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Supra-physiological levels of cortisol suppress lysozyme but not the antibody response in Atlantic salmon, *Salmo salar* L., following vaccine injection

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ABSTRACT

Using key immunological parameters including lysozyme activity and specific antibody titres, we examined the influence of chronic, supra-physiological levels of cortisol on the vaccine-induced immune responses of farmed Atlantic salmon, *Salmo salar* L. Individual fish were injected with a polyvalent, oil-adjuvanted bacterial vaccine 400 degree days prior to sea water entry, and subsequently injected with a cortisol implant at either 53 or 212 degree days post-vaccine injection. All fish were sampled 74 degree days post-cortisol injection (at 127 and 286 degree days post-vaccine injection). Separate groups of Atlantic salmon were injected with a DNA vaccine against infectious haematopoietic necrosis virus (IHNV) either by itself or concurrently with the polyvalent, oil-adjuvanted bacterial vaccine and treated as described above. Because we were unable to detect a significant increase in lysozyme activity as a result of the DNA vaccine, we cannot determine whether there was a cortisol-induced suppression of this innate immune response. Our results indicate, however, that if Atlantic salmon are exposed to significant elevations in plasma cortisol following the injection of a polyvalent, oil-adjuvanted bacterial vaccine alone or concurrently with the IHNV DNA vaccine, lysozyme activity is suppressed while induction of pathogen-specific antibody titres is unchanged. We conclude, therefore, that chronically elevated, supra-physiological levels of plasma cortisol suppress the lysozyme-mediated innate immune response in Atlantic salmon injected with a polyvalent, oil-adjuvanted bacterial vaccine, but do not affect antibody production associated with the adaptive immune response.

1. Introduction

One of the primary indicators of both acute and chronic physiological stresses in fish is the increased presence of cortisol in the plasma (Wendelaar Bonga, 1997). Studies in salmonid fish have shown that increased amounts of cortisol, both injected and naturally induced, can and do affect immune responsiveness and ultimately disease susceptibility (Angelidis et al., 1987; Maule et al., 1989; Pickering and Dustin, 1983; Pickering and Pottinger, 1985, 1989; Ruane et al., 1999; Salonius and Iwama, 1993; Thompson et al., 1993; Wiik et al., 1989). Recent studies have suggested that the observed differences in immune responsiveness and disease susceptibility in relation to elevated cortisol levels are specific to species, strain, antigen type, and possibly to the timing of the stressor (Eggset et al., 1999; Engelsma et al., 2003; Espelid et al., 1996; Funk et al., 2004; Lovy et al., 2008; Singer et al., 2003; Wiik et al., 1989). The purpose of this study, therefore, was to examine the effects of cortisol administration on the immune response of Atlantic salmon (*Salmo salar* L.) following vaccine injection.

Farmed salmonids are routinely vaccinated prior to seawater entry as a way to ensure they are protected against the various bacterial and viral diseases they might encounter in a net pen environment. The exact timing of vaccine injection is important as it ensures that pathogen-specific antibodies (Ab) are present before the pathogen is encountered. Intraperitoneal injection with a polyvalent, oil-adjuvanted bacterial vaccine (AV) used in aquaculture has been shown to elicit a strong physiological stress response in salmonids similar to that produced by a chronic stressor, including elevated levels of plasma cortisol, a temporary suppression of the immune response, and a short-term increase in disease susceptibility (Eggset et al., 1999; Fevolden et al., 1994; Funk et al., 2004; Wedemeyer, 1997).

Recent studies have suggested that if plasma cortisol levels are elevated after initiation of the innate and adaptive immune responses, overall disease susceptibility and the production of pathogen-specific Abs are unaffected (Espelid et al., 1996; Eggset et al., 1999; Funk et al., 2004; Lovy et al., 2008). These findings have important implications in aquaculture with respect to the timing of vaccine injections. The specific objective of this study was, therefore, to examine vaccine-

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induced innate and adaptive immune parameters in farmed Atlantic salmon following the injection of supra-physiological levels of cortisol. Specifically, this study sought to determine if lysozyme activity and specific Ab production were negatively impacted when cortisol was injected 53 and 212 degree days (dd) after individuals were injected with a polyvalent, oil-AV alone and/or concurrently with a newly licensed rhabdovirus DNA vaccine (DV) specific to the infectious haematopoietic necrosis (IHN) virus. Supra-physiological levels of cortisol were used in this study to mimic an extreme event. It has previously been shown that small increases in cortisol have minimal effect on Ab production, (Espelid et al., 1996; Eggset et al., 1999; Funk et al., 2004; Lovy et al., 2008), but the combined and interactive effects of chronic stress and the parr-to-smolt transformation of Atlantic salmon can lead to unpredictable, and sometimes large increases in plasma cortisol level (Singer et al., 2003). Thus, we chose to test the effect of very high levels of plasma cortisol on Ab production in order to determine whether, under these extreme conditions, any effect could be detected.

2. Materials and methods

2.1. Fish stock and rearing conditions

Juvenile Atlantic salmon (approximately 30 g each) were obtained from Big Tree Creek Hatchery (Marine Harvest Canada, Campbell River, BC, Canada) and maintained at the Department of Fisheries and Oceans — University of British Columbia Centre for Aquaculture and Environmental Research (DFO-UBC CAER). Unvaccinated individuals (640 fish) were randomly divided into 20×200 L indoor tanks (32 fish per tank) that continuously received well water at a constant temperature (10.6 °C). Fish were maintained under natural photoperiod ranging from 10:14 to 13:11, light:dark over the course of the experiment. With the exception of the 24 h period preceding tagging, vaccination, and sampling protocols, fish were fed to satiation twice daily with a commercially available pellet-food (Bio-Olympic Fry®; Bio-Oregon, Vancouver, Canada). Fish were acclimated to these conditions for 28 days prior to tagging and vaccination protocols.

2.2. Vaccination procedure

At the time of vaccination, fish were netted and transferred to a small fresh-water filled container where they were individually anaesthetized with a non-lethal dose of aerated tricaine methane sulphonate (MS222; Syndell Laboratories, Vancouver, BC, Canada), buffered with sodium bicarbonate ($NaHCO_3$; Sigma Aldrich, Oakville, ON, Canada) in a 1:2 ratio (100 mg L^{-1} MS222 to 200 mg L^{-1} $NaHCO_3$). Fish were randomly divided into four vaccine groups (8 fish per vaccine group in each tank) and injected both intramuscularly (IM) and intraperitoneally (IP). The IM injection was placed immediately anterior and lateral to the dorsal fin (i.e. in the epaxial muscle), while the IP injection was one fin length ahead of the pelvic fins, along the midline of the fish as follows. Fish from the control group were injected with 50 µL of 0.02 M phosphate-buffered saline (PBS) IM, and 100 µL of PBS IP. Fish from the adjuvant vaccine (AV) group were injected with 50 µL of PBS IM, and 100 µL IP of a commercially available, polyvalent, oil-adjuvanted vaccine containing formalin inactivated, whole-cell bacterins for *Aeromonas salmonicida*, *Listonella anguillarum* serotype O1 and O2, *Vibrio ordalii*, and *Vibrio salmonicida* (Lipogen Forte®; Novartis Aqua Health, Charlottetown, PE, Canada). Fish from the DNA vaccine (DV) group were injected with 50 µL IM of a rhabdovirus DNA vaccine containing 10 µg of plasmid encoding the glycoprotein (G) gene from the IHN virus (APEX IHN®; Novartis Aqua Health), and 100 µL of PBS IP. Fish from the combined group were injected with 50 µL of the DV IM, and 100 µL of the oil-AV IP. Our vaccination protocol including doses and timing of injection was carried out as suggested by the vaccine manufacturers. Because many immunological and physiological responses of salmonid fish are temperature sensitive, all data referring to the experimental design and to the timing of the immune response are stated in terms of degree days (dd) where 1 dd is accumulated by a fish while in water held at 1 °C for 24 h (Rombough, 1988).

Concurrent with the vaccination procedure, all fish were tagged with alphanumeric visible implant tags (Northwest Marine Technology, Shaw Island, WA, USA) for individual identification and visible implant elastomer tags (Northwest Marine Technology) for vaccine group identification. At completion of the vaccination and tagging procedures, all fish were returned to their respective 200 L holding tanks and allowed to recover from anaesthesia in well aerated fresh-water.

2.3. Experimental design

2.3.1. Experiment #1 — 53 degree days post-vaccine injection

To determine the effect of chronic, supra-physiological levels of cortisol on the innate immune response post-vaccine injection (pvi), ten 200 L holding tanks were divided into two experimental treatments: control and cortisol injected (five replicate tanks in each treatment). Fifty-three degree days (dd) pvi, once the innate immune response had been fully established, fish from the cortisol treatment were individually anaesthetized with a non-lethal dose of aerated MS222 buffered with sodium bicarbonate, as above, and injected IP with a cortisol implant [50 µg cortisol (Sigma Aldrich) g^{-1} body weight in a 1:1 vegetable oil:vegetable shortening vehicle (Crisco®, Smucker Foods of Canada Co., Markham, ON, Canada)] as described previously (Singer et al., 2003; Specker et al., 1994). Cortisol implants have been shown to produce a slow release of cortisol into the circulation of teleosts thereby simulating a chronic stressor (Maule and Schreck, 1987; Maule et al., 1989; Specker et al., 1994; Vijayan et al., 1994). In addition, previous studies have indicated that the level of cortisol present in the serum is relatively constant for at least one month following implant of the vehicle (Specker et al., 1994). Fish were returned to their holding tank and allowed to recover from anaesthesia in well aerated fresh-water. At 74 dd post-cortisol injection (pci) (127 dd pvi) all fish were lethally sampled as described below.

2.3.2. Experiment #2 — 212 degree days post-vaccine injection

To determine the effect of chronic, supra-physiological levels of cortisol on the adaptive immune response pvi, ten 200 L holding tanks were divided into two experimental treatments: control and cortisol injected (five replicated tanks in each treatment). Two-hundred-twelve dd pvi, following initiation of antibody production and the adaptive immune response, fish from the cortisol treatment were individually anaesthetized with a non-lethal dose of aerated MS222 buffered with sodium bicarbonate, as above, and injected IP with the cortisol implant described above. Fish were returned to their holding tank and allowed to recover from anaesthesia in well aerated fresh-water. At 74 dd pci (286 dd pvi) all fish were lethally sampled as described below.

2.4. Blood sampling

On the day of sampling, each of the 5 cortisol and 5 control tanks were randomly sampled by transferring the fish into an aerated, fresh-water filled holding container. Individual fish were removed from this holding container and subsequently anaesthetized with a lethal dose of MS222 buffered with sodium bicarbonate (500 mg L^{-1} MS222 to 1000 mg L^{-1} $NaHCO_3$). Following weight (wt) measurements (to the nearest 0.1 g), blood samples were drawn from caudal venepuncture using a non-heparinised syringe. All blood removal was accomplished on individual fish in less than 2 min to reduce any possible sampling stress effect. Whole blood was placed at 4 °C for 4 h after which it was