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Short communication

Evaluation of different stocking densities in a Senegalese sole (*Solea senegalensis*) farm: Implications for growth, humoral immune parameters and oxidative status

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ABSTRACT

Fish are usually exposed to farm operations that can activate the stress system, which in turn diverts energy from normal metabolic processes (i.e. growth or immune defence) to deliver it into the physiological systems activated to cope with stress. The main goal of this study was to assess the effect of high stocking densities on growth performance of Senegalese sole (*Solea senegalensis*) juveniles reared under intensive farming conditions. The impact of high densities on plasma cortisol and metabolites, humoral immune parameters and hepatic oxidative status was evaluated. Senegalese sole juveniles (45 ± 5 g average body weight) were stocked at three different initial densities (7, 17 and 24 kg m^{-2}) throughout a 60 days rearing period. Final stocking densities were 13, 31 and 40 kg m^{-2} with no differences in terms of growth performance. Similar levels of plasma cortisol, circulating leucocytes and metabolites and humoral innate immune parameters suggest that stocking densities do not affect the hematological and biochemical status of Senegalese sole juveniles, at least under the current experimental conditions. Overall, this study presents the possibility for Senegalese sole on-growing at high stocking densities, strengthening the needs for its intensive commercial farming.

1. Introduction

Farm operations such as grading, transport, crowding and vaccination can produce undesired side-effects and cause stress (Wendelaar Bonga, 1997). Stocking density in particular may affect growth performance of cultured fish: growth reduction has been demonstrated in several finfish species including Atlantic cod (*Gadus morhua*) and turbot (*Scophthalmus maximus*) (Irwin et al., 1999; Lambert and Dutil, 2001). High stocking density is considered an aquaculture related chronic stressor that produces an initial elevation of plasma cortisol which appears to decrease in time, probably due to a negative feedback of cortisol at the level of the hypothalamus and pituitary axis, thus modulating adrenocorticotrophic hormone (ACTH) secretion and consequently cortisol production (Mommsen et al., 1999; Wendelaar Bonga, 1997). The suppressive effect of stress on the immune system is highly disputable and does not necessarily translate into decreased resistance to infection in both mammals and fish (Dhabhar, 2009; Verburg-van Kemenade et al., 2009). Stress may compromise the defence against pathogens and increase susceptibility to diseases (Ellis, 2001; Tort, 2011).

Oxidative stress occurs due to imbalance between the production of reactive oxygen species (ROS) and the activity of antioxidants, which protect biological macromolecules from oxidation (Halliwell, 1999). The antioxidant enzymes include superoxide dismutase (SOD), which detoxifies O_2^-, catalase, which reduces H_2O_2, glutathione peroxidase (GPX), which reduces both H_2O_2 and organic peroxides by a glutathione-dependent reaction, and glutathione reductase (GR), which catalyses the NADPH-dependent regeneration of glutathione from the oxidized form generated by GPX (Halliwell and Gutteridge, 2007). In fish, ROS generation may be influenced by aquaculture-related factors such as variations in the concentration of dissolved oxygen (Pérez-Jiménez et al., 2012), feed deprivation (Morales et al., 2004), changes in temperature (Castro et al., 2012; Ibarz et al., 2010) or crowding (Pérez-Sánchez et al., 2013; Trenzado et al., 2009).

Senegalese sole (*Solea senegalensis* Kaup, 1858) has been extensively studied in the last two decades, due to its high-value and market demand, as well as the adaptability of existent facilities to accommodate

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its rearing (Imsland et al., 2003). While there have been important technical developments in rearing and feed technologies during recent years, advances in the state of knowledge of the behaviour of Senegalese sole in captivity and nutritional requirements, are still important bottlenecks for its cultivation (Morais et al., in press). Grow-out is an important step with regard to economic viability, and nowadays most of the new sole farms in Spain and Portugal use recirculation aquaculture systems for on-growing to better control environmental conditions, especially water temperature and nitrogen wastes (Morais et al., in press). In intensive culture systems, profitability depends on the volumes of production, which is a function of growth rate of the fish and the stocking density. Densities of up to 30 kg m^{-2} have been tested with no effects on growth (Salas-Leiton et al., 2008) whereas a relationship has been found between high stocking densities and stress in the fish (Costas et al., 2008, 2013; Salas-Leiton et al., 2010). However, there is little information regarding the growth potential of Senegalese sole in commercial culture given that the growth data available is taken from rearing experiments designed to test nutritional requirements or on-growing conditions. The present study was designed to evaluate the effect of different stocking densities on growth performance of Senegalese sole juveniles reared under intensive conditions at the facilities of a local farm. The influence of different rearing densities on plasma cortisol and metabolites, humoral immune parameters as well as hepatic oxidative status was also evaluated to give an overall picture of some physiological processes being affected. This is particularly relevant since stress-related physiological changes affect metabolism and cell processes (including the immune cells), compromising defence mechanisms and thereby increasing the outcome of diseases (Verburg-van Kemenade et al., 2009).

2. Materials and methods

2.1. Fish and experimental conditions

The experiment was carried out in the facilities of a local fish farm (Aveiro, Portugal). This company produces Senegalese sole in a hyper-intensive production concept based in a shallow raceway system in conjunction with a recirculated aquaculture system. The production system is characterized by shallow tanks stacked in a rack system with several levels per rack. The water level in each tank is about 8 to 20 cm depending on the size of the fish. The water renewal constitutes 10–15% of the total daily volume. This water comes from wells installed about 500 m off the facility.

Senegalese sole juveniles (45 ± 5 g average body weight) provided by the farm were stocked in 1 m^2 fiberglass tanks to achieve three different initial densities: 7, 17 and 24 kg m^{-2} (156, 378 and 534 individuals, respectively), being 7 and 24 considered as low and high stocking densities according to the standards of aquaculture industry, respectively. After an initial acclimation period (15 days), all treatments were performed in triplicate tanks for a period of 60 days (May-June 2012). During the experimental period, mean temperature was 19.1 ± 1.1 °C, photoperiod 12 L:12D, salinity 24‰ and pH 6.7 ± 0.1. During the light phase, light intensity on water surface was 180 lx. A flow was used to ensure a water inflow of 300 L h^{-1}. This water exchange maintained dissolved oxygen above 95% saturation level under all tested densities. Ammonia and nitrite levels in water were measured twice a week and never exceeded 0.02 and 0.1 mg L^{-1}, respectively. Fish were hand-fed *ad libitum* with a commercial dry feed (LE-3 Elite, Skretting, Spain; Protein: 57%; Fat: 18%; Ash: 11.5%; Digestible energy: 19.9 MJ kg^{-1}) 4 times a day (6:00, 12:00, 18:00 and 00:00 h). No antibiotic treatments were used at any time and an exhaustive cleaning protocol was performed daily.

Experimental procedures were directed by trained scientists (following FELASA category C recommendations) and were conducted according to the guidelines on the protection of animals used for scientific purposes from the European directive 2010/63/UE.

2.2. Sampling procedures

One hundred and fifty Senegalese sole per tank were individually weighed and measured at the beginning of the experiment, whereas two sampling periods were performed at days 30 and 60. Since experimental sampling procedures were identical during both periods, they will be described once. Sampling time started at 10 a.m. and finished at 12 a.m. Following fasting for 24 h, four fish were quickly removed from each tank at a time ($n = 12$ per treatment) and anaesthetized with a lethal dose of 2-phenoxyethanol (1.5 mL L^{-1}, Merk, Germany). Blood was withdrawn from the caudal vein of each fish using heparinised syringes. Blood collection lasted less than 3 min in order to avoid cortisol increase due to manipulation during sampling. Fresh blood was transferred to a heparinised eppendorf tube for hematocrit determination (microcentrifugation 10,000 ×*g* for 10 min at room temperature) and blood smears preparation. The remaining blood was used for plasma collection. Plasma was obtained by centrifugation (5000 ×*g* for 5 min at room temperature), frozen in liquid nitrogen and stored at −80 °C for further analysis. After blood collection, fish were individually weighed and measured. Moreover, liver from each fish was also dissected and weighed for hepatosomatic index (HSI) determination, frozen in liquid nitrogen and kept at −80 °C for further analysis.

2.3. Analytical procedures

2.3.1. Plasma cortisol and metabolites

The levels of different constituents in plasma (i.e. cortisol, glucose, tryglicerides and proteins) have been used as indices for evaluating the physiological and health condition of fish (Maita, 2007). Plasma cortisol was measured by radioimmunoassay as described by Metz et al. (2005), and already validated for Senegalese sole (Arjona et al., 2009). Glucose and triglycerides analyses were performed on plasma samples using commercially available Spinreact kits (St. Esteve, Spain; Glucose HK Ref. 1001200; Triglycerides Ref. 1001311), adapted for 96-well microplates. Plasma protein was determined in 1:50 (v/v) diluted plasma samples using the bicinchoninic acid (BCA) Protein Assay Kit (Pierce #23225, Rockford, USA) for microplates. Bovine serum albumin served as a standard. These assays were run on a Power-Wave™ microplate spectrophotometer (BioTek Instruments) using KCjunior Data Analysis Software for Windows.

2.3.2. Blood smears

Observations obtained from examination of blood smears are also able to provide much information about the physiological status of fish (Maita, 2007). Therefore, blood smears were prepared from fresh blood, air dried, and stained with Wright's stain (Haemacolor, Merck) after fixation for 1 min with formol–ethanol (10% of 37% formaldehyde in absolute ethanol). Detection of peroxidase activity to label neutrophils was done by a procedure described elsewhere (Afonso et al., 1998). The slides were examined under oil immersion (1000×) and at least 200 leucocytes were counted and classified as thrombocytes, lymphocytes, monocytes and neutrophils. The relative percent of each leucocyte type was calculated.

2.3.3. Humoral immune parameters

Alternative complement pathway (ACP) activity was estimated as described by Sunyer and Tort (1995) using rabbit red blood cells. The ACH50 units were defined as the concentration of plasma giving 50% haemolysis. Lysozyme activity was measured using a turbidimetric assay as described elsewhere (Costas et al., 2011). Total peroxidase activity in plasma was measured following the procedure described by Quade and Roth (1997). The peroxidase activity (unit's mL^{-1} plasma) was determined defining one unit of peroxidase as that which produces an absorbance change of 1 optic density. These innate immune parameters have been chosen as indicators of immunocompetence since