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Disease resistance and health parameters of growth-hormone transgenic and wild-type coho salmon, *Oncorhynchus kisutch*

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ABSTRACT

To extend previous findings regarding fish health and disease susceptibility of growth-enhanced fish, hematological and immunological parameters have been compared between growth hormone (GH) transgenic and wild-type non-transgenic coho salmon (*Oncorhynchus kisutch*). Compared to non-transgenic coho salmon, transgenic fish had significantly higher hematocrit (Hct), hemoglobin (Hb), mean cellular hemoglobin (MCH), mean cellular volume (MCV), and erythrocyte numbers, and lower white cell numbers. In addition, resistance to the bacterial pathogen *Aeromonas salmonicida* (causal agent of furunculosis) has been assessed between the strains. Higher susceptibility of transgenic fish to this disease challenge was observed in two separate year classes of fish. The present findings provide fundamental knowledge of the disease resistance on GH enhanced transgenic coho salmon, which is of importance for assessing the fitness of transgenic strains for environmental risk assessments, and for improving our understanding effects of growth modification on basic immune functions.

1. Introduction

Global aquaculture production has been growing rapidly throughout the past four decades. Annually, almost half of the fish and seafood consumed by humans is supplied by aquaculture [1]. Modification of growth rate in fish has been achieved through domestication, directed selection, and, more recently, by growth hormone (GH) transgenesis [2]. GH transgenic fish have been proposed as a means to enhance production efficiency in aquaculture by shortening production times and enhancing feed conversion efficiency. While enhancement of growth rate is important, it is equally critical that survival of fish be enhanced, or at least not impaired by new technologies. In general, diseases are among the greatest problems facing aquaculture; they can reduce the availability and quality of the product, and can affect animal welfare. Thus, a potential benefit of gene transfer in fish will be enhancement of disease resistance [3,4].

Salmonids fishes are among the most economically important fish cultured globally, and further are an excellent model species for transgenic fish research. GH-transgenic salmonids have been found to show a strong stimulation of growth rate (>3 fold increases in weight gain per day, depending on the developmental stage) which can result in very large differences in body sizes at specific ages [5,6]. Such large changes in growth are also correlated with significant physiological differences from wild type, including metabolic changes such as protein, carbohydrate, and lipid use [7–9], liver and muscle enzymology [10–12], oxygen use and demand [13–15], and the stimulation of glutathione anti-oxidation systems [16]. Thus, stimulation of growth rate by transgenesis is seen to be associated with a complex series of cellular and organismal changes. Such physiological demands may have important consequences to immune function as well. Understanding the effects of enhanced growth on immune function has important ramifications for risk assessments of transgenic fish in nature because fish health affects survival and fitness, and potentially transgene prevalence and ecosystem function [17,18].

The fish immune system has both innate and adaptive responses [19]. In fish, food not only provides 'fuel' for growth and development; there is an increasing body of evidence that diet or dietary components can have a significant impact on fish health, and nutrient deficiencies can directly affect immune function and disease resistance [20]. The requirements for nutrients by the immune system must be co-ordinated with other system including reproduction and somatic growth, and needs to be matched with energy availability. Thus, a growth-hormone-enhanced fish may drive

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allocation of available nutritional resources away from other essential physiological processes, such as immune function, shifting the trade-off between such systems away from that which evolved for best fitness in wild-type fish.

To examine how rapid growth rates may affect susceptibility to pathogens in salmon, previous research examined disease resistance of GH transgenic coho salmon to infection by *Listonella anguillarum* [21]. That study found impaired resistance to the pathogen at the smolt but not fry stages. Further, smolts surviving initial infections showed high resistance to a second exposure. Together, these data suggest the innate immune system of the transgenic strain was less effective against the pathogen, but that the acquired immune response was functioning well. However, it may not be reasonable to draw general conclusions from studies on one disease agent, since pathogen-specific responses may not reflect broad-spectrum effects on immune function. Thus, to extend previous findings, the disease resistance of the same strain of GH transgenic coho salmon to a different bacterial pathogen, *Aeromonas salmonicida* (Asal), has been examined. Asal is responsible for causing the disease furunculosis, and can induce high levels of mortality in cultured and wild populations of salmonids in freshwater, brackish water and sea-water [22], and its effects are influenced by several factors including environmental conditions, fish age and genetics [23]. In an effort to identify mechanisms affecting innate immunity in this transgenic salmon strain, various immunological and hematological analyses were performed to compare responses between transgenic and non-transgenic fish. The results of the present study are important to improve our understanding of the relationship between hormonal influences and disease resistance.

2. Materials and methods

2.1. Experimental fish and sampling

The experiments were performed at the Centre for Aquaculture and Environmental Research (CAER), Fisheries and Oceans Canada, West Vancouver, Canada. This non-commercial research facility has multiple containment screening systems to prevent the escape of genetically-modified fish to the natural environment. Fast-growing genotypes of coho salmon (*Oncorhynchus kisutch*, Walbaum) were initially produced by microinjecting the OnMTGH1 GH gene construct into eggs from wild parents from the Chehalis River in British Columbia [24]. Two different year classes of GH transgenic coho salmon and non-transgenic coho salmon were used. Both populations were reared under similar conditions in fiberglass tanks supplied with fresh well-water (10 ± 1 °C) under natural photo periods. To attain fish of similar size, non-transgenic salmon from the previous brood year were used to compare to transgenic salmon.

The fish were starved 24 h prior to sampling, at which time fish were euthanized in a bath containing a lethal concentration of buffered tricaine methanesulphonate (200 mg/L; Syndel Laboratories Ltd., Vancouver, BC, Canada; 400 mg/L sodium bicarbonate). Duplicated studies were performed at different time schedules. A total of 120 fish were sampled for Study A (60 transgenics and 60 controls), and a total of 80 fish for Study B (40 transgenics and 40 controls). All fish were weighed, fork length measured, and condition factor (CF) calculated [CF = Weight (g)/Fork Length (cm)^3]. To collect blood, tails were severed using sterile blades and blood was sampled immediately for hematological analyses. Head kidney tissue was aseptically removed to determine phagocytic respiratory burst activity.

2.2. Hematological analysis

Hematological analyses were performed using a standard methodology [25]. Serum was collected from fresh blood that had settled overnight at 5 °C, then stored at −80 °C for later measurement of lysozyme and bactericidal activity. Fresh whole blood was used to measure hematocrit (Hct), in duplicate and expressed as mean percent packed cell volume (% PCV). Fresh blood was also collected to later measurement of hemoglobin (Hb) using the cyanmethemoglobin method (Sigma Diagnostics Procedure No. 525), and the number of erythrocytes (RBCs) using manual hemacytometer counts [26]. In addition, fresh blood was dropped onto a clean glass slide, smeared and stained with a modified Wright-Giemsa stain according to manufacturer's instructions. Twenty-five fields were systematically examined from each slide (one slide per fish) under oil immersion (1000× magnification). The number of lymphocytes, neutrophils, thrombocytes, monocytes, and erythrocytes were recorded, and the leukocyte:erythrocyte ratio calculated. The number of circulating leucocytes/mm^3 was determined by multiplying the above ratio, with the number of erythrocytes determined from the hemacytometer counts. Mean cellular hemoglobin (MCH, μg/cell), mean cellular volume (MCV, nm^3), and mean cellular hemoglobin content (MCHC, g/100 mL) were also calculated using the Hct, RBC numbers, and Hb data.

2.3. Phagocyte respiratory burst activity

Phagocyte respiratory burst activity was measured with 60 samples of each group from Study A. The glass-adherent nitroblue tetrazolium (NBT) assay was performed on suspension of head kidney tissue to measure phagocytic respiratory burst activity [27]. Briefly, head kidney tissue was aseptically removed, placed into 3 mL sterile, chilled cultured medium containing Leibovitz medium (L-15) containing 2% heat-inactivated (45–50 °C, 45 min) fetal bovine serum, heparin (10 U/mL), penicillin-streptomycin (100 U/mL), and homogenized through repeatedly aspirating the tissue until a homogeneous suspension was produced. The homogenate was washed twice (200× g, 20 min, 5 °C) and then suspended in 5 mL culture media. The cell preparation was incubated overnight at 15 °C with gentle rotation. The following morning the tubes were centrifuged, and the pellet/homogenate slurry (approx. 40 μL) was dropped onto duplicate wells of a multiwelled pre-cleaned glass slide. The slides were incubated in a moist chamber at room temperature (18–20 °C) for 30 min. Non-adherent slides were washed off the glass slide by gently rinsing with sterile phosphate buffered saline (PBS; pH 7.4). The NBT solution (0.2% NBT in 0.85% saline) was added onto the wells (approx. 50 μL), and the slides incubated for an additional 1 h. Following incubations, coverslips were placed over the slide wells, and the adherent cells examined microscopically under oil immersion (1000× magnification). Those cells with morphological characteristics of monocytes, macrophages, and neutrophils were counted by systematically examining each well. A total of 200 cells were counted and the number of cells with blue halos recorded as NBT positive.

2.4. Serum lysozyme activity

Serum lysozyme concentration was determined with 60 samples of each group from Study A using a modification (analysis using microtitre plates) of the method described by Litwack [28]. Standards were simultaneously analyzed using known concentration of hen egg white lysozyme (HEWL; Sigma L6876), and serum lysozyme concentration expressed as mean μg/mL.

2.5. Serum bactericidal activity

Serum bactericidal activity was determined for the transgenic and control coho groups, by randomly selecting 15 of the 60 frozen serum samples for each group. Serum bactericidal activity was