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Short sequence report

Effect of PRL, GH and cortisol on the serum complement and IgM levels in gilthead seabream (*Sparus aurata* L.)

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That the endocrine system regulates the immune response in mammals has been demonstrated and is widely accepted, while in fish this interaction is still under investigation. The expression of neuropeptides/hormones and cytokines, as well as of their receptors, has been demonstrated in both neuroendocrine and immune system cells in mammals [1–3]. The role of cortisol in the immune system is the best characterized because of its implication in the stress response. It is known, for example, that this hormone decreases the phagocytic response, mitogenesis, antibody-producing cells, circulating immunoglobulin M (IgM) titres, lymphocyte numbers and resistance to pathogens [4–10]. On the other hand, PRL and GH are able to enhance leucocyte mitogenesis, phagocytosis, respiratory burst, cell-mediated cytotoxicity and antibody production in several teleost fish [11–16]. These results clearly illustrate the relation between endocrine and immune systems in fish although the mechanisms involved are still unclear.

While the influence of such hormones on fish cellular immune responses has been widely studied, their effect on the humoral response has been more scarcely considered despite the great importance of these responses. IgM and complement activity are major adaptive and innate humoral responses, respectively, and are both regulated by the endocrine system [17]. Circulating IgM levels reflect the immune system status without exposing the fish to a specific antigen [14], while the alternative complement activity is the most standard parameter for determining the innate humoral immune system. Previous results have shown that cortisol administration decreases the circulating IgM levels in non-infected fish [18,19]. Other studies, however, have shown depressed complement activity after stress as a probable consequence of the high

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circulating levels of cortisol [8,9,20]. On the other hand, PRL and GH activate the immune system, an effect that has also been related to the osmoregulatory responses. Thus, using different strategies (seawater acclimation, hypophysectomy or hormone implants), the positive effects of PRL and GH on the fish humoral immune responses have been documented [12–15, 21–26]. The same studies point to a link between the neuroendocrine and immune systems in teleosts. Taking into consideration the known effects of PRL, GH and cortisol on the immune system and the importance of neuroendocrine control of the immune system, we developed the present study.

Immature male gilthead seabream (*Sparus aurata*, 100–150 g bw) were provided by Planta de Cultivos Marinos (C.A.S.E.M., University of Cádiz, Puerto Real, Cádiz, Spain) and transferred to the laboratories at the Faculty of Marine Science (Puerto Real, Cádiz). They were maintained in 400 l seawater aquaria with an open system, natural photoperiod and at constant temperature (18 °C). The fish were fed daily with 1% body weight using commercial dry pellets (Dibaq-Diprotg SA, Segovia, Spain). They were fasted for 24 h before hormone injection. Four different groups (11 fish per group) of seawater-acclimated seabream were used. Hormone treatments were selected from the literature and previous works [27–30]. Fish were anaesthetized with 2-phenoxyethanol (0.5 ml l^{-1} water), weighed, and intraperitoneally (ip) implanted with slow-release coconut oil implants. For this, fish were injected with 5 μl g^{-1} body weight of coconut oil alone (controls) or coconut oil containing ovine PRL (oPRL, NIADDK-oPRL-21, National Institute of Health, Bethesda, MD, USA) (5 μg g^{-1} bw), recombinant bovine GH (rbGH, Monsanto Lot#M010-001, distributed by National Institute of Health, Bethesda, MD, USA) (5 μg g^{-1} bw) or cortisol (H-2882, hydrocortisone 21-hemisuccinate, Sigma, Madrid, Spain) (50 μg g^{-1} body weight). Fish were sampled 7 days after implant. No mortality was observed during the experiment.

Plasma was obtained by standard protocols and stored at −80 °C until used. Total IgM levels and alternative complement activity were determined. Plasma total IgM levels were measured by an indirect enzyme-linked immunosorbent assay (ELISA) [31]. Briefly, flat-bottomed 96-well plates were coated overnight with seabream plasma (plasma diluted 1/500 in 50 mM carbonate-bicarbonate buffer, pH 9.6). Samples were blocked with bovine serum albumin and incubated for 1 h with the primary antibody (mouse anti-gilthead seabream IgM monoclonal antibody; Aquatic Diagnostics Ltd., 1/100 in blocking buffer). After incubation with the secondary antibody anti-mouse IgG-HRP (1/1000 in blocking buffer), samples were developed with 3,3′,5,5′-tetramethylbenzidine hydrochloride (TMB, Sigma) and H_2O_2. The plates were read at 450 nm in a plate reader (BMG, Fluoro Star Galaxy). Negative controls consisted of samples without plasma or primary antibody, and these OD values were subtracted for each sample value. Finally, the activity of the alternative complement pathway was assayed using sheep red blood cells (SRBC, Biomedics) as targets [32]. SRBC were washed in phenol red-free Hank's buffer (HBSS) containing Mg^{2+} and EGTA and resuspended at 6% (v/v) in HBSS. Aliquots (100 μl) of test plasma as complement source, diluted in HBSS, were added to 100 μl of SRBC in a flat-bottomed 96-well plate to give final plasma concentrations ranging from 40 to 0.31%. After incubation for 90 min at 22 °C and the removal of unlysed erythrocytes, the optical density was read at 550 nm in a plate reader. The values of maximum (100%) and minimum (spontaneous) haemolysis were obtained by adding 100 μl of distilled water or HBSS to 100 μl samples of SRBC, respectively. The degree of haemolysis (Y) (percentage of haemolytic activity with respect to the maximum) was estimated and the lysis curve for each specimen was obtained by plotting $Y/(1-Y)$ against the volume of plasma added (ml) on a $\log_{10}$-$\log_{10}$ scaled graph. The volume of plasma producing 50% haemolysis (ACH_{50}) was determined and the number of ACH_{50} units ml^{-1} was obtained for each specimen. Both parameters were represented as means + SE and analysed by one-way analysis of variance (ANOVA, $P \leq 0.05$) and a test of comparison of means.

The results show that a single ip injection of pituitary (PRL and GH) and interrenal (cortisol) hormones changes the adaptive and innate humoral immune parameters of gilthead seabream after 7 days. Compared with the control (injected with coconut oil alone) the circulating IgM levels (Fig. 1) decreased in fish treated with rbGH and oPRL, but only to a statistically significant extent ($P < 0.05$) with the latter. In contrast, the

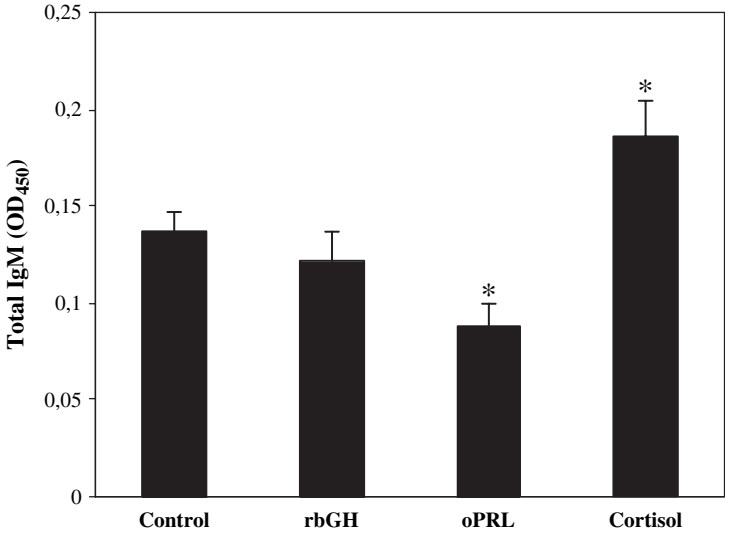

Fig. 1. Circulating IgM level, expressed as optical density at 450 nm, in plasma of gilthead seabream 7 days after intraperitoneal injection of oPRL (5 μg g^{-1} bw fish), rbGH (5 μg g^{-1} bw fish) or cortisol (50 μg g^{-1} bw fish). Data represent mean+SE ($n=11$). Asterisks denote statistically significant differences (ANOVA one-way, $P \leq 0.05$) with respect to the control group (injected with coconut oil alone).

injection of cortisol produced a statistically significant increase in circulating IgM levels after 7 days (Fig. 1). The alternative complement activity, however, was significantly decreased in fish injected with rbGH and oPRL ($P<0.1$), while cortisol treatment did not produce any alteration in this activity (Fig. 2).

In fish, the mammalian hypothalamus-pituitary-adrenal (HPA)-axis has its equivalent in the hypothalamus-pituitary-interrenal (HPI)-axis, which interacts with the immune system. Within the HPI-axis, the effects of PRL, GH and cortisol have been studied although mechanisms involved in the neuroendocrine-immune system interactions are still under investigation and some evidence exists of a bi-directional effect. The synthesis of hormones by the immune cells and of cytokines by the neuroendocrine cells, as well as their respective receptors, has been documented. Thus, several HPA-axis hormones (ACTH, PRL, GH, CRH, endorphins, etc.) are produced in mammalian and fish leucocytes [2,15,33,34]. Moreover, pituitary cells, for example, are able to express cytokines (IL-1, TNF, IFN, IL-6) and respond to them, implying the presence of specific receptors [3]. These findings suggest some degree of interaction between hormones and cytokines in both endocrine and immune organs. Although the production of cytokines by endocrine cells and hormones by leucocytes is quite low their effects are amplified due to the paracrine and autocrine actions [3].

We have studied for the first time the influence of HPI-axis hormones on the humoral parameters of the Mediterranean teleost gilthead seabream. Our previous results in seabream indicated that, following acute stress, the plasma levels of cortisol and glucose increased, which correlated well with the general immunodepression seen in several humoral (complement activity) and cellular innate activities (phagocytosis, respiratory burst and cytotoxicity) [7–9,35]. In these studies, the circulating cortisol concentrations of resting fish (around 5 ng cortisol ml^{-1} serum) increased up to 70–140 ng ml^{-1} in stressed specimens. In this work and, in the same specimens, the circulating cortisol levels showed up to a 10-fold increase (50–70 ng/ml) 7 days after ip injection with 50 μg g^{-1} bw fish of cortisol [28,29]. Our results also show that cortisol implants (50 μg g^{-1} bw) result in an increase in the humoral immune parameters, especially the circulating IgM levels, which no other study has documented. Moreover, cortisol implants

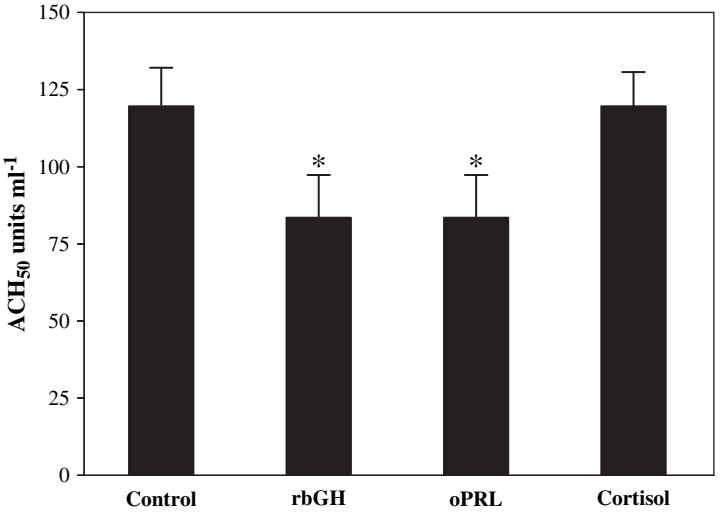

Fig. 2. Alternative complement activity, expressed as ACH_{50} units ml^{-1}, in plasma of gilthead seabream 7 days after intraperitoneal injection of oPRL (5 μg g^{-1} bw fish), rbGH (5 μg g^{-1} bw fish) or cortisol (50 μg g^{-1} bw fish). Data represent mean + SE ($n = 11$). Asterisks denote statistically significant differences (ANOVA one-way, $P \leq 0.1$) with respect to the control group (injected with coconut oil alone).

of 100 μg g^{-1} bw produced very similar effects (data not shown). According to previous data, fish transferred to hypersaline water will increase cortisol release to act as hypoosmoregulatory hormone [28,29]. Similar findings in our lab showed that in seabream specimens adapted to hypersaline water (55 ppt) for 14 days the circulating IgM levels increased but there was no effect on the complement activity [36]. More studies should be carried out to ascertain whether these plasma cortisol levels reached after stress or osmotic shock affect adaptive and innate immune responses, such as circulating levels of total IgM or alternative complement activity, in different ways.

On the other hand, the use of oPRL and rbGH hormones decreased the alternative complement activity as well as the levels of circulating IgM in seabream. Some mammalian hormones have been successfully used in fish, including *S. aurata*, where they show similar functions but with a lower efficiency [27,30]. However, recent advances have made available some piscine hormones, for which the effects described are similar to those found when using mammalian hormones [14–16]. In euryhaline teleosts, including gilthead seabream, PRL is involved in acclimation to hypoosmotic environments [30,37]. In addition, adaptation to low salinities increases PRL mRNA expression and plasma levels in several fish species, including gilthead seabream [29,38,39]. However, the role of GH in osmoregulation is confusing since it behaves differently depending on the fish species and the water salinity [30,40]. To date, no clear osmoregulatory role of GH has been reported in gilthead seabream [30]. With respect to the immune system, similar effects have been noted for PRL and GH, both HPI-axis hormones acting as immunostimulants. Strikingly, our results contradict the literature showing that the humoral parameters are depressed. Moreover, these results agree well with our previous data, in which these two humoral parameters, together with the peroxidase content, in seabream specimens acclimated to hypoosmotic media decreased or were not affected [36]. Interestingly, these conditions increased PRL mRNA expression in seabream pituitary cells but reduced the GH expression and perhaps its circulating levels (Laiz-Carrión et al., unpublished data). Unfortunately, a method to measure the plasma PRL and GH levels in gilthead seabream is not available yet. These contradictory findings in seabream could reflect the great differences seen, for example, in several fish species after osmotic media changes. More studies should be carried out to ascertain the role of PRL and

GH in the gilthead seabream immune system and to clarify whether they affect the humoral and cellular immune functions differently.

To conclude, we have shown the in vivo effects of oPRL, rbGH and cortisol on the circulating IgM levels and alternative complement activity of seabream, as two major immune status indicators. Cortisol increased whilst PRL and GH decreased, respectively, both immunological parameters, which agrees with previous results in seabream and links the endocrine–immune system interactions with the osmoregulatory system. In light of the results obtained the interactions between endocrine–immune–osmoregulatory systems and stress seem to be very complex and deserve deeper investigations.

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