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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