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Short-term crowding stress in Atlantic cod, *Gadus morhua* L. modulates the humoral immune response

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ABSTRACT

Considering overcrowding as a stressor, the effects of a short-term exposure on the immune responses of Atlantic cod, *Gadus morhua* L. were determined at 2, 24 and 72 h post-crowding. While plasma glucose and total antioxidant capacity were quantified as stress responses, immune responses were based on humoral indices. Bactericidal capacity of plasma was also assessed. There was a significant increase in plasma glucose and total antioxidant capacity at 2 h post-crowding, however the former returned to pre-stress levels 24 h later and the latter remained elevated until 72 h. The plasma protein did not reveal any significant change, but there was a transient upregulation of globulin at 24 h post-crowding. The myeloperoxidase activity increased at 2 h after the stress, but returned to its pre-stress levels at 24 h. The lysozyme activity was upregulated even from the first time point after stress, although the differences were significant only at 24 and 72 h compared to the activity prior to stress. At 72 h post-crowding there was a significant increase in alkaline phosphatase activity while haemolytic activity showed a significant decrease. An increase in the proliferation of the bacterial pathogens, *Vibrio anguillarum* and *Aeromonas salmonicida* was seen from 24 h in the plasma of the stressed fish. Thus, our finding suggests that short-term crowding stress modulates the immune responses in Atlantic cod and therefore husbandry procedures should consider these effects while addressing better management strategies.

1. Introduction

Immunity in teleosts, similar to that in other vertebrates, has a major role in protection against invading pathogens. It can either be an innate mechanism which constitutes the first line of defense or an acquired specific immunity. Fish, in contrast with mammals, rely more on their innate non-specific defense mechanisms (Ellis, 2001).

During a stress episode, fish exhibits an organized set of responses to deal with the stressor (Barton, 2002). One of the primary responses is mediated by the hypothalamus-pituitary-interrenal (HPI) axis, which releases cortisol, the dominant steriod in stress physiology, into the circulatory system (Wendelaar Bonga, 1997). There is a bidirectional communication between the neuroendocrine and immune system that is mediated by hormones and cytokines, respectively (Fast et al., 2008). Stress, depending on the severity modulates the immune response (Ortuño et al., 2001), whereas cortisol release may exert stimulatory effects (Butts and Sternberg, 2008; Fast et al., 2008).

Studies on the effects of a variety of stressors on the fish immune system have been well documented in several species of fish. Stress reduced the number of circulating B-lymphocytes and decreased the antibody response following vaccination in carp (Verburg-van Kemenade et al., 1999); depressed phagocytic and complement activities in head kidney and blood of gilthead seabream (Ortuño et al., 2001), modulated the expression of immune-related genes in cod (Caipang et al., 2008a; Pérez-Casanova et al., 2008), affected the corticosteroid receptors and proinflammatory cytokines in carp (Stolte et al., 2008) and decreased leukocyte survival following bacterial challenge in salmonids (Fast et al., 2008).

Several operations in intensive aquaculture can be classified as physiologically stressful to the farmed animal. Stress due to increased stocking density and repeated handling has been reported to result in prolonged elevation of plasma cortisol levels (Pickering and Pottinger, 1989), leading to serious physiological consequences in fish (Barton and Iwama, 1991). High density can also suppress growth (Rowland et al., 2006) and decrease feed consumption (Papoutsoglou et al., 1998). Crowded conditions could impose increased energy demands in fish and they cope with metabolic adjustments through gluconeogenesis and glycogenolysis (Vijayan et al., 1997). Other serious consequences of crowding stress are immune suppression and higher susceptibility to pathogens (Gornati et al., 2004; Costas et al., 2008; Di Marco et al., 2008).

Although the preceding effects of crowding stress in fish are generally accepted, information relating the type and duration of crowding to the modulation of the innate immune system is rare. As the farming of Atlantic cod, *Gadus morhua* L. becomes widespread, it is imperative to study its stress physiology in relation to the husbandry conditions. Previously, we developed an acute (short-term) crowding

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stress protocol for this fish (Caipang et al., 2008a) and examined its effect on selected innate immune parameters and *in vitro* susceptibility to bacterial pathogens. We also quantified the secondary effects of stress for this species by determining plasma glucose and total antioxidant capacity (Caipang et al., 2008b). In the present report which is linked to those mentioned above, we describe the effects of short-term crowding stress on humoral immune response of Atlantic cod and proliferation of bacterial pathogens in the plasma of the stressed fish.

2. Materials and methods

2.1. Crowding experiment

Atlantic cod weighing 500–750 g were employed in this study conducted at the Mørkvedbukta Research Station, Bodø University College, Norway. The fish were stocked in 500 L tanks supplied with flow-through seawater (7–8°C) and were allowed to acclimatize for 3 weeks ahead of the stress study. There were eight tanks in the set-up; two each for a particular sampling point — initial, 2 h, 24 h and 72 h. The protocol used for the crowding stress and blood sampling were based on our previous description (Caipang et al., 2008a). Briefly, the water level in the tanks was lowered to increase the fish density from 10 to 100 kg m^{-3} and the fish were kept at the high density for 1 h. The fish were exposed to this pattern of overcrowding three times over a 12-h interval period. Attention was paid to avoid other compounding stress factors such as hypoxia. Seven fish from each of the two replicate tanks representing one of the aforementioned time points ($N = 14$ fish) were sampled after subjecting them to the stressor. The initial group was sampled prior to applying the stressor. In order to avoid stressing the fish during sampling, the fish were anaesthetized inside the rearing tank using 5 mg L^{-1} of metomidate (Marinil™, Wildlife Laboratories, Fort Collins, CO, USA). After ensuring that the fish were adequately sedated, they were taken out and euthanized immediately by a strong blow on the head. Blood was rapidly collected from the caudal vein. The procedures for the crowding experiments had the approval of the National Animal Research Authority (FDU) in Norway.

2.2. Plasma samples

Plasma was collected from both experimental and control (initial) groups after centrifugation of the heparinized blood at 750 × *g* for 10 min, aliquoted into 1 mL microfuge tubes and stored at -20°C until use.

2.3. Assays for stress-related effects

Plasma glucose and total antioxidant capacity were quantified using commercial kits (Biovision, Mountain View, CA, USA) following the manufacturer's procedures.

2.4. Assays for humoral immune response

Plasma protein was analysed using the Quant-iT™ protein assay kit (Q33210; Molecular Probes, Eugene, OR, USA) and the fluorescence was measured following the manufacturer's protocols as described by Caipang et al. (2008c). Plasma globulin was determined using the above-mentioned protein assay kit (Molecular Probes) after the addition of saturated ammonium sulphate solution and dissolving in carbonate-bicarbonate buffer as described by Swain et al. (2007).

Myeloperoxidase activity in the plasma was determined following the procedure described by Quade and Roth (1997) with some modifications. Briefly, 10 μL of plasma was diluted with 90 μL of 1× phosphate buffered saline (PBS; Ca^{+2} and Mg^{+2} free) in 96-well microtiter plate to which 35 μL of 20 mM 3,3',5,5'-tetramethyl benzidine hydrochloride (Sigma, Steinheim, Germany) and 5 mM H_2O_2 were added. After a 2-min incubation, 35 μL of 4 M sulphuric acid was added to stop the reaction and the color developed was determined spectrophotometrically (Fluostar Optima; BMG Labtech GmbH, Offenburg, Germany) at 450 nm. Stimulation index was derived following the procedures described by Diaz-Rosales et al. (2006).

Plasma lysozyme activity was measured using a previously described method (Lange et al., 2001) with some modifications. A 1:2 dilution (100 μL) of the plasma was mixed with a similar volume (100 μL) of 0.3 mg mL^{-1} suspension of *Micrococcus lysodeikticus* (Sigma) in sodium phosphate buffer. After incubating for 0 and 10 min at 15°C the optical density was measured at 570 nm using a microplate reader (Fluostar Optima). A unit of lysozyme activity was defined as the amount of plasma causing a decrease in absorbance of 0.001 min^{-1}.

Alkaline phosphatase activity was determined by the addition of *p*-nitrophenyl phosphate (Sigma) to 15 μL of plasma sample that was incubated at 15°C for 30 min followed by the addition of 3 N NaOH to terminate the reaction (Apines-Amar et al., 2004). Optical density was read at 405 nm using a microplate reader (Fluostar Optima).

The total haemolytic activity (classical, alternative or lectin pathway) of the plasma was determined using a modification of the procedure described by Blazer and Wolke (1984). It should be emphasized that measurements of total haemolytic activity is a rather insensitive measure of complement activation; nevertheless it is applied to obtain comparative values for the experimental set-up. Serial two-fold dilution of plasma in PBS was added into each well of the 'U' shaped microtiter plates and mixed with an equal volume of freshly prepared 2.5% rabbit erythrocyte suspension. The plates were incubated at 15°C for 1 h and haemolysis was observed in each well. Haemolytic titer was expressed as the reciprocal of the highest dilution of plasma showing complete haemolysis of rabbit red blood cells (RBC) and was expressed as geometric mean titer following Nusbaum et al. (2002).

2.5. Bacterial proliferation

The capability of plasma in controlling proliferation of *Vibrio anguillarum* VI-F-258-3 and *Aeromonas salmonicida* NCIMB 1102 were determined using a co-incubation assay (Caipang et al., 2008b). The bacterial activity was measured by the addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 2 mg mL^{-1}) to the plasma–bacteria solution after a 24-h incubation at 15°C. The MTT reduction was measured with a microplate reader (Fluostar Optima) at 630 nm and bactericidal index was determined as absorbance of each sample divided by the absorbance of the control (bacteria incubated in culture medium only; Budiño et al., 2006). Each plasma sample was run in duplicates during the analysis.

2.6. Statistical analysis

The observations on the plasma samples ($N = 14$) from fish in two replicate tanks were combined for statistical analyses as there were no variations between the tanks. All assays were performed in triplicate and the mean ± S.D. was calculated for each group. Statistical analyses were carried out using Graphpad Prism 5 (Graphpad Software, Inc., La Jolla, CA, USA). The data were checked for normality (D'Agostino & Pearson omnibus normality test) and for equal variance (Bartlett's test) and were then analysed using ANOVA. Transformation was done wherever necessary. If significance was noted, Dunnett's multiple comparison tests were used to compare the values from different time groups and the initial group. The differences were considered significant at $P < 0.05$.

3. Results

3.1. Stress responses

The plasma glucose level in the pre-stress (initial) group was 2.01 ± 1.2 nmol μL^{-1} (Fig. 1A). Its level significantly increased at 2 h post-