ELSEVIER

Fish & Shellfish Immunology 18 (2005) 255–261

Fish & Shellfish Immunology

www.elsevier.com/locate/fsi

Short communication

Salinity influences the humoral immune parameters of gilthead seabream (*Sparus aurata* L.)

Alberto Cuesta[a], Raúl Laiz-Carrión[b], M.P. Martín del Río[b], José Meseguer[a], J. Miguel Mancera[b], M. Ángeles Esteban[a,*]

[a]*Department of Cell Biology, Faculty of Biology, University of Murcia, 30100 Murcia, Spain*
[b]*Department of Biology, Faculty of Marine Science, University of Cádiz, 11510 Puerto Real, Cádiz, Spain*

Received 13 April 2004; accepted 13 July 2004
Available online 13 October 2004

Keywords: Salinity; Complement; Peroxidases; IgM; Immune system; Gilthead seabream (*Sparus aurata* L.)

Environmental salinity is a very important factor for aquatic organisms and any changes in salinity seriously affect physiological processes. In fish, studies on the effect of salinity have mainly looked at changes in osmoregulatory organs and their hormonal control, plasma parameters, energy metabolism, growth, etc. [1–3]. However, little is known about the changes in the fish immune system after salinity disturbance despite the fact that salinity is one of the most important environmental factors in the aquatic medium. The transfer from freshwater to seawater decreases rainbow trout (*Salmo salar*) specific-antibody titers [4], but in brown trout (*Salmo trutta*) it increases or has no effect on serum lysozyme activity, or leucocyte phagocytosis and cytotoxicity [5]. In the grouper fry (*Epinephelus* sp.) it has been demonstrated that specimens exposed to either hypo- or hyper-osmotic shock increase their susceptibility to infectious pancreatic necrosis virus (IPNV) [6].

Gilthead seabream is the most important commercial fish for Mediterranean aquaculture. In some cases the fish farms are situated in waters with extreme salinity levels or which suffer salinity alterations (bays, natural ponds, places with freshwater inputs, etc.). This is why several studies have focused on the effects of salinity on some physiological responses, mainly osmoregulation and energy metabolism [7–14], as well as providing some information about the negative impact of several stressors on the immune system of this teleost species [15–18]. However, there are no data on the impact of salinity disturbance on the seabream immune system. This led us to carry out the present study.

* Corresponding author. Tel.: +34 968367665; fax: +34 968363963.
E-mail address: aesteban@um.es (M. Ángeles Esteban).

1050-4648/$ - see front matter
doi:10.1016/j.fsi.2004.07.009

Specimens of gilthead seabream (*Sparus aurata* L.) were maintained in laboratory aquaria with full seawater (SW, 38 p.p.t.) and fed daily with 1% body weight using commercial dry pellets (Dibaq-Diprot, Segovia, Spain). In a first experiment, fish (100–150 g body wt) were randomly divided into three groups (12 fish/group), maintained in brackish water (BW, 12 p.p.t.), hypersaline water (HSW, 55 p.p.t.) or SW (control) and sampled after 2 weeks. In a second experiment, fish (20 g body wt) were randomly divided into three groups (12 fish/group), maintained in low saline water (LSW, 6 p.p.t.), BW or SW (control) and sampled after 100 days. In both experiments, the salinity was changed by two salinity points per hour by either adding natural marine salt or mixing with dechlorinated tap water. Salinity was checked everyday and adjusted when necessary. No mortality was observed during the experiments.

Plasma was obtained by standard protocols and stored at −80 °C until used. Total IgM levels, peroxidase content and alternative complement activity were determined. Plasma total IgM levels were measured by an indirect enzyme-linked immunosorbent assay (ELISA) [19]. 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 with or without plasma or primary antibody, and these OD values were subtracted for each sample value. The total peroxidase content present in plasma was measured according to Quade and Roth [20]. Briefly, 15 μl of plasma was diluted in 35 μl of HBSS without Ca^{2+} or Mg^{2+} in flat-bottomed 96-well plates. Then, 50 μl of 2.5 mM TMB and 5 mM H_2O_2 (both substrates prepared daily) were added. The colour-change reaction was stopped after 2 min by adding 50 μl of 2 M sulfuric acid. The optical density was read at 450 nm in a plate reader. Standard samples without plasma were also analysed. Finally, the activity of the alternative complement pathway was assayed using sheep red blood cells (SRBC, Biomedics) as targets [21]. 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. Data were represented as means + S.E. and analysed by one-way analysis of variance (ANOVA, $P < 0.05$) and a test of comparison of means.

The results show that seabream acclimation to HSW for 14 days produced a statistically significant enhancement of the total IgM levels compared with SW-acclimated fish (Fig. 1) but did not affect the other immune parameters studied. On the other hand, BW acclimation for 14 or 100 days did not affect the total IgM levels in plasma (Figs. 1 and 2). However, the effect on the humoral innate immune parameters differed according to the acclimation time. Alternative complement activity increased in fish acclimated to BW for 14 days (Fig. 1), but presented similar values to the control group after 100 days of acclimation (Fig. 2). Finally, the plasma peroxidase content did not vary in fish acclimated to BW for 14 days (compared with the control group levels) but decreased when the acclimation time was longer (Figs. 1 and 2). Finally, after LSW-acclimation for 100 days, both peroxidase content and alternative complement activity were significantly lower compared to the control group maintained in SW, while total IgM levels in plasma were not affected by LSW-acclimation (Fig. 2).

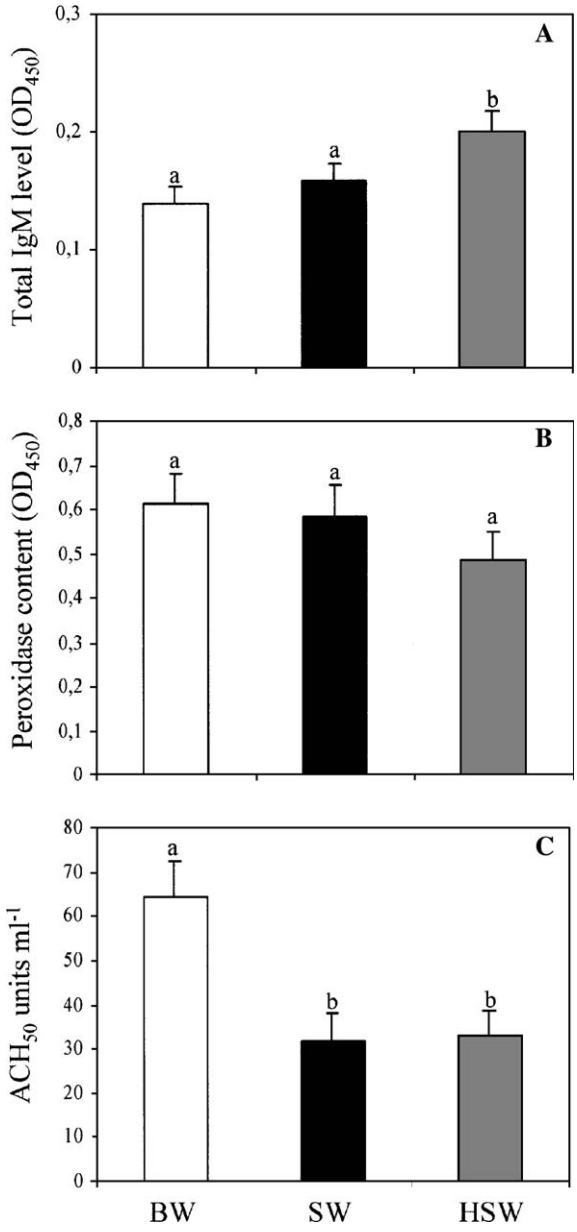

Fig. 1. Total IgM level (A) and peroxidase content (B), both expressed as optical density (450 nm), and alternative complement activity (C), expressed as ACH_{50} units ml^{-1}, in plasma of gilthead seabream specimens kept in brackish water (BW, 12 p.p.t.) (□), seawater (SW, 38 p.p.t.) (■) or hypersaline water (HSW, 55 p.p.t.) (▨) for 14 days. Data represent mean + S.E. ($n = 10–12$). Different letters indicate statistically significant differences (ANOVA one-way, $P < 0.05$) between groups.

It is well known that stress and disease may result in underproduction in aquaculture. However, the effects of environmental factors, such as temperature or salinity are not well documented despite their importance in fish biology. We aimed to study for the first time the effects of salinity disturbance on the gilthead seabream immune system. Previous studies have only focused on the effects of salinity on seabream

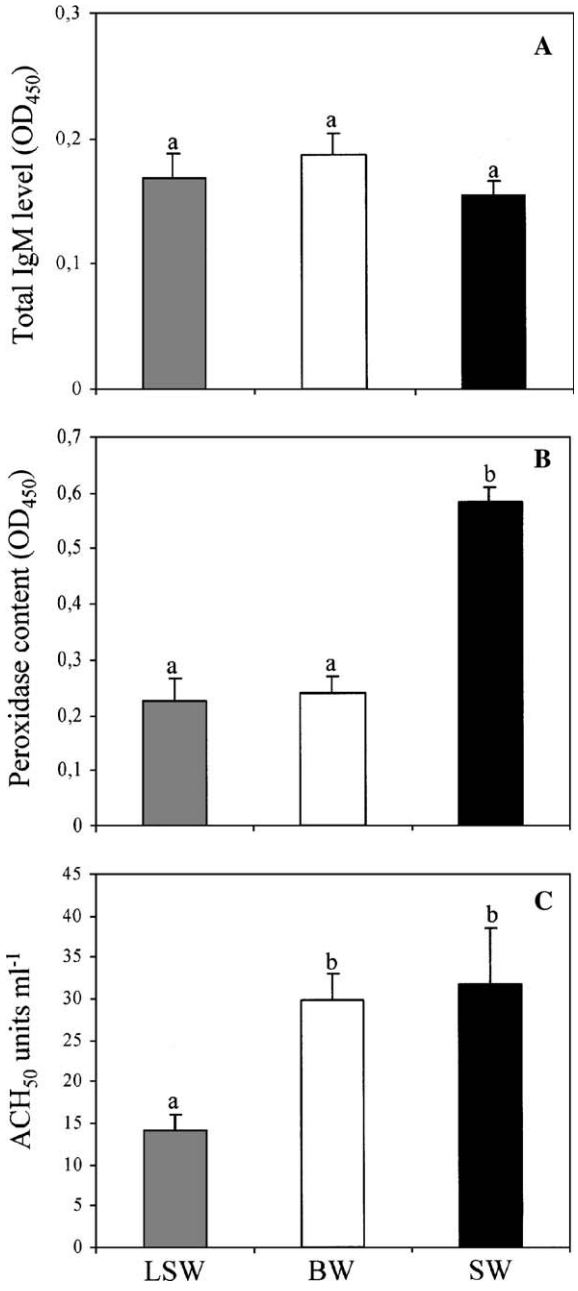

Fig. 2. Total IgM level (A) and peroxidase content (B), both expressed as optical density (450 nm), and alternative complement activity (C), expressed as ACH_{50} units ml^{-1}, in plasma of gilthead seabream specimens acclimated to low salinity water (LSW, 6 p.p.t.) (▩), brackish water (BW, 12 p.p.t.) (□) or seawater (SW, 38 p.p.t.) (■) for 100 days. Data represent mean + S.E. ($n = 10–12$). Different letters indicate statistically significant differences (ANOVA one-way, $P < 0.05$) between groups.

osmoregulatory and metabolic responses [7–14]. Thus, only after HSW-acclimation, fish present increased plasma glucose and lactate levels, suggesting that fish were stressed as a consequence of the high salinity. However, LSW- and HSW-acclimated fish were under stress, as confirmed by their increased energy metabolism and poorer growth than observed in SW-acclimated fish [12–14]. Finally, those acclimated to BW showed much better growth than LSW- and SW-acclimated fish. Previous results reported by us correlate the stress (detected by increased plasma glucose and cortisol) caused by several factors (anaesthetics, crowding, air exposure and agitation) with a general depressed status of the innate immune parameters in seabream [16–18]. All the above prompted us to further investigate the interactions between osmoregulation–stress–immunity after salinity disturbance in fish farms.

In this way, we have studied the effects of salinity disturbance on several humoral immune parameters of the gilthead seabream. Our results show that LSW-acclimation for 100 days significantly decreased the peroxidase content and complement activity compared to the SW-maintained fish. On the other hand, BW acclimation statistically enhanced the complement activity after 14 days of treatment but the activity returned to the control level in those fish acclimated for 100 days. In contrast, the peroxidase content was not affected by 2 weeks of treatment but it was decreased to a significant extent after longer exposure. Finally, the HSW-acclimation for 2 weeks significantly increased the total IgM levels while the other parameters were not affected. Few studies have evaluated the salinity effects on fish immune parameters. In salmonids, for example, transference from freshwater to seawater (hyper-osmotic shock) decreased specific-antibody production, but increased or had no effect on serum lysozyme and leucocyte phagocytosis and cell-mediated cytotoxicity [4,5]. Other studies have demonstrated that the grouper fry increases its susceptibility to IPNV [6], while cod and winter flounder increase their survival after pathogenic challenge when exposed to hypo-osmotic shock [22,23]. However, the data indicate that the resistance to pathogens is not due to an enhanced immune response but, more likely, to a lower degree of pathogenicity and a reduced occurrence of opportunistic parasites in the water. More studies unifying the fish treatments and the immune parameters evaluated should be carried out in an effort to ascertain how hypo- or hyper-osmotic shock affects the fish immune system.

Based on the available data, there is no proof concerning which mechanisms influence the fish immune system after salinity disturbances. However, all the evidence points to a modulation of the immune system by osmoregulatory hormones, including PRL, GH and cortisol [15–17,24–34]. In general, PRL is involved in hypo-osmotic media adaptation while GH and cortisol are antagonists [8–12,35]. PRL and GH have been shown to enhance the IgM levels, lysozyme and leucocyte phagocytosis, cytotoxicity, mitogenesis and survival [24–28]. On the other hand, cortisol plays a role in hyper-osmotic media adaptation and has also been seen to be responsible for immunosuppressive effects after stress [23,16,17,29–34]. Thus, in several fish species, stressed specimens or those treated with cortisol, circulating lymphocytes, phagocytosis, leucocyte mitogenesis, antibody producing cells and circulating IgM levels decreased while the apoptosis of B cells increased. In agreement with this, stressed seabream presented high circulating cortisol levels and decreased immune parameters [16,17]. On the other hand, in vitro incubation of seabream leucocytes with cortisol only decreased the immune parameters when pharmacological concentrations were used and it did not affect all the studied responses [34]. The only available data on seabream indicate that: (a) fish acclimation to BW or LSW increases the expression of PRL mRNA and, probably, its circulating levels; (b) acclimation to LSW decreases GH mRNA expression and (c) cortisol increases in HSW-acclimated fish [8,35]. Overall, our results seem to contradict the literature concerning the effect of osmoregulatory hormones upon the immune system. While the fish acclimated for 100 days to LSW and BW have higher levels of PRL [35], the peroxidase content and alternative complement activity were depressed. However, BW acclimation for 2 weeks increased the complement activity though we have no information about the PRL expression. On the other hand, fish acclimated to HSW increased their circulating IgM levels, while the other parameters were not affected even though the increased plasma levels of glucose, lactate and perhaps cortisol, indicated that they are really stressed. This finding does not support the immunosuppressive role for cortisol. Considering

all these data, the participation of osmoregulatory hormones in the immune regulation of seabream exposed to different salinities may not be the only phenomenon. Other phenomena must be taking place and the effects seen could be due to many factors. For example, the fact that the osmoregulatory response is differently affected in organs such as brain, gills, head-kidney or liver could also influence the immune system [35].

To conclude, acclimation of gilthead seabream to LSW, BW or HSW altered the humoral immune parameters studied. In general, hypo-osmotic acclimation has a negative (or no) effect, while hyper-osmotic acclimation has a beneficial (or no) effect on seabream humoral immune parameters. These results might be explained by the effects of osmoregulatory hormones and the involvement of different organs in the immune and osmoregulatory responses. The stress–osmoregulation–immunity interaction deserves deeper study and the role played by hormones needs to be clarified.

Acknowledgments

This research was supported in part by grants BOS2001-4031-C02-01/02 and PTR95-0431-OP (Ministerio de Ciencia y Tecnología-Spain) to J.M.M. The authors are grateful to ACUINOVA S.A. (San Fernando, Cádiz, Spain) and Laboratories Húmedos CASEM (Universidad de Cádiz, Puerto Real, Cádiz, Spain) for providing experimental fish.

References

[1] Maetz J. Aspects of adaptation to hypo-osmotic and hyper-osmotic environments. In: Malins DC, Sargent JR, editors. Biochemical and biophysical perspectives in marine biology. New York: Academic Press; 1974. p. 1–167.
[2] McCormick SD. Hormonal control of gill Na^+, K^+-ATPase and chloride cell function. In: Wood CM, Shuttlewoth TJ, editors. Ionoregulation: cellular and molecular approaches, Fish physiology, vol. 14. San Diego: Academic Press; 1995. p. 285–315.
[3] McCormick SD. Endocrine control of osmoregulation in fish. American Zoologist 2001;282:290–300.
[4] Betoulle S, Troutaud D, Khan N, Deschaux P. Rèsponse anticorps, cortisolèmie et prolactinèmie chez la truite arc-en-ciel. Comptes Rendus de l'Academie des Sciences de Paris 1995;318:677–81.
[5] Marc AM, Quentel C, Severe A, Le Bail PY, Boeuf G. Changes in some endocrinological and non-specific immunological parameters during seawater exposure in the brown trout. Journal of Fish Biology 1995;46:1065–81.
[6] Chou HY, Peng TY, Chang SJ, Hsu YL, Wu JL. Effect of heavy metal stressors and salinity shock on the susceptibility of grouper (*Epinephelus* sp.) to infectious pancreatic necrosis virus. Virus Research 1999;63:121–9.
[7] Mancera JM, Pérez-Fígares JM, Fernández-Llebrez P. Osmoregulatory responses to abrupt salinity changes in the euryhaline gilthead sea bream (*Sparus aurata*). Comparative Biochemistry and Physiology A 1993;106:245–50.
[8] Mancera JM, Fernández-Llebrez P, Grondona JM, Pérez-Fígares JM. Influence of environmental salinity on prolactin and corticotropic cells in the euryhaline gilthead sea bream (*Sparus aurata* L.). General and Comparative Endocrinology 1993;90: 220–31.
[9] Mancera JM, Pérez-Fígares JM, Fernández-Llebrez P. Effect of cortisol on brackish water adaptation in the euryhaline gilthead sea bream (*Sparus aurata*). Comparative Biochemistry and Physiology A 1994;107:397–402.
[10] Mancera JM, Pérez-Fígares JM, Fernández-Llebrez P. Effect of decreased environmental salinity on growth hormone cells in the euryhaline gilthead sea bream (*Sparus aurata* L.). Journal of Fish Biology 1995;46:494–500.
[11] Laiz-Carrión R, Martín del Río MP, Miguez JM, Mancera JM, Soengas JL. Influence of cortisol on osmoregulation and energy metabolism in gilthead seabream *Sparus aurata*. Journal of Experimental Zoology A 2003;298:105–18.
[12] Mancera JM, Laiz-Carrión R, Martín del Río MP. Osmoregulatory action of PRL, GH, and cortisol in the gilthead seabream (*Sparus aurata* L.). General and Comparative Endocrinology 2002;129:95–103.
[13] Sangiao-Alvarellos S, Laiz-Carrión R, Guzmán JM, Martín del Río MP, Miguez JM, Mancera JM, et al. Acclimation of *S. aurata* to various salinities alters energy metabolism of osmoregulatory and nonosmoregulatory organs. American Journal of Physiology. Regulatory, Integrative and Comparative Physiology 2003;285R:897–907.

[14] Laiz-Carrión R, Sangiao-Alvarellos S, Guzmán JM, Martín del Río MP, Soengas JL, Mancera JM. Growth performance of gilthead sea bream *Sparus aurata* in different osmotic conditions: implications on osmoregulation and energy metabolism. Aquaculture, submitted for publication.
[15] Tort L, Sunyer JO, Gómez E, Molinero A. Crowding stress induces changes in serum haemolytic and agglutinating activity in the gilthead seabream *Sparus aurata*. Veterinary Immunology and Immunopathology 1996;51:179–88.
[16] Ortuño J, Esteban MA, Meseguer J. Effects of short-term crowding stress on the gilthead seabream (*Sparus aurata* L.) innate immune response. Fish and Shellfish Immunology 2001;11:187–97.
[17] Ortuño J, Esteban MA, Meseguer J. Effects of four anaesthetics on the innate immune response of gilthead seabream (*Sparus aurata* L.). Fish and Shellfish Immunology 2002;12:49–59.
[18] Cuesta A, Esteban MA, Meseguer J. Effects of different stressor agents on gilthead seabream natural cytotoxic activity. Fish and Shellfish Immunology 2003;15:433–41.
[19] Cuesta A, Esteban MA, Meseguer J. Total serum immunoglobulin M levels are affected by immunomodulators in seabream (*Sparus aurata* L.) specimens. Veterinary Immunology and Immunopathology 2004;101:203–10.
[20] Quade MJ, Roth JA. A rapid, direct assay to measure degranulation of bovine neutrophil primary granules. Veterinary Immunology and Immunopathology 1997;58:239–48.
[21] Ortuño J, Esteban MA, Mulero V, Meseguer J. Methods for studying the haemolytic, chemoattractant and opsonic activities of seabream (*Sparus aurata* L.) serum. In: Barnes AC, Davidson GA, Hiney MP, McIntosh D, editors. Methodology in fish diseases research. Aberdeen: Fisheries Research Services; 1998. p. 97–100.
[22] Knudsen KK, Sundness G. Effects of salinity on infection with *Lerneaocera branchialis* (L.) (Copepoda: Pennelidae). Journal of Parasitology 1998;84:700–4.
[23] Plante S, Audet C, Lambert Y, de la Noüe J. The effects of two rearing salinities on survival and stress of winter flounder broodstock. Journal of Aquatic Animal Health 2002;14:281–7.
[24] Harris J, Bird DJ. Modulation of the fish immune system by hormones. Veterinary Immunology and Immunopathology 2000;77: 163–76.
[25] Narnaware YK, Kelly SP, Woo NY. Stimulation of macrophage phagocytosis and lymphocyte count by exogenous prolactin administration in silver sea bream (*Sparus sarba*) adapted to hyper- and hypo-osmotic salinities. Veterinary Immunology and Immunopathology 1998;61:387–91.
[26] Pérez-Sánchez J. The involvement of growth hormone in growth regulation, energy homeostasis and immune function in the gilthead sea bream (*Sparus aurata*): a short review. Fish Physiology and Biochemistry 2000;22:135–44.
[27] Yada T, Misumi I, Muto K, Azuma T, Schreck CB. Effects of prolactin and growth hormone on proliferation and survival of cultured trout leucocytes. General and Comparative Endocrinology 2004;136:298–306.
[28] Yada T, Uchida K, Kajimura S, Azuma T, Hirano T, Grau EG. Immunomodulatory effects of prolactin and growth hormone in the tilapia, *Oreochromis mossambicus*. Journal of Endocrinology 2002;173:483–92.
[29] Pickering AD. Stress responses of farmed fish. In: Black KD, Pickering AD, editors. Biology of farmed fish. Sheffield: Sheffield Academic Press; 1998. p. 222–55.
[30] Wendelaar Bonga SE. The stress response in fish. Physiological Reviews 1997;77:591–625.
[31] Nagae M, Fuda H, Ura K, Kawamura H, Adachi S, Hara A, et al. The effect of cortisol administration on blood plasma immunoglobulin M (IgM) concentrations in masu salmon (*Oncorhynchus masou*). Fish Physiology and Biochemistry 1994;13: 41–8.
[32] Wang R, Belosevic M. The in vitro effects of estradiol and cortisol on the function of a long-term goldfish macrophage cell line. Developmental and Comparative Immunology 1995;19:327–36.
[33] Espelid S, Løkken GB, Sterio K, Bgwald J. Effects of cortisol and stress on the immune system in Atlantic salmon (*Salmo salar* L.). Fish and Shellfish Immunology 1996;6:95–110.
[34] Esteban MA, Rodríguez A, García-Ayala A, Meseguer J. Effects of high doses of cortisol on innate cellular immune response of seabream (*Sparus aurata* L.). General and Comparative Endocrinology 2004;137:89–98.
[35] Laiz-Carrión R, Bedruello B, Fuentes J, Guzmán JM, Martín del Río MP, Power DM, Mancera JM. Prolactin, growth hormone and somatolactin pituitary expression in gilthead sea bream Sparus aurata under different salinities, high stocking density and food-deprivation. Proceedings of the 5th International Symposium of Fish Endocrinology. 2004.