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Physiological response of hybrid striped bass subjected to *Photobacterium damselae* subsp. *piscicida*

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ABSTRACT

This study consisted in the analysis of the physiological response through metabolic, immune and molecular indicators over time in the hybrid striped bass (HSB) affected by pasteurellosis, a bacterial infection caused by *Photobacterium damselae* subsp. *piscicida* (PDP). Affected fish showed statistically lower hematocrit, glucose values tended to increase and lactate levels decreased, compared to control values. Regarding the immunological parameters studied, lysozyme and complement showed a reduced response after the bacterial infection. Cortisol, a main indicator of stress response showed that animals were stressed during the first weeks of infection, reaching a 2.5-fold increase. Tissue glucocorticoid receptor (GR) expression was also analyzed to test its responsiveness in front of bacterial infection in several tissues and also its efficiency as an early predictor of health state in fish under infection by PDP. The GR expression was time-specific for each tissue and showed an earlier response in liver, adipose tissue, intestine and head kidney. These alterations suggest a chronic stress situation characterized by a metabolic, energetic and immune imbalance in affected HSB.

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1. Introduction

Morone hybrids have become an important component of US aquaculture industry and later its production has been introduced into other countries, including Taiwan, Israel and Europe (Luidwig, 2004). There are two commonly produced *Morone* hybrids in commercial aquaculture, the palmetto bass and the sunshine bass (Harrell and Webster, 1997). Hybrids are apparently more robust, faster growers and more resistant to disease and to environmental extreme conditions than pure striped bass (Harrell and Webster, 1997). Between both *Morone* hybrids, sunshine bass, reciprocal cross produced more recently by crossing a female white bass (*M. chrysops*) with a male striped bass (*M. saxatilis*) (Harrell and Webster, 1997), is the most common hybrid produced for aquaculture because white bass is easier to hold and spawn and these females produce more viable eggs in captivity than striped bass (Luidwig, 2004). Sunshine bass, as a hybrid between marine and freshwater, also shows resistance to a wide range of water quality characteristics under culture conditions (Hodson 1989).

However, during production fish may be eventually disturbed because of aquaculture practices such as handling, transportation or confinement, and it may suffer stress (Davis et al., 2002) and become more susceptible to some kind of diseases (Ashley, 2007).

Fishes respond in different ways to maintain homeostasis after stress and many physiological changes are involved in such a stress response including hematology (Dethloff et al., 1999), osmolality (McDonald and Milligan, 1997), energetic metabolism (Barton and Iwama, 1991, Carragher and Rees, 1994) and hormone release. Stressors activate the hypothalamic pituitary interrenal axis (HPI) in fish, and therefore the release of cortisol, the final hormone of the axis. Cortisol is the most important corticosteroid in teleost fish, and its level in plasma has been used as the principal indicator of stress (Barton, 2002; Mommsen et al., 1999; Rotllant et al., 2003).

The majority of physiological effects of cortisol in vertebrates are mediated through two intracellular receptors that act as ligand-dependent transcription factors. They are called receptor type I or mineralocorticoid receptor (MR) and receptor type II or glucocorticoid receptor (GR) (Beato et al., 1996). The molecular characterization of cortisol receptors in fish has resulted in two classes (GR and MR) and splicing forms as well. Thus, until now, GR has been analyzed in gilthead seabream, *Sparus aurata* (Acerete et al., 2007), rainbow trout, *Oncorhynchus mykiss* (Bury et al., 2003, Ducouret et al., 1995), fathead minnow, *Pimephales promelas* (GenBank accession no. AY533141), Burton's haplo (tilapia), *Haplochromis burtoni* (Greenwood et al., 2003), zebrafish, *Danio rerio* (GenBank accession no. AB218424), European sea bass, *Dicentrarchus labrax* (Terova et al., 2005) and bastard halibut, *Paralichthys olivaceus* (GenBank accession no AB013444). MR was recently studied in rainbow trout, (*O. mykiss*) (Colombe et al., 2000, Sturm et al., 2005) and Burton's haplo, *Haplochromis burtoni* (Greenwood et al., 2003). However, only two of these studies have also analyzed the cortisol receptor response, specifically the glucocorticoid

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receptor response, at functional level *in vivo* after subjecting the animals to stressors (Acerete et al., 2007; Terova et al., 2005). Therefore in summary the actions of cortisol via GR in teleosts seem to be better characterized than MR actions (Bury and Sturm, 2007).

As a result of aquaculture practices, stressors can provoke maladaptative responses and induce serious diseases in fish that can result in important production losses. One of these diseases in wild and farmed marine fish species can be, for instance, a bacterial septicemia called pasteurellosis or photobacteriosis. Halophilic bacterium *Photobacterium damselae* subsp. *piscicida* (formerly *Pasteurella piscicida*) is the etiological agent of pasteurellosis, also referred to as pseudotuberculosis because, in chronic cases, diseased fish show, in several internal organs, whitish tubercles consisting of bacterial accumulations (Romalde, 2002; Toranzo et al., 2005). The natural hosts of the pathogen are a wide variety of fish species; the disease was first described in wild populations of white perch (*Morone americanus*) and striped bass (*M. saxatilis*) in 1963 in Chesapeake Bay (USA) (Snieszko et al., 1964) and a few years later, pasteurellosis became a problem in other species of the Mediterranean region as the gilthead seabream (*S. aurata*), the European sea bass (*D. labrax*) and the hybrid striped bass (*M. saxatilis*×*M. chrysops*) (Magariños et al., 1996; Toranzo et al., 1991).

Hybrid striped bass have been identified as potential candidates for aquaculture in several European countries, as previously mentioned. Although some studies have analyzed the HSB response after different infections (Evans et al., 2001; McNulty et al., 2003; Shoemaker et al., 2001; Udomkusonsri et al., 2004), a few of them have actually analyzed the physiological parameters (Buchanan, 2008). Moreover, few studies have focused in the physiological response of HSB to different stressors (Bielmyer et al., 2006; Davis and Small, 2006; Davis, 2004; Davis and Griffin Billy, 2004). Finally, there are no molecular studies evaluating the GR expression in HSB.

Thus, the aim of the present study was to analyze the specific response of the HSB to a bacterial infection provoked by *P. damselae* subsp. *piscicida* through the dynamics of immune, energetic and metabolic variables. We also wanted to check the stress response through cortisol levels and the molecular GR response to PDP infection in several tissues.

2. Materials and methods

2.1. Animals and experimental design

Ten thousand and four hundred sexually immature hybrid striped bass (HSB) of 0.5 g body weight were obtained from a fish farm in Israel (Madan Ma'agan Michael). Fish were transported to a Spanish fish farm (Tarragona) keeping the water conditions as follows: constant oxygenation, 15 °C temperature and low density (8 kg/m^3). They were partially anaesthetized to diminish the transport stress. They arrived to the farm facilities and immediately distributed in 4 tanks of 900 L with a semi-closed circulation system, equipped with biological and mechanical (200 μm) filters and a constant flow of 40–50 L/s. Moreover, water was treated with ozone, oxygenated and passed through a heat exchanger and ultraviolet filters before entry to the tanks. Water salinity was 4‰, pH 7.56 ± 0.4 and oxygen levels were maintained at 6–8 ppm. Animals' density was kept at approximately 1.5 kg/m^3. The water temperature was 20.2 ± 2.2 °C and the photoperiod was maintained at 14-h light/10-h dark cycle (226 lx). Three months later, with 6 g of body weight approximately, animals were transferred to two tanks of 9000 L (but the same water conditions). Animals' density in these tanks was kept at approximately 3.5 kg/m^3. Fish were fed four times a day (ration level of 3%) with a commercial diet (Skretting) until 24 h before the experiments, as feeding could interfere with measurements.

Six months after distributing the fish, when they were 30 g approximately, high mortalities were recorded in one of the tanks, practically reaching 50%. Veterinary analyses were made in several animals of different tanks by the Ichtyopathological Laboratory of Skretting Company and by the Service of Fish Pathology Diagnostics (Universitat Autònoma de Barcelona). From these animals, samples of head kidney, brain, spleen and liver of 8 fish were streaked onto 1% NaCl brain heart infusion agar (BHI) plates and incubated for 48 h at room temperature (22 °C). Gills and skin were also processed for microscopy. Both analyses confirmed the presence of *P. damselae* subsp. *piscicida* in fish tissues of the tank with high mortalities and they were studied to test the eventual effects of the infection after the detection of the bacteria as explained below.

2.2. Sampling procedure

Eight animals ($n = 8$) were used for each one of the sampling times after diagnosis of infection: 1st week (t1), 2nd week (t2), 3rd week (t3), 4th week (t4) and 5th week (t5). Control group consisted in not infected animals of the same stock, cultured in a different tank, and previously checked for inexistence of infection.

Fish were anaesthetized with a non lethal dose of 2-phenoxyethanol (Sigma) and blood was collected with a hypodermic syringe from the caudal vein. The blood collection lasted less than 3 min in order to avoid cortisol rise induced by the manipulation during sampling. The extracted blood was divided into two sets of eppendorf tubes. One set contained heparin (*Lithium heparin*, Deltalab) for the hematological measurements (hematocrit). The second set, without anticoagulant, was left to clot at 4 °C and centrifuged at 9000 r.p.m for 5 min at room temperature. The collected serum was stored at −80 °C for further assays (osmolality, glucose, lactate, lysozyme, complement and cortisol).

Six animals were used in each sampling to collect several tissues: brain, liver, spleen, adipose tissue, gills, intestine and head kidney. Fish were anaesthetized with a lethal dose of 2-phenoxyethanol and tissues were carefully dissected out of fish and immediately frozen in liquid nitrogen and stored at −80 °C until use for RNA and DNA extraction. In total, 48 fish of the batch were sampled.

2.3. Physiological measurements

Hematocrit values (Hc, %) were immediately determined after sampling by placing fresh blood in glass capillary tubes (NRRI-Modulohm I/S) and centrifuging for 5 min in a microhematocrit centrifuge (Sigma_202M). Osmolality (Osm/kg) in serum was assessed by measuring freezing point in an osmometer (Osmomat 030 Gonotec, Berlin). Glucose and lactate were determined by enzymatic colorimetric analysis in ELISA plates using commercial kits (Biomérieux, Marcy L'Etoile, France). Lysozyme activity assays (kU/mL) were performed by a turbidimetric method that uses the lysis of *Micrococcus luteus* (Sigma) for determination of the lyzozyme activity using egg-white lysozyme (Sigma) as standard. Haemolytic assays were performed following the technique described by Sunyer et al. (1995) with minor modifications for ELISA plates. The results are expressed in ACH50 units, as the titre at which 50% hemolysis is produced.

Serum cortisol levels were measured by radioimmunoassay (R.I.A.) (Rotllant et al., 2001). The antibody used for the assay was purchased from

Table 1
Specific or degenerated primers used for the RT-PCR reactions and real-time PCR reactions for hybrid striped bass (HSB) glucocorticoid receptor (GR) and *Photobacterium damselae* subsp. *piscicida* (PDP) and 18S (reference).

GR-e forward	5′-AGTGCTCCTGGCTGTTYCTNATG-3′
GR-e reverse	5′-TTTCGGTAATTGGTTGCTGATGAT-3′
PDP5 forward	5′-TTGCGATGACGACAGCTATG-3′
PDP6 reverse	5′-TCTGGCGACCAACAAACGTA-3′
QT-GR forward	5′-GAGCAGATGCTGAAGATTTCCA-3′
QT-GR reverse	5′-ATAGAAACGCTGCCAGTTCT-3′
18S forward	5′-CGAGCAATAACAGGTCTGTG-3′
18S reverse	5′-GGCAGGGACTTAATCAA-3′

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