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Blood Lymphocyte and Neutrophil Response of Cultured Rainbow Trout, O*ncorhynchus mykiss*, Administered Varying Dosages of an Oral Immunomodulator – 'Fin-Immune™'

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Abstract**—**In a 10-week (May – August, 2008) Phase I trial, 840, 1+ rainbow trout, *Oncorhynchus mykiss*, received a commercial oral immunomodulator, Fin Immune™, at four different dosages (0, 10, 20 and 30 mg g-1) to evaluate immune response and growth. The overall objective of was to determine an optimal dosage of this product for rainbow trout that provides enhanced immunity with maximal growth and health. Biweekly blood samples were taken from 10 randomly selected fish in each tank (30 samples per treatment) to evaluate the duration of enhanced immunity conferred by Fin-Immune™. The immunological assessment included serum white blood cell (lymphocyte, neutrophil) densities and blood hematocrit (packed cell volume %). Of these three variables, only lymphocyte density increased significantly among trout fed Fin-Immune™ at 20 and 30 mg g-1 which peaked at week 6. At week 7, all trout were switched to regular feed (lacking Fin-Immune™) and by week 10, lymphocyte levels decreased among all levels but were still greater than at week 0. There was growth impairment at the highest dose of Fin-Immune™ tested (30 mg g-1) which can be associated with a physiological compensatory mechanism due to a dose-specific threshold level. Thus, our main objective of this Phase I study was achieved, the 20 mg g-1 dose of Fin-Immune™ should be the most efficacious (of those we tested) to use for a Phase II disease challenge trial.

Keywords**—**Blood Lymphocyte, Neutrophil Response of Cultured Rainbow Trout, Oncorhynchus mykiss, Oral Immunomodulator – 'Fin-Immune™.

I. BACKGROUND

A common health concern in finfish aquaculture is whether hatchery-reared fish develop an immune system comparable to that of wild fish. Given that hatchery fish are raised in an artificial environment during the critical larval growth stages, do these fish receive the necessary stimuli and/or nutrients to develop a fully competent immune response? In commercial fish production, vaccines have traditionally and successfully been used to boost the immune system of hatchery fish. However, some methods of administration (e.g., injection) can be quite laborious, costly and highly stressful to the fish and yet confer only a temporary resistance to disease. Another limitation of most commercial vaccines is that they usually enhance resistance to only one (or few) specific pathogen(s). An alternative method of promoting fish health is to use oral immunostimulants/immunomodulators, pre-mixed in fish feeds. Oral immunostimulants/immunomodulators are easy to administer and boost the general, *non-specific* immune response of an animal, thus should provide protection against a variety of pathogens. These products do not contain any chemicals or antibiotics (thus no safety withdrawal period) and have been successfully used on cultured fish and shrimp to improve their general immune response and enhance growth rate [1]-[3]. Phase I of this project measured white blood cell (lymphocyte, neutrophil) response among cultured rainbow trout fed three different dosages of a commercial (Aloha Medicinals Inc.) oral immunomodulator (product name 'Fin- Immune'). The product (beta-glucans + vitamins and essential minerals) is partly derived from the *Cordyceps* mushroom which has been shown to provide enhanced immunity for 5-7 months, based on studies with livestock [4]. The overall objective was to determine an optimal concentration of this product for domesticated rainbow trout (*Oncorhynchus mykiss*) that provides enhanced immunity (measured via changes in white blood density) with maximal growth and health.

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II. METHODS

2.1. Feed preparation – Aloha Medicinals Inc provided 2.0 kg of the powder immunomodulator, Fin-Immune™, which was mixed with a 3 mm dry pellet commercial trout feed (Bio-Oregon, Skretting™). To mix the immunomodulator all feed was ground and re-pelleted in the nutrition lab of the Centre for Aquaculture & Environmental Research (CAER) of the Fisheries & Oceans Canada West Vancouver Lab.

The pellets were ground into a fine powder and were reformed (extruded at 3 mm) with specific dosages of Fin-Immune™, using steam-pelleting (80 °C for 5 s). In this

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procedure, the Fin-Immune™ product was incorporated inside the feed rather than top-coating to ensure a more uniform mixture. Four different dosages were prepared: 0, 10, 20 and 30 mg Fin-Immune™ per g of feed. The 0 mg g-1 feed was a control and was ground and re-pelleted in the same manner as the Fin-Immune™ added feeds. Feed for the entire trial was prepared simultaneously to avoid batch variation and remained in cold storage (2°C) prior to use.

2.2 Fish Husbandry - Pre-smolt, hatchery-reared rainbow trout (1+ yr), *Oncorhynchus mykiss*, (n = 840; 60-80g), raised in the Vancouver Island University (VIU; formerly Malaspina University-College), Fisheries & Aquaculture trout hatchery. All fish were initially weighed and randomly allocated to 12, 420L tanks (70 fish per tank, density ~ 13.3 g L-1). For all weighing and handling fish, the anesthetic TMS was used (100 mg L-1) according to VIU hatchery SOPs. During the initial weighing (week 0), a small sample of blood (~ 0.1 ml) was removed for analysis and the fish were checked for any signs of parasites or chronic stress. All fish were fed at a rate of 2% biomass per day for ten weeks. After each two-week sampling period the amount of feed (2% biomass) was re-adjusted to reflect the increased growth. Throughout the trial, all trout received ambient freshwater in a 90% recirculation system. All tanks were monitored for critical water parameters (e.g., oxygen, temperature, flow) daily with a staff member on 24-hour callback status. Prior to handling any fish, this experimental protocol was evaluated and approved by the VIU institutional Animal Care Committee.

2.3 Treatment groups - We used three replicate tanks per treatment with 70 trout per tank, using a blind feeding and sampling regime (i.e., codes were used so the dose of Fin-Immune™ was unknown until the end of the trial). Using a randomized block design, the following treatments were assigned to the 12 tanks:

1. Control (Tanks 2, 4, 9) – 0 mg immunomodulator per 1 g of feed for 10 weeks.

2. Treatment A (Tanks 3, 8, 10) - 10 mg immunomodulator per 1 g of feed for 7 weeks, + 3 weeks regular feed.

3. Treatment B (Tanks 1, 7, 11) - 20 mg immunomodulator per 1 g of feed for 7 weeks, + 3 weeks regular feed.

4. Treatment C (Tanks 5, 6, 12) - 30 mg immunomodulator per 1 g of feed for 7 weeks, + 3 weeks regular feed.

(NOTE: In the original proposal, we planned to feed the immunomodulator for 8 weeks; however, after 7 weeks the fish had grown so large that they had exhausted our supply of immunomodulator-treated feed. Consequently, we had to switch to regular feed one week earlier than planned.)

2.4 Immunological Assessment - To evaluate the duration of enhanced immunity conferred by Fin-Immune™, bi-weekly blood samples (0.1 ml per fish) were taken from 10 randomly selected fish in each tank (30 samples per treatment). While under TMS anaesthesia, less than 0.1 ml of blood was removed from the caudal vein using a sterile, heparinized needle + syringe. During each sample period, length (0.1 cm) and weight (0.1 g) were recorded and the fish were checked for any signs of parasites and stress.

From each blood sample (n = 120 per sample), a monolayer blood smear was prepared on a glass slide and stained (Diff-Quik™). In addition, heparinized micro-capillary tubes (75 mm, Sure -Prep™, BD Clay Adams™) were filled with blood (~ 0.065 ml) and centrifuged at 11,700 rpm using a micro-centrifuge (Autocrit Ultra 3™, BD Clay Adams™) to measure blood hematocrit (packed cell volume = % volume of blood cells to blood plasma). The stained blood slides were examined using a compound microscope (1000X) attached to a computer with image analysis software (Motic Image Plus™) to count the number of lymphocytes (per 100 erythrocytes) and neutrophils (per 1000 erythrocytes). Three random fields of view were used for each slide and the results were averaged as per[5] and [6].

2.5 Biological Assessment – Bi-weekly length and weight data were used to calculate condition factor [100 x (weight · length-3)] and mean specific growth rate: 100 x [(lnWf - ln Wi) time-1], where Wf is final mean weight, Wi initial mean weight and time is a specified period in days.

2.6 Data Analysis - Mean variables were analysed biweekly using a one-way analysis of variance (ANOVA) using the factor feed or overall by a two-way ANOVA using the factors feed and time (week). If the data were non-normal, the Kruskal-Wallis nonparametric test was used.

III. RESULTS AND DISCUSSION

Lymphocytes and neutrophils are often used in studies evaluating general immune response due to ease of sampling and cost-efficiency [7]. Other techniques exist to examine immune response (e.g., serum immunoglobulin/antibody levels, gene expression, etc.); however, these are better suited to an evaluation of the *specific* immune response (e.g., vaccine trial). Given that we were testing an immunomodulator, designed to boost the *non-specific* immune response, general responses (e.g., serum lymphocyte, neutrophil, lysozyme, heat shock proteins, etc.) are preferred. Lymphocytes are key white blood cells involved with coordination of specific and non-specific immune components and their abundance in the blood is usually a long-term (chronic) response [7]. Lymphocytes densities in salmonids range 1.6-4.57 cells per 100 erythrocytes [7]. Conversely, neutrophils are white blood cells associated with a short-term (acute) response, and have a lower density (2.0-2.85 cells per 1000 erythrocytes) associated with a somewhat ephemeral existence [7]. The hematocrit is a measure of the percent of blood that is cells (i.e., hematocrit of 25% = 25 ml of blood cells in 100 ml of blood). Normal

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ranges for hematocrit are dependent on age, stress and disease. A low hematocrit (> 15% for salmonids) is anemia, which can be the result of bleeding from trauma/injury, nutritional deficiencies or diseases. A high hematocrit (> 50% for salmonids) can be an indicator of dehydration or blood cell production disorders (cancer).

3.1 Immunological Assessment -Mean lymphocyte density was affected by time and dose (P = 0.001; ANOVA). The mean lymphocyte density increased for all treatment groups during the first two-weeks of the trial; however during the next four weeks, those fish fed Fin-Immune™ had an increased density of lymphocytes (Fig. 1). At week 6, the mean lymphocyte density of the fish fed the 20 and 30 mg g-1 dosages was significantly (P = 0.02; ANOVA) higher than the control fish and those fed 10 mg g-1. During the trial, some individual fish fed Fin-Immune™ had very high lymphocyte densities (7.5-8.5 cells per 100 erythrocytes), basically twice that recorded for salmonids [7]. During weeks 8-10, there were no differences in mean lymphocyte density and the values began to decrease, but the mean density at week 10 was still significantly greater than at week 0 (P < 0.001; ANOVA). This decrease was associated with the switch to regular (non-immunomodulator) feed and further indicates the efficacy of the product. These data also suggest a minimum feeding period of four weeks to achieve an increased immune response but the elevated response may be of short duration. Conversely, there were no trends among the neutrophil density data (Fig. 2) and limited trends among the hematocrit data (Fig. 3). There was variation among mean neutrophil density data as indicated by the large error bars in Figure 2. As with the lymphocyte data, some individual fish fed Fin-Immune™ had very high neutrophil densities (7.5-13.5 cells per 1000 erythrocytes); much greater than previously reported. This implies that these cells were activated (or 'primed'); however, their ephemeral nature contributed to data variation. Consequently, their role in a disease challenge is unpredictable from these data. After week 0, the blood hematocrit values for all groups decreased associated with the first blood sampling trial. Throughout the trial, all hematocrit values were well within normal values (25-40 %) reported for salmonids [7]. From week 0 to week 4 there were some significant differences in the mean hematocrit values; however, there were no apparent trends in these differences, thus a tank effect may have occurred. For example, between weeks 0 - 4, the mean hematocrit trends reversed twice and from week 6 - 10, all values were not significantly different. Although highly speculative, the hematocrit data might indicate that the fish fed Fin-Immune™ (20 and 30 mg g-1) were initially able to build up their blood cell density at a slightly faster rate (4 weeks) than the control fish and those fed 10 mg g-1.

3.2 Biological Assessment - During the initial health assessment (week 0) all fish were disease-free and of good health status. Water quality variables throughout the trial were within optimal ranges: oxygen (6.9- 8.7 mg L-1), temperature (10.3-16.6 °C) and pH (3.6-6.8). Mortality rates throughout the experiment were variable with an overall mortality rate of 4.76%. Trout fed the highest dose of Fin-Immune™ (30 mg g-1) had the highest mortality rate (5.24%); however, the lowest mortality rate was from the trout fed 20 mg g-1. Of the four highest mortality rates per tank, one was associated with each treatment, which suggests a tank effect rather than being related to the feed. Each mort was tested for parasites and bacteria and all were negative. As with the lymphocyte data, growth was affected by time and feeding dose (P < 0.001; ANOVA). At the start of the trial, there were no significant differences in mean length and weight (Fig. 4); however, the mean condition factor (g cm-3) was significantly greater (P < 0.038; ANOVA) in the 10 mg g-1 treatment group than the 30 mg g-1(Fig. 5). All fish grew well during the trial, with the highest growth rates (2.16-2.48 g day-1) during the first two weeks of the experiment and the lowest growth rates (1.54-1.97 g day-1) during the last two weeks of the experiment (Table 1). As a general trend, the growth was better among the control trout. At week 4, the mean weight of control fish was significantly greater (P < 0.015; ANOVA) than those fed Fin-Immune™ at 20 or 30 mg g-1(Fig. 4). Similarly, during week 8, the mean condition factor (g cm-3) of control fish was significantly greater (P < 0.05; ANOVA) than those fed Fin-Immune™ at 30 mg g-1 (Fig. 5). Furthermore, at week 10, the mean weight of control fish was significantly greater (P < 0.05; ANOVA) than those fed Fin-Immune™ at 30 mg g-1 (Fig. 4). This trend indicates that the trout responded to a threshold dosage (30 mg g-1), such that, their immune system was essentially 'saturated.' Feeding these trout any higher dosage would result in further growth impairment. Growth reduction is a consequence of allocating more biochemical resources into producing immune cells rather than muscle or somatic growth. This saturation effect associated with a threshold dosage has also been reported by [6] and in the reviews of [3] and [2] for a variety of aquatic species. These growth data provide further evidence that 20 mg g-1 is a preferred dosage than 30 mg g-1 to use with cultured rainbow trout.

3.3 Conclusions - Studies reviewing the efficacy of using immunostimulants/immunomodulators in preventing/mitigating disease occurrence among cultured fishes [2], [3], [8] have demonstrated equivocal results (variable immune responses and protection). Unfortunately, many of these studies were simple trials that lacked repeatability and they used crude extracts of products (e.g., yeast or bacteria cell wall products). Furthermore, many studies used a vaccine approach by targeting the specific immune response (e.g., antibody production). The present *Phase I* study assessed the efficacy of several dosages of the commercial oral immunomodulator, Fin-Immune™ when added to a diet for trout. Two (20 & 30 mg g-1) of the three doses showed very promising results when examining blood lymphocyte density. However, growth was compromised at

the higher dosage, thus 20 mg g-1 is the preferred dose to use for a *Phase II* disease challenge.

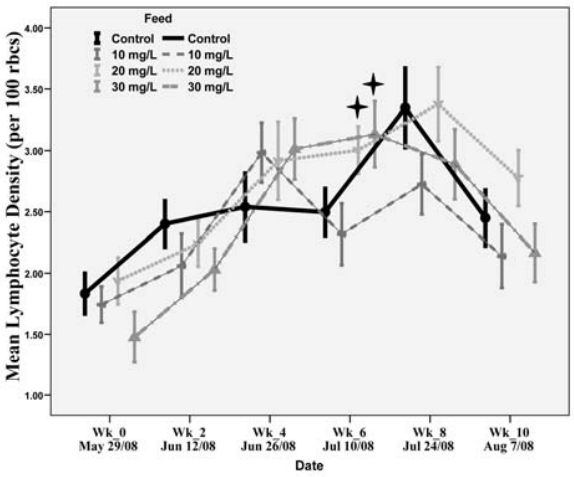


Fig. 1 Mean (+ S.E.) blood lymphocyte density (per 100 erythrocytes) of rainbow trout fed varying dosages of Fin-Immune™ at week 0 (start) to week 6. At week 7, all fish received the control diet (0 mg Fin-Immune™) until week 10. Symbols (✦) indicate significant difference from others at that week.

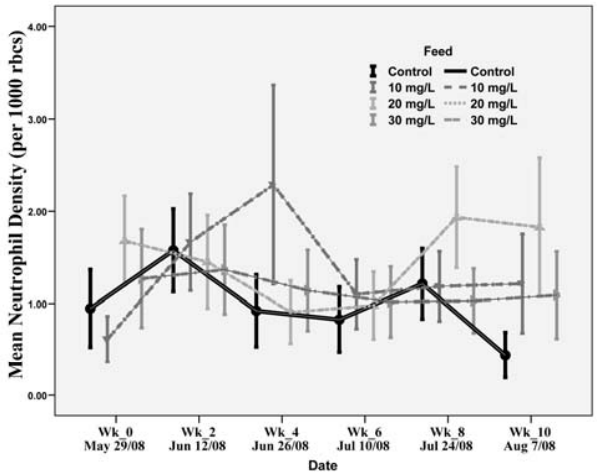


Fig. 2 Mean (± S.E.) blood neutrophil density (per 1000 erythrocytes) of rainbow trout fed varying dosages of Fin-Immune™ at week 0 (start) to week 6. At week 7, all fish received the control diet (0 mg Fin-Immune™) until week 10.

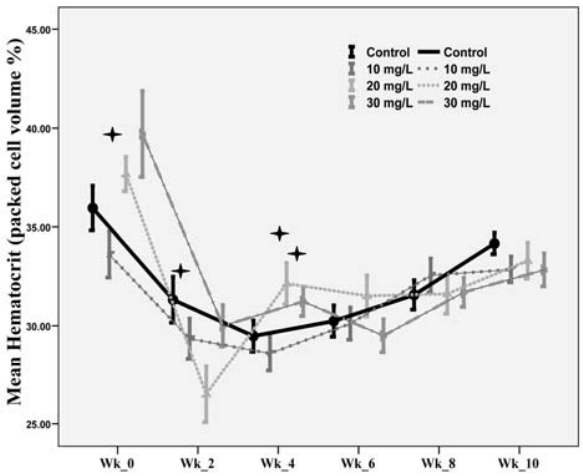


Fig. 3 Mean (± S.E.) blood hematocrit (% packed cell volume) of rainbow trout fed varying dosages of Fin-Immune™ at week 0 (start) to week 6. At week 7, all fish received the control diet (0 mg Fin-Immune™) until week 10. Symbols (✦) indicate significant difference from others at that week.

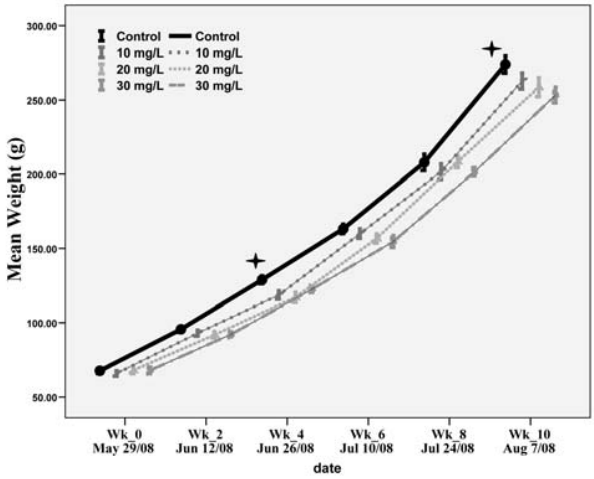


Fig. 4 Mean (± S.E.) weight (g) of rainbow trout fed varying dosages of Fin-Immune™ at week 0 (start) to week 6. At week 7, all fish received the control diet (0 mg Fin-Immune™) until week 10. Symbols (✦) indicate significant difference from others at that week.

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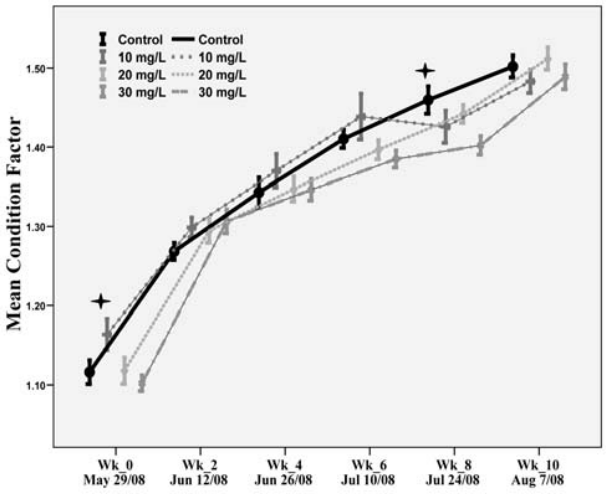


Fig. 5 Mean (± S.E.) condition factor [100 (weight · length $^{-3}$)] of rainbow trout fed varying dosages of Fin-Immune^{TM} at week 0 (start) to week 6. At week 7, all fish received the control diet (0 mg Fin-Immune^{TM}) until week 10. Symbols (✚) indicate significant difference from others at that week.

TABLE I
MEAN SPECIFIC GROWTH RATE (G · DAY^{-1}) OF RAINBOW TROUT FED VARYING DOSAGES OF FIN-IMMUNE^{TM} AT WEEK 0 (START) TO WEEK 6. AT WEEK 7, ALL FISH RECEIVED THE CONTROL DIET (0 MG FIN-IMMUNE^{TM}) UNTIL WEEK 10.

Feed	Wk 0-Wk 2	Wk 2-Wk 4	Wk 4-Wk 6	Wk 6-Wk 8	Wk 8-Wk 10
Control	2.48	2.13	1.69	1.73	1.97
10 mg g^{-1}	2.47	1.74	2.14	1.66	1.89
20 mg g^{-1}	2.16	1.72	2.09	2.04	1.54
30 mg g^{-1}	2.19	2.04	1.66	1.89	1.63
Avg. Total	**2.32**	**1.92**	**1.89**	**1.83**	**1.76**

IV. FUTURE STUDIES

Research on oral immunomodulators has many applied aspects. For example, such compounds provide an alternative, stress-free method of enhancing *non-specific* immunity of cultured fish. In addition, efficacy trials, such as the present study, will assist in preventing any overuse/underuse of this product when used commercially at a fish farm or hatchery. Using the most efficacious concentration of immunomodulator (20 mg g-1) defined in this Phase I study, the next logical testing step would be to try a bacterial disease (e.g., Bacterial Kidney Disease from *Renibacterium salmoninarum* or furunculosis from *Aeromonas salmonicida*) challenge as a Phase II study. In a Phase II study, the fish should be fed Fin-Immune^{TM} for a minimum of six weeks prior to bacterial challenge and it would be useful to test three groups of fish: (i.) continuously fed Fin-Immune^{TM}; (ii.) fed Fin-Immune^{TM} only until the onset of disease challenge and (iii.) control group, not fed Fin-Immune^{TM}.

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