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RESEARCH PAPER

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Relationship between Non-Specific Immune Response and Body Size in Cultured Rainbow Trout

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Abstract

The aim of this study was to evaluate some blood parameters involved in non-specific immunity in order to assess the possible correlation with biometric indices (weight and length) in cultured rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792). For this purpose sixty Italian cultured trout (350.00-950.00 g weight range, 28.00-40.00 cm length range) reared in a Sicilian farm were utilized in this research. In the farm, physico-chemical characteristics of water were measured.

From each fish, blood samples were collected to evaluate White Blood Cells Count (WBC), Thrombocyte Count (TC), Ceruloplasmin (Cp), Total Protein (TP), serum albumin and serum protein fractions.

Linear regression analysis was used to evaluate the relationship between the biometric indices and non-specific immune parameters. Results showed a statistically significant correlation between weight and length with ceruloplasmin. Our study suggests that non-specific immune response in fish changes in relation to body size; this result is useful in order to obtain a wider knowledge of the non-specific immune parameters that may allow diversified vaccination plans in relation to the different size.

Introduction

Cultured Rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) is a species of fish with high commercial value. In the past, Italy and France were considered the most important producers of trout and with 39,700 tonnes/year trout is still the major species farmed in Italy (Manfrin, Bovo, Selli, & Ceschia, 2009). However the commercialization of this fish is constantly subjected to damage caused by several marketing problems and by increasing management costs. In cultured rainbow trout culture more resources must be investedto maintain the health status of this fish. Fish need a good nutrition and adequate water quality for their welfare and to be protected against infectious diseases. The immune system maintain the health of fish and is susceptible to a variety of stressors in the aquaculture environment. The continuous expansion of intensive aquaculture caused a proportional increase of disease problems in fish. In fact in most culturing farms, occasional mass mortalities caused by different diseases such as bacterial infective diseases, is one of main reasons for decreasing the degree of its production. Therefore, fish diseases representa serious threat to economic viability in this and any other aquaculture practice becoming a major limiting factor. To control infectious diseases current methods used are represented by vaccination, strengthening hygienic measures, drug therapy and eradication of infected populations. In fish, several elements of non-specific immunity play an important role in preventing infection (Stosik & Deptuła, 1990). Recent studies of fish immunity are mainly focused on understanding on response mechanisms of immune system to foreign agent.

The immune system of fish and other vertebrates can be divided into non-specific and specific arms. The non-specific, or innate immune system provides an immediate response to several invading pathogens. WBC are the most important body's non-specific defenses. Macrophages and granulocytes are particularly important in inflammation; they migrate to sites of pathogen infection and are then active in pathogen destruction.

As regards the thrombocytes, their possible roles of in immunity (including phagocytosis) it was detected

in previous works (Wigley, Hulme, & Barrow, 1999; Stosik, Deptuła, Travnicek, & Baldy-Chudzik, 2002).

Cp is an acute phase protein with important anti-inflammatory roles which is released in response to inflammation and infection processes. Cp is a parameter of the non-specific immunity that, as other acute phase proteins, modulates an immune response (Dunier, Siwicki, & Demael, 1991; Siwicki *et al*., 2000; Bayne & Gerwick, 2001). It is also involved in the transport of copper from hepatocytes to other tissues and in the regulation of hepatic iron mobilization. In fish, the Cp levels could be an important tool in the early detection of disease caused by agents of infection such as viruses and bacteria.

In organisms under toxic conditions, TP, albumin, globulin and the other protein fractions play an important role in the immunity and in the processes of osmotic balance. Because serum proteins include humoral elements of the non-specific immune system, high concentrations of TP, albumin and globulin could be the result of an improvement of non-specific immune response of fishes. Globulins are the precursors for the synthesis of immunoglobulins that play important functions in the immunity of the organisms towards diseased and toxic conditions. In previous study (Johnson *et al*., 2012) it was hypothesized that species with rapid development, rapid growth, and with a short life span, invest relatively little resources in defenses, whereas species characterized by a slowly development, a gradual growth, and with a longer life spans invest into costly defenses because they have a higher likely to encounter parasites. This would suggest some possible correlation between size and immune response in fish.

This research aims to study some blood parameters involved in non-specific immunity White Blood Cells Count (WBC), Thrombocyte Count (TC), Ceruloplasmin (Cp), Total Protein (TP), serum albumin and serum protein fractions in Italian farmed trout in order to evaluate the possible correlation of these parameters with biometric indices (weight and length). This could represent a contribut ionto knowledge of the immune system of cultured fish and to optimize the conditions of hygiene and the procedures for disease control.

Materials and Methods

Rainbow trout is a typical fish of cold water. A normal adult rainbow trout weighs about 2-3 kg; the maximum weight and length are 25.4 kg and 120 cm, respectively (Parisi *et al*., 2014).

The study was carried out on 60 rainbow trout (525.10±137.00 g weight,33.28±2.27 cm total length) provided from an Italian farmin Palazzolo Acreide (Siracusa, Italy) that consists of rectangular tanks (total number 11). The tank measured was 20 m in length, 5 m in width and 3.5 m deepwith volumes of 80 m^3. Fish were subjected at stocking densities of 25 kg/m^3 and natural photoperiod (11L/13D). All fish were fed daily at 08.00 h and 18.00 h, with standard commercial dry food. All fish were considered clinically healthy at the time of sampling (external examination for any signs of abnormalities or infestation).

In the farm, physico-chemical parameters (temperature, salinity, pH and dissolved oxygen) of water were measured using a multi parameter instrument (YSI 556 MPS - USA) and these parameters are shown in Table 1.

All fish in each tank were randomly captured in order to evaluated blood parameters and biometric data. Blood samples were collected from individual fish, and each sample was analyzed separately. Each fish per tank were randomly captured, anesthetized with tricaine methanesulfonate (MS-222, Sigma,St. Louis, MO, USA) (0.7 g L^{-1}), and blood samples were taken from the caudal vein. The sample was transferred into 2 different tubes, one containing ethylene diamine tetraacetic acid EDTA (Miniplast 0.6 mL; LP Italiana Spa, Milano, 1.26 mg/0.6 mL) as anticoagulant agent for the assessment of leucocytes and thrombocytes and the other without anticoagulant agent (Terumo Corporation, Japan) for the evaluation of biochemical parameters (ceruloplasmin, total plasma protein, serum albumin and serum protein fractions). During anesthesia, the fish were measured for their total length using an ictiometer (Scubla SNC, 600 mm, Italy) and weighted using anelectronic balance (Kern 440-49 N, Germany). The conditionfactor (K) was calculated with this formula $W \times 100/L^3$ where W is the weight of the fish in grams (g), L is the length of the fish in centimeters (cm). As reported by Davis & Lebourdais, 2007, for salmonids, K values usually fall in the range 0.8 to 2.0. For haematological analysis, blood sample with EDTA was gently mixed on a roller for 10 minutes at room temperature before automated analysis (HeCo Vet C, SEAC, Florence, Italy) for WBC and TC count. For biochemical analysis, blood samples were allowed to clot at room temperature, and then centrifuged at 2000 g for 10 min to separate serum. The resultant serum was pipetted into a sample cup before automated analyzer UV Spectrophotometer (SEAC, Slim, Florence, Italy) for evaluation of total protein. Serum protein fractions were

Table 1 Water quality values (Mean±SD) for the farm assessed during the experimental period

Water Parameters	Mean±SD
Temperature (°C)	16.60±0.46
Water salinity (gL^{-1})	0.30±0.10
Dissolved Oxygen (mgL^{-1})	7.33±0.15
pH	8.23±0.15

separated by zone electrophoresis on a buffered agarose gel at pH 8.8 on an automated electrophoresis system (Sel Vet 24, SELEO Enginering, Naples, Italy) according to the procedure described by the manufacturer.Serum proteins were separated into the following fractions in order of fastest to slowest mobilities: albumin, α, β, and γ-globulins. The relative concentrations (%) of the protein fractions were determined as the percentage of the optical absorbance. Albumin:globulin ratios (A/G) were computed from the electrophoretic scan.

Ceruloplasmin were determined colorimetrically (Florence, Italy) using the commercial test kit (Giesse Diagnostic, Italy).

All analysis were performedin triplicate and repeated three times with similar results.

Protocols of animal husbandry and experimentation were approved in accordance with the standards recommended by the Guide for the Care and Use of Laboratory Animals and Directive 2010/63/EU for animal experiments.

Statistical Analysis

Analytical data, represented as mean ± standard deviation (DS) are the averages of three analyses carried out by the same operator.

A one-sample Kolmogorov-Smirnov test was used to determine if the data was normally distributed. Relationships between variables (biometric indices) and blood parameters were determined using the Spearman correlation analysis. *P* values less than 0.05 were considered statistically significant. All data were analyzed using statistical software Prism v.5.00 (Graphpad Software Ltd., USA, 2003).

Results

Temperature, salinity, pH and dissolved oxygen (DO) values are shown in Table 1.

Tables 2 shows the statistical results of weight, length, condition factor, WBC, TC, ceruloplasmin, total protein, serum albumin and serum protein fractions obtained.

Regression analysis showed a linear relationship between a blood parameter and biometric indices for the studied species, in particular, Cp showed a significant positive correlation (P<0.0001) with weight and length. Person's correlation coefficientsare shown in Table 3. The other parameters did not show statistically significant correlation between weight and length.

Discussion

The values of the water quality parameters (temperature, salinity, pH and dissolved oxygen) obtained, and showed in table 1, results particularly suitable for this species.

In this study, WBC, TC, TP, serum albumin and serum protein fractions exhibit a variability in trout with different size (weight and length) as showed in Table 2; but did not show statistically significant correlations between weight and length. In contrast with previous research some authors showed a correlation between biometric indices and WBC, TC and TP in two species of farmed fish (Gilthead sea bream *Sparus aurata* and European sea bass *Dicentrarchus labrax*) (Fazio,Saoca, Casella, Fortino, &Piccione, 2015).Their study showed that in *Sparus aurata* WBC were negatively relatedto weight and length, while TC were positively related to weight. TP were negatively related to weight and length in *S. aurata* and positively in *D. labrax*. These results emphasize the importance of fish size in the interpretation of blood parameters in order to evaluate correctly the health status of the fish. It was reported that some blood parameters changed in fish in relation to the biometric indices (Jawad, Al-Mukhtar, & Ahmed, 2004; Adam & Agab, 2008). Even if these differences

Table 2 Statistical results for the evaluated parameters in rainbow trout (*Oncorhynchus mykiss*) (n = 60)

Parameters	Range	Mean ± SD	Median	95% confidenceinterval	25^{th}-75^{th} percentile
Weight(g)	350.00-950.00	525.10±137.00	480.00	489.70±560.50	440.00±562.50
Length (cm)	28.00-40.00	33.28±2.27	33.00	32.69±33.86	32.00±34.00
Conditionfactor (K)	1.110-1.820	1.40±0.14	1.40	1.36±1.43	1.30±1.48
WBC (x 10^3/µL)	18.20-22.55	20.36±0.80	20.33	20.16±20.57	19.77±20.93
TC (x 10^3/µL)	31.00-84.00	48.55±10.97	47.00	45.72±51.38	43.00±55.75
Ceruloplasmin (mgL^{-1})	10.00-50.00	26.50±9.30	30.00	24.10±29.00	20.00±30.00
Total Protein (gL^{-1})	23.00-40.20	30.90±3.40	30.90	30.00±31.80	28.50±32.80
Albumin (gL^{-1})	04.80-10.50	8.30±1.30	8.50	8.00±8.60	7.40±9.40
α-globulins (gL^{-1})	11.90-26.00	16.20±2.20	16.20	15.70±16.80	14.60±17.20
β-globulins (gL^{-1})	2.20-7.50	4.80±1.20	4.70	4.50±5.10	4.00±5.60
γ-globulins (gL^{-1})	0.40-2.80	1.50±0.50	1.40	1.30±1.60	1.10±1.70
Rapporto A/G (gL^{-1})	2.00-5.20	3.70±0.60	3.70	3.50±3.90	3.20±4.10

*Note: K (Condition Factor); WBC (White Blood Cells Count); TC (Thrombocyte Count).

could be due to the higher metabolic rate of bigger fish respect to smaller ones (Chaudhuri, Pandit, & Benerjee, 1986), are genetically established (Raizada, Jain & Raizada, 1983). This is an important aspect, infactnon-specific immune system provides an immediate response to an invading pathogen, so it's possible to have a different response in relation to fish size. As indicated in Table 3, Cp showed a significant positive correlation (P<0.0001) and a coefficient of correlation of 0.87 with the weight, and equally a correlation with length with an higher value of r (0.91), as showed in Figure 1a, b.Cp is an acute phase protein that together with the c-reactive and serum amyloid A proteins assumes considerable importance in the monitoring of particularly widespread infections in aquaculture. The application of acute phase proteins in veterinary clinical practice is a field which has raised an increasing interest in the last years; their functions and influences on the organism are showed in several previous studies (Murata, Shimada, & Yoshioka, 2004; Petersen, Nielsen & Heegard, 2004).

The measurement of the concentrations of acute phase proteins can detect the presence of infection or pathological lesion because these values are influenced by inflammatory conditions. However the use of these analytes as indicators of animal health and in the detection of diseases in farm animal is not widely documented. Therefore, in cultured rainbow trout and

Table 3. Correlation matrix among the evaluated parameters of rainbow trout (*Oncorhynchus mykiss*) (n = 60)

	WEIGHT	LENGTH
WBC	0.20	0.19
TC	-0.05	-0.06
CERULOPLASMIN	0.87*	0.91*
TOTAL PROTEIN	-0.11	-0,17
ALBUMIN	-0.18	-0.19
α-GLOBULIN	-0.10	-0.17
β-GLOBULINE	-0.03	-0.03
γ-GLOBULINE	0.20	0.14
RAPPORTO A/G	-0.14	-0.11

When the asterisk appears, the correlation is significant for P<0.0001
Note: WBC (White Blood Cells Count);TC (Thrombocyte Count).

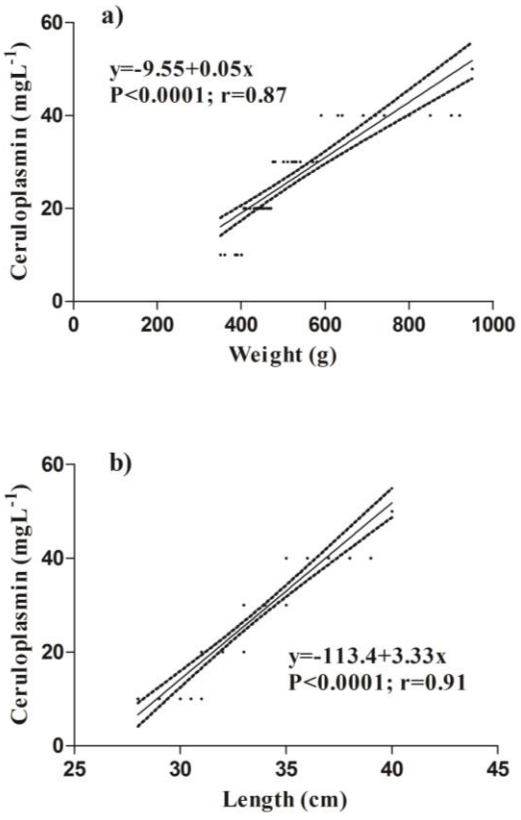

Figure 1. Positive correlation between weight and ceruloplasmin(*r* = 0.87, P< 0.0001) (a), and between length and ceruloplasmin(*r* = 0.91, P< 0.0001) (b) in rainbow trout *Oncorhynchus mykiss*.

in the other fish species where some diseases increase the acute phase proteins is important to know the relationship between biometric indices and Cp value in fish. Some study were previously conducted about ceruloplasmin in fish and in particular, in trout. Yonar, Sağlam and İspir (2010) examined the plasma Cp level in *O. mykiss* feed on a diet with different doses of sulfamerazine. In general, sulfamerazine and its derivates are often used in fish farm against several fish diseases. Their study showed that sulfamerazine has an immuno suppressive effect on non-specific immunity resulting in an increase in plasma ceruloplasmin level. Another research conducted by Yildiz, Meric and Ergonul (2009) reported the possible effects of the exposure to formalin and chloramines on some non-specific immune parameters in rainbow trout. Formalin is used to treat ectoparasitic infections, particularly protozoa; chloramine-T is used as a disinfectant and as a treatment for bacterial gill disease and occasionally fin-rot. Their results did not show any significant changes of Cp in fish exposed to both formalin and chloramine-T.

Yada, Muto, Azuma and Ikuta (2004) evaluated the effects of prolactin and growth hormone on plasma levels of lysozyme and ceruloplasmin in *O. mykiss*. Our results, showed a positive correlation between ceruloplasmin levels and growth indices highlighting the linkage between body size an serum ceruloplasmin.

Liu *et al*. (2011) and Sahoo *et al*. (2013) studied the ceruloplasmin gene in order to established its potential for physiological antioxidant responses in channel catfish after bacterial infection with *E. ictaluri* and iron treatment and its association with resistance to *Aeromonas hydrophila* in rohu *Labeo rohita* respectively.

Despite the presence of these and other previous research about ceruloplasmin in fish, in literature there is not report about a relationship between Cp and biometric indices.

In conclusion, our results showed that biometric indicescan influence in a directly proportional the serum concentration of Cp, while for WBC, TC, TP, serum albumin and serum protein fractions were not correlation with a weight and length in cultured rainbow trout. Our results showed the importance of fish size in the interpretation of some non-specific immune parameters in order to evaluate correctly the immunity assessment in cultured fish.

Further investigation is necessary to deepen this research using other farmed species and other immune parameters, in order to obtain a wider knowledge of the non specific immune parameters that may allow diversified vaccination plansin relation to the different size.

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