,13] and changes in the number of circulating leucocytes [14] are generally observed in fish blood. However, cortisol implication in the regulation of innate immunity remains less documented and appears somewhat controversial. In sea bass, *Dicentrarchus labrax*, cortisol implantation induced a rise of serum lysozyme activity but did not induce any changes in channel catfish, *Ictalurus punctatus* [15,16]. Also, the regulation by cortisol of complement activity and superoxide production, usual indicator of the respiratory burst activity of macrophages, is still unclear [2,12,17,18]. Once more, main of these studies focused on plasma immune parameters while the knowledge about intra-tissue regulation is limited so far.

The actions of cortisol in vertebrates are mediated through nuclear receptors that act as ligand-dependent transcription factors. Corticosteroid receptors (CRs), i.e. glucocorticoid (GR) and mineralocorticoid (MR) receptors, have been cloned and characterized in some fish species and their molecular characterization suggested that cortisol would be their primary physiological ligand [19,20]. Nevertheless, the information about CRs implication in fish immunity control is scarce. GRs were detected in leucocytes and spleen [21,22]. LPS treatment or infection with blood parasite induced variations in the GRs mRNA expression in the head kidney phagocytes or in the spleen, supporting GR implication in the immune regulation in gilthead seabream, *Sparus aurata* and common carp, *Cyprinus carpio* [23–25].

The objective of the present study is to assess the effects of handling stress on plasma and splenic immune parameters in Eurasian perch and to evaluate if cortisol/CRs regulate such splenic response. To address these questions, (1) we preliminary cloned the CRs and characterized one of the GR, the GR1. We studied its gene expression pattern and spleen gene regulation by piscine physiological steroids. (2) We measured plasma cortisol and spleen CRs mRNA expression after an acute stress concomitantly with plasma and splenic innate immune parameters and the expression of relevant immune genes. (3) Finally, we studied the *in vitro* effects of cortisol on the spleen immune parameters measured *in vivo* after the stress.

2. Materials and methods

2.1. Fish and in vivo handling stress experiment

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 Eurasian perch, *Perca fluviatilis* (112 ± 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 FUNDP (Facultés Universitaires Notre-Dame de la Paix, University of Namur, Belgium) experimental fish facilities at 20 °C under constant photoperiod (12L:12D) at a density of 15 kg/m^3 in recirculated water systems. Fish were fed *ad libitum* every day with a commercial diet and allowed to acclimatise for 2 months before the experiments. A group of perch was subjected to an acute handling stress. Fish were caught with a net and subsequently maintained out of water for 1 min (stressed fish, 4 tanks). Another group of perch was not handled (control fish, 4 tanks). 9 fish sampled before stress served as initial control. 12 fish in each condition (stress and control) were sampled 1, 6, 24 and 72 h after stress (3 fish per tank at each sampling time). Before sampling, fish were anaesthetized in MS-222 (120 mg/l) and blood was collected within 5 min by caudal vein puncture using a 1 ml heparinized syringe. Then, blood was centrifuged at 4500 rpm for 10 min to collect plasma. Spleen was collected and weighted. For 6 fish in each condition (stress and control), spleens were divided into 2 pieces using dissection scalpels. A piece of spleen served for superoxide production assay carried out the same day and the other piece was immediately frozen (−80 °C) for further lysozyme and complement assays. For 6 other fish in each condition, spleens were also divided into 2 pieces (one for immunoglobulin assay and the other for gene expression analysis), frozen in liquid nitrogen and stored at −80 °C pending analysis.

From the same batch of fish (unstressed), several organs (skin, muscle, gill, liver, spleen, stomach, heart, intestine, anterior kidney, posterior kidney, ovary, brain) were rapidly collected using scalpels, dissection scissors and tweezers in order to we analyse the pfGR1 gene expression pattern. Skin and muscle were collected under the first dorsal fin. Gill filaments were separated from the arches by cutting just above the septum. The whole heart and brain were collected while the middle part of intestine was selected. In addition, after depressing and removing the air bladder, we collected the posterior kidney by gently scratching with tweezers along the vertebral column. A part of the anterior kidney was also sampled at the interrenal level. In this group of fish, we also analysed the *in vitro* effects of steroids on the pfGR1 transcript abundance, immune gene expression and immune parameters in the spleen.

2.2. Immune parameters measurement

2.2.1. Lysozyme assay

The lysozyme activity protocol was adapted from Ellis et al. [26]. In microplates 96 wells, the lysozyme activity assay was initiated by mixing 2 µl of spleen lysate or 10 µl of plasma with 250 µl of lyophilized *Micrococcus lysodeikticus* (Sigma) suspension at a concentration of 0.6 mg ml^{-1} in phosphate buffer, pH = 6.2. Using sterile potter homogenizers, the spleen lysate was obtained by homogenizing for 30 s, 1 g of spleen in 2.33 ml of the following buffer containing protease inhibitor cocktail: sodium phosphate buffer 0.067 M and triton X-100 0.1%/ethanol 95% and acetic acid 1%, v/v, pH = 6.2. The difference in absorbance at 450 nm was monitored between 0 min and 15 min (between 0 and 2–4 h for the spleen) and used to calculate lysozyme activity in units. One unit represents the amount of lysozyme that caused a 0.001 decrease in absorbance per minute. The lysozyme activity was calculated either in unit per µg of spleen proteins or in unit per ml of plasma. The quantity of proteins in spleen lysate was measured thanks to Bradford protein assay [27].

2.2.2. Intracellular superoxide ($O2^{\bullet-}$) production

The procedure of spleen intracellular superoxide ($O2^{\bullet-}$) production was adapted from Fatima et al., [28] with some modifications. Briefly, the spleen tissue was cut into 1 mm^3 pieces and gently mashed in presence of 0.5 ml L-15 medium using the back of a syringe piston and 100 µm nylon mesh grid into a Petri dish containing 0.5 ml supplemented medium. The cell suspension obtained was placed in 1.5 ml polypropylene tubes and centrifuged at 500 g for 5 min. The supernatant was discarded and 0.5 ml of fresh culture medium was added under the cell suspension which, finally, was gently mixed. This washing operation was performed twice. Aliquots (100 µl) of the cell suspension were then added to