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Dexamethasone modulates expression of genes involved in the innate immune system, growth and stress and increases susceptibility to bacterial disease in Senegalese sole (*Solea senegalensis* Kaup, 1858)

E. Salas-Leiton [1], O. Coste [1], E. Asensio, C. Infante, J.P. Cañavate, M. Manchado*

IFAPA Centro El Toruño, Junta de Andalucía, Molecular Biology Laboratory, Apartado 16, 11500 El Puerto de Santa María, Cádiz, Spain

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ABSTRACT

Cortisol, the main glucocorticoid in fish, undertakes pleiotropic biological effects in response to stressors to maintain homeostasis. It can exert several actions on the immune system, growth and cellular metabolism, establishing a fine-tune regulation stress response and cross-talk interactions with other regulatory pathways. In this study, we investigated a causal relationship between high levels of glucocorticoids and susceptibility to pathogens and modification of gene expression profiles in Senegalese sole. For this purpose, we carried out two experiments using post-metamorphic individuals (21 days after hatching) that were exposed to dexamethasone (DXM), a potent glucocorticoid, in order to mimic cortisol effects. We quantified transcript levels of a wide set of genes involved in innate immune system (g-type lysozyme and hepcidin (*hamp1*)), HPI axis (*crf*, *crfbp*, *pomcα*, *pomcβ*, *gr1* and *gr2*), HPT axis (*tgb*), cellular stress defense system (*hsp70* and *hsp90aa*), GH/IGF axis (*igf-I* and *igf-Ir*) and the neuropeptide *trh*. Short-term exposure to 0.1, 1 and 10 ppm DXM provoked a reduction *of pomcβ* transcripts and an increase of *crfbp* mRNAs in a dose-dependent manner at 48 and 72 h after treatment. Moreover, g-type lysozyme transcript levels decreased significantly at 72 h whereas *hamp1* mRNA levels increased at 48 h after exposure. Long-term DXM treatment (10 ppm DXM) affected negatively weight of soles (~20% lower than controls). Moreover, reduced mRNA levels were observed for *pomcβ* after 1 week and *igf-I* and *hamp1* after 2 weeks. In contrast, *crfbp* and *crf* increased mRNA levels after 2 weeks. *hsp70* exhibited a dual response increasing transcript levels at 1 week after treatment and reducing thereafter. No significant changes in gene expression were observed at any time during this study for *tgb*, *trh*, *hsp90aa*, *pomcα*, *gr1* and *gr2*. Finally, a challenge experiment using the pathogen *Photobacterium damselae* subsp *piscicida* confirmed earlier and higher mortalities in DXM-treated animals. Taken together, these data indicate that a prolonged exposure to DXM increases the susceptibility to pathogens and reduces growth. Moreover, DXM can trigger a wide cellular response modulating the expression of genes involved in the innate immune system, HPI and GH/IGF axes as well as cellular stress defense. These results are highly valuable to evaluate responses associated to aquaculture stressful conditions and discriminate specific glucocorticoid-mediated effects.

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1. Introduction

Cortisol, the main gluco- and mineralocorticoid in teleosts, is a hormone secreted by interrenal cells from head kidney [1]. Integrated in the hypothalamic-pituitary-interrenal (HPI) axis, cortisol is released to blood in response to a wide variety of stressors such as changes in salinity [2,3] and temperature [4], social stress [5], crowding [6–8] and husbandry [9]. To overcome the stressor, cortisol promotes an energy redistribution and regulates the hydromineral balance [10]. Moreover, the HPI establishes a cross-talk with other axis such as the hypothalamic-pituitary-thyroid (HPT), growth hormone/Insulin growth factor (GH/IGF) and other regulatory peptides (i.e. thyrotropin releasing hormone (TRH)) to cope with stress and exert a fine-tune control on essential physiological functions such as growth, osmoregulation, metabolism and immune system [1,11–13]. At this regard, different cellular and humoral components of innate [14–16] and adaptive [17,18] immune responses are down-regulated after exogenous cortisol treatments. Moreover, a higher susceptibility to disease induced by

* Corresponding author. Tel.: +34 956 01 13 37; fax: +34 956 01 13 24.
E-mail address: manuel.manchado@juntadeandalucia.es (M. Manchado).
[1] Both should be considered as first authors.

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cortisol administration has been reported in commercial species as brown trout (*Salmo trutta*, L.) [19], Atlantic salmon (*Salmo salar*) [20], coho salmon (*Oncorhynchus kisutch*) [21] and channel catfish (*Ictalurus punctatus*) [22].

Synthesis of cortisol and glucocorticoid-mediated responses are tightly regulated at different steps. Following a stressful condition, the neurons from hypothalamus release corticotrophin-releasing hormone (CRF) to the pituitary (rostral *pars distalis*) promoting the synthesis of proopiomelanocortin (POMC) and release of the adrenocorticotropic hormone (ACTH), which, in turn, activates corticosteroid biosynthesis, mainly cortisol. To regulate its own synthesis, this hormone acts at three main steps: (i) CRF bioavailability. Cortisol can inhibit CRF transcription, and also modulates the synthesis of CRF receptors and CRF binding protein (CRFBP). The formers mediate CRF actions whereas the latter interferes cortisol release by blocking CRF [23–25]; (ii) Synthesis and release of POMC from the pituitary corticotrophs. The glucocorticoid dexamethasone (DXM) selectively suppresses POMC expression in the anterior pituitary [26]; iii) Abundance of glucocorticoid receptor (GRs). These nuclear receptors bind effectively to cortisol and DXM to trigger glucocorticoid-mediated cellular responses [27–29]. Their transcription can be up- or down-regulated by cortisol depending of the tissue, exposure conditions and species [30–33]. A better knowledge of the mechanisms of cortisol to auto-regulate systemic responses and the interrelationship with other regulatory pathways is critical to understand and evaluate the effects of glucocorticoids during stress.

Senegalese sole, *Solea senegalensis*, is a high interest aquaculture species in Southern Europe. Successful growth results related to stocking density have been recently achieved in juvenile fish [7,8,34]. A recent work of our group demonstrated that this species tolerated high stocking densities (30–45 kg m^{-2}) with adequate growth rates. However, the high levels of cortisol and the reduced transcript levels of *igf-I*, heat shock protein 70 (*hsp70*), g-type lysozyme and hepcidin (*hamp1*) made us to hypothesize that this situation might compromise the immunocompetence status [7] increasing the susceptibility to bacterial outbreaks that nowdays limits the industrial production of sole [35,36]. The goal of present work was to demonstrate a causal relationship between corticosteroids and the immunocompetence status of *Solea senegalensis* and their effects on the expression of a wide set of genes involved in innate immune system (g-type lysozyme and *hamp1*), HPI axis (*crf*, *crfbp*, *pomcα*, *pomcβ*, *gr1* and *gr2*), HPT axis (thyroglobulin (*tgb*)), cellular stress defense system (*hsp70* and *hsp90aa*), GH/IGF axis (*igf-I* and *igf-Ir*) and the neuropeptide *trh*. For that purpose, we selected the glucocorticoid DXM, a potent corticosteroid synthetic analogue with a high affinity to bind GRs and able to trigger corticosteroid-specific mediated cellular responses [28,37,38]. For easy management, immersion experiments using benthic postlarvae (three weeks after hatching) were performed. Short- and long-term DXM treatments were applied to study the effects of exposure times on gene expression. A challenge experiment using the pathogen *Photobacterium damselae* subsp *piscicida* was also carried out to evaluate the effects of corticosteroids on the susceptibility to this important pathogen. This knowledge is relevant for *S. senegalensis* farming since consequences invoked by potentially stressing rearing conditions are evaluated.

2. Material and methods

2.1. Short-term exposure to DXM

A total of 40 post-metamorphic individuals (20 days after hatching (DAH)) were stocked in each of twelve plastic experimental jars (2 L as working volume). They were adapted for 48 h prior to DXM treatments. Three different concentrations of DXM (Sigma–Aldrich, Ref D1756) were assayed in triplicate: 0.1, 1 and 10 ppm (0.25, 2.5 and 25 μM respectively). As DXM was dissolved in absolute ethanol (>99.5% v/v), the control group (0 ppm) received the same quantity of ethanol. Sampling times were at 6, 12, 24, 48 and 72 h after treatment; nevertheless, in our study, only specimens from the last three sampling points were analyzed. Two individuals from every jar (six for each experimental group) were carefully taken and transferred to new bowls containing a relaxing dose of tricaine (MS-222, Sigma–Aldrich A5040). Soles were individually introduced in eppendorf tubes, frozen in liquid nitrogen and stored at −80 °C until use. During the experimental period, fish were kept under starvation conditions in order to keep optimal water quality (physical–chemical parameters). Additionally, temperature was kept at 20–21 °C, dissolved oxygen ranged between 5 and 6 mg/L and salinity fixed in 39‰. Fish were exposed to weak light (200–300 lx) and natural photoperiod (12L: 12D), while no chemical was used at any time.

2.2. Long-term exposure to DXM

From dose-dependent results obtained in previous short-term exposure, a second assay was designed to study the effects caused by a chronic exposure to DXM. Fifty post-metamorphic larvae (21 DAH) (mean dry weight of 1.53 ± 0.31 mg) were grown in each of four assay trays (7.5 L as rearing volume) throughout two weeks of experimental period. Half of trays were treated with 10 ppm of DXM and the other half was kept as control and added the same ethanol quantity as the DXM-treated group. A 20% of water content was replaced every 48 h adding the corresponding dose of DXM and ethanol (10 and 0 ppm conditions, respectively). First sampling was performed only in one replicate at 7 days after starting DXM exposure leaving the other one untouched for the second sampling at 14 days. A total of 7 specimens were extracted from every experimental group and processed as described above. Fish were daily fed with artemia (*Artemia salina*) (2000 metanauplii d^{-1}), previously enriched according to manufacturer's protocol (Easy Selco, INVE). Preceding trials had revealed such amount allowed for optimum growth of fish while no excess food usually remained on assay trays, reducing thus siphoning and subsequent stress. Moreover, with the goal of reducing handling as much as possible, the slight siphoning of bottom of trays was coincident with water renewals. Physical-chemical parameters were routinely controlled to guarantee cultivation conditions were similar to those of short-term exposure.

2.3. Pathogen resistance challenge

Once concluded the long-term exposure experiment (day 14), we performed a challenge experiment to evaluate the resistance to the *P. damselae* subsp. *piscicida*. A total of fifty individuals of each treatment were transferred to five new assay trays (ten soles per tray and 1 L of working volume). Two different doses of such pathogen, 5×10^4 and 5×10^6 CFU mL^{-1}, were assayed. Both doses and experimental groups (DXM and control) were assayed in duplicate. Moreover, two control trays (one from control and other from DXM group) were added phosphate buffer saline (PBS) in the same quantity that challenged groups. The bacterial challenge was performed by immersion during 1 h after which all water was fully replaced. Strain used was DI-91 [39]. Fish were kept under water-closed system and physical–chemical parameters were the same as reported above (Section 2.1). No food was supplied to fish during the challenge. Mortality was checked daily during 1 week.

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