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Article

Rainbow Trout Hematology Coinciding with Metabolic Requirement

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Abstract

Rainbow trout (*Onchorhynchus mykiss*) are an important aquaculture species yet there are few diagnostic tools available to assess their health. There is little published information regarding age-related changes in blood values of juvenile fish. It is important to evaluate juvenile fish, as this is the time they are raised in aquaculture settings. Determining age-related changes in the blood values of fishes would further develop clinical pathology as a diagnostic tool, enhancing both fish medicine and the aquaculture industry. The results of standard hematology and clinical chemistry analysis were evaluated in juvenile rainbow trout at 4, 6, 9, 15 and 19 months of age. Values for PCV and RBC indices were significantly lower, and plasma protein concentration was significantly higher in younger fish. Total WBC and lymphocyte counts were significantly higher in fish at 6 and 9 months of age, while neutrophil and monocyte counts were higher at 6, 9 and 15 months. Eosinophil counts were significantly higher in 9 month old fish. The majority of hematologic values fell within previously established reference intervals, indicating that only slight modification to the intervals is necessary for evaluating hematologic results of rainbow trout at different ages. The following analytes deviated sufficiently from adult reference intervals to warrant separate reference values: plasma protein concentration at 4 months, WBC and lymphocyte counts at 15 and 19 months, and thrombocyte-like-cells at 9 months of age. Values for most biochemical analytes were significantly different among age groups except for creatinine and potassium concentrations. Comparisons with reference intervals were not made for biochemical analytes, because established reference intervals were not available.

Keywords: Age, fish, hematology, plasma chemistry, rainbow trout

Introduction

Rainbow trout (*Onchorhynchus mykiss*) are an important aquaculture species raised for food and sport release, yet little is known about their response to diseases. There are few tools available to diagnose and monitor disease in these and other species of fish. One such tool, the analysis of blood, can detect acute and chronic pathophysiologic changes attributable to nutrition, water quality, toxicants, and diseases. However, routine diagnostic hematology and clinical chemistry analyses are not used extensively in fish medicine due to the lack of reference intervals for various fish species. Also, external factors such as water quality, diets, culture conditions, and age or sex of the fish can affect some blood values [1-8]. Numerous studies dealing with factors that influence blood values have been published, yet there is little consensus on the specific influence of these factors. In many of these studies, only a few fish were used or only a few analytes were measured. Additionally, many studies were field experiments, in which variations in values were associated with the capture of wild fish and transportation of the blood to the laboratory for analysis [1,6-8].

Fish age is often cited as a factor affecting blood values. A review of the literature revealed few studies where age changes were systematically documented. Often, differences in analytes were compared between young juvenile fish and sexually mature fish, the latter with a seasonal reproductive cycle. These data are of limited use for fish health professionals because the majority of production fish are juveniles, which are harvested before or as they reach sexual maturity. In order to document age-related changes in hematologic and plasma biochemical values of juvenile fish, we sampled a single population of rainbow trout between 4 months (fingerlings) and 19 months (market size) of age.

Materials and methods

Water quality

Water quality was monitored using a commercial water analysis kit (Hach Company, Loveland, CO, USA) as follows: pH, temperature, and ammonia concentration were measured daily; alkalinity, hardness, nitrite, nitrate and dissolved oxygen concentrations were measured twice weekly. Water quality values on the days that the fish were sampled are shown in **Table 1**, and were representative of water quality over the duration of the 19 months study.

Table 1 Water quality values and mean weights and lengths of fish on sampling days at different ages throughout the 19 months study.

Parameter*	**Age (months)**				
	4	**6**	**9**	**15**	**19**
Temperature	18	20	18	20	20
pH	7.5	7.4	7.3	7.6	7.6
NH_3 non-ionized (mg/L)	0.006	0.005	0.005	0.006	0.004
NO_2-N (mg/L)	0.09	0.03	0.04	0.03	0.10
NO_3-N (mg/L)	8	6	3	5	4
Alkalinity (mg/L)	130	58	86	86	84
Hardness (mg/L)	253	232	180	242	187
Dissolved oxygen (mg/L)	7.8	8.0	7.3	7.4	7.7
Fish Weight (g)	33	42	64	125	166
Fish total length (cm)	14.0	15.1	17.0	23.9	26.2

* Hematological study

Parameter*	**Age (months)**			
	6	**9**	**15**	**19**
Temperature	20	18	20	20
pH	7.1	7.3	7.7	7.5
NH_3 non-ionized (mg/L)	0.001	0.003	0.006	0.003
NO_2-N (mg/L)	0.01	0.07	0.05	0.01
NO_3-N (mg/L)	6	6	5	5
Alkalinity (mg/L)	42	83	68	68
Hardness (mg/L)	215	112	291	214
Dissolved oxygen (mg/L)	7.5	7.3	7.2	7.5
Fish Weight (g)	44	76	131	215
Fish total length (cm)	15.4	19.7	24.0	27.7

* Biochemical study

Fish

Original rainbow trout (*Onchorhynchus mykiss*), were obtained from a Laribal trout hatchery as 3 day old fry. The fish were raised in a 3,600 L recirculating system with a high pressure sand filter and a trickle biofilter. Stocking density (approximately 13 g/L) remained constant throughout the study as fish were removed for other experiments during the 19 month growth period. The fish used in this study were not bled for at least 2 wk after the removal of fish from the system. All fish were fed a pelleted diet (prepared at Kokernag feed mill) for the duration of the study, at approximately 2 % of body weight per day. By the start of the study, the fish were accustomed to routine procedures such as daily cleaning, water changes, water sampling, and periodic netting in order to minimize any hematologic or biochemical changes associated with routine handling. Each fish was weighed and measured at the time of sampling (**Table 1**).

Blood collection

Fish were bled at the following ages: 4, 6, 9, 15 and 19 months. Hemoglobin concentration and biochemical values were not determined on 4-month-old fish due to insufficient sample volume. All fish were fasted 24 h prior to sampling and appeared clinically healthy and in good body condition. During sampling, care was taken to minimize stress in the netted fish and in fish remaining in the tank. All fish were netted within 20 sec and were immediately anesthetized with aerated and buffered tricaine methanesulfonate (150 mg/L MS-222, Sigma Chemical Co, Saint Louis, Mo, USA). Blood samples were collected from the caudal tail vessels with 21 or 23 gauge needles and 1 or 3 cc syringes as soon as the fish lost equilibrium and did not respond to handling, but before ventilatory response was noticeably depressed (–after ~30 seconds of anesthesia). To prevent repeated sampling, fish were not returned to the original tanks after blood collection.

Hematologic analysis

Collected blood was placed into individual EDTA-containing tubes. For each age group, 20 fish were sampled for hematologic analysis. Packed cell volume (PCV) was determined in microhematocrit tubes after centrifugation for 5 min. Plasma protein concentration was determined on the plasma in the microhematocrit tube with a clinical refractometer (Reichert-Jung, Buffalo, NY, USA). Total RBC and WBC counts were determined manually with a Neubauer hemacytometer using Natt-Herrick's solution as diluents [9]. Cell counts were determined manually because nucleated RBCs prevent accurate enumeration of cell counts on automated analyzers [10]. Since thrombocytes could not be distinguished from WBC on the hemacytometer, both were counted together; thrombocytes were enumerated and subtracted from the WBC count during the differential count. Blood smears were made using EDTA anticoagulated blood and stained with Wright's-Giemsa for differential counts. Leukocytes and thrombocytes were counted until 200 WBC were enumerated. Hemoglobin was determined using the cyanomethemoglobin method (Sigma Chemical Co); samples were centrifuged prior to reading the absorbance to remove nuclear debris. The MCV, MCH and MCHC were calculated by standard formulas.

Biochemical analysis

Blood was collected as described for hematologic analysis and placed into individual heparinized tubes held on ice. The tubes were centrifuged immediately at 2,750 g and the plasma promptly removed and frozen at –10 °C. Plasma collected from 6 and 9 month old fish was of insufficient volume, such that samples from 2 fish of the same age were pooled to provide composite samples of sufficient volume for analysis. For each age group, 20 samples (composite or individual) were analyzed. Samples were analyzed within 10 days of collection and thawed immediately prior to analysis. Samples were analyzed using an automated dry chemistry system (Kodak Ektachem 700, Rochester, NY, USA) for the following analytes: total protein, albumin, creatinine, and total bilirubin concentrations, alkaline phosphatase (ALP) and aspartate aminotransferase (AST) activities, and glucose, cholesterol, ammonia, sodium, chloride, potassium, calcium, magnesium, and phosphorus concentrations. The concentration of globulins was calculated (total protein minus albumin concentrations).

Statistical analysis

Statistical analysis was conducted using a commercial statistical program (Statistix, version

4, Tallahassee, FL, USA). Differences in results among the 5 age groups were determined with a Kruskal-Wallis one-way nonparametric ANOVA. When significant differences ($P < 0.05$) between groups were observed, a Tukey's means comparison test was used to identify which groups were different.

Results

Results of hematologic analysis at different ages are shown in **Table 2**. The PCV and MCV values were significantly lower in 4 month old fish compared to fish at other ages. The hemoglobin concentration, MCH, and MCHC were significantly lower in 6 month old fish than in fish at other ages. Plasma protein concentration was significantly higher at 4 and 6 months of age. Erythrocyte counts were significantly higher at 9 and 15 months of age. Total WBC, small lymphocyte, large lymphocyte, and thrombocyte-like cell (TLC) counts were significantly higher in 6 and 9 month old fish, while neutrophil and monocyte counts were higher at 6, 9 and 15 months of age. Eosinophil counts were significantly higher at 6 months of age.

Table 2 Mean hematologic values of rainbow trout between 4 and 19 months of age (n = 20 for each age group).

Analyte	Age (months)				
	4	**6**	**9**	**15**	**19**
PCV (%)	26	33	35	35	32
Hemoglobin (g/dL)	ND	6.8	8.7	8.6	7.9
RBC ($\times 10^6/\mu L$)	3.33	3.59	4.05	4.06	3.68
MCV (fL)	78	93	86	87	88
MCH (pg)	ND	18.9	21.4	21.2	21.7
MCHC (g/dL)	ND	20	25	25	25
WBC ($\times 10^3/\mu L$)	36.7	56.7	71.5	34.4	23.5
Small lymphocytes ($\times 10^3/\mu L$)	31.6	44.4	55.3	26.7	18.1
Large lymphocytes ($\times 10^3/\mu L$)	2.3	5.3	7.8	1.6	1.5
Neutrophils ($\times 10^3/\mu L$)	0.5	1.3	1.6	2.0	0.7
Monocytes ($\times 10^3/\mu L$)	0.4	1.6	2.2	1.7	1.0
Eosinophils ($\times 10^3/\mu L$)	0.8	1.0	0.2	0.5	0.6
Thrombocyte like cells ($\times 10^3/\mu L$)	1.0	3.2	4.4	1.9	1.7
Thrombocytes ($\times 10^3/\mu L$)	25.2	46.5	45.9	35.3	40.8
Plasma protein (g/dL)	6.5	6.1	5.2	4.9	5.1

ND = not determined.

The majority of hematologic values for individual fish fell within previously established reference intervals for original cross, rainbow trout (**Table 3**) [2]. However, there were a few age-analyte combinations for which a number of values fell outside the reference interval, including PCV at 6, 9 and 15 months; hemoglobin concentration at 9 and 15 months; plasma protein concentration at 4 months; WBC and lymphocyte counts at 15 and 19 months; and TLC count at 9 months. Results of plasma biochemistry analysis are shown in **Table 4**. Concentrations of total protein, globulins, phosphorus, and cholesterol were significantly lower in fish at 9 and 15 months of age. At 6 months, ammonia concentration and AST activity were significantly higher, and sodium concentration was lower. Significant differences among groups also were noted for ALP activity, and chloride, glucose, and bilirubin concentrations (**Table 4**). Biochemical values at various ages were not compared with reference intervals as biochemical reference intervals have not been determined for original cross hybrid striped bass.

Table 3 Range (minimum - maximum) of hematologic values for rainbow trout at different ages (n = 20 for each age group), with comparison to established reference intervals.

Analyte	Age (months)					
	4	6	9	15	19	Reference intervals
PCV (%)	20-32	28-37	27-41	32-41	29-36	20-34
Hemoglobin (g/dL)	ND	4.6-8.0	6.5-9.9	7.4-10.3	7.3-9.4	4.2-8.4
RBC ($\times 10^6/\mu L$)	2.18-4.00	2.77-4.42	3.07-4.87	3.49-4.58	3.15-4.22	2.42-4.96
MCV (fL)	58-95	79-113	70-97	74-92	78-102	65-117
MCH (pg)	ND	16-24	18-24	19-24	19-25	16.2-24.8
MCHC (g/dL)	ND	16-25	20-26	22-27	22-27	19-26
WBC ($\times 10^3/\mu L$)	22.9-69.6	24.8-84.3	33.4-105.9	20.0-53.5	12.1-13.0	32.6-118.2
Small lymphocytes ($\times 10^3/\mu L$)	19.1-69.2	18.7-65.9	27.2-76.3	13.7-45.0	9.2-21.8	22.5-115.1
Large lymphocytes ($\times 10^3/\mu L$)	0.8-6.8	1.2-10.0	1.6-14.6	0.6-3.3	1.6-3.0	1.2-10.0
Neutrophils ($\times 10^3/\mu L$)	0-2.3	0-3.6	0-5.3	0.5-4.4	0.1-2.4	0-6.8
Monocytes ($\times 10^3/\mu L$)	0-1.1	0-4.2	0-5.7	0.8-2.3	0.2-2.5	0-3.2
Eosinophils ($\times 10^3/\mu L$)	0-2.7	0-2.0	0-3.0	0-2.0	0-1.6	0-2.7
Thrombocyte like cells ($\times 10^3/\mu L$)	0.1-1.9	0.7-5.1	0.8-8.7	0.7-4.8	0.5-4.3	0-5.3
Thrombocytes ($\times 10^3/\mu L$)	7.9-54.0	22.1-77.8	21.5-82.9	20.0-48.2	19.5-74.7	21.3-104.3
Plasma protein (g/dL)	5.9-7.4	5.5-6.4	4.5-5.7	4.2-5.5	4.5-6.0	4.1-6.2

Table 4 Mean plasma chemistry values of rainbow trout between 6 and 19 months of age (n = 20 for each group).

Analyte	Age (months)			
	6	9	15	19
Total protein (g/dL)	3.4	3.1	3.0	3.6
Albumin (g/dL)	1.3	1.2	1.3	1.4
Globulins (g/dL)	2.1	1.9	1.7	2.2
Creatinine (mg/dL)	0.2	0.2	0.2	0.2
Ammonia (µg/dL)	471	357	365	292
Total bilirubin (mg/dL)	0.1	0	0.2	0.2
Alkaline phosphatase (mU/mL)	72	66	72	58
Aspartate aminotransferase (mU/mL)	209	94	39	33
Sodium (mEq/L)	144	150	151	154
Potassium (mEq/L)	3.38	3.21	3.22	3.23
Chloride (mEq/L)	144	146	150	149
Calcium (mg/dL)	10.66	11.02	10.89	11.53
Magnesium (mg/dL)	2.0	2.0	2.4	2.1
Phosphorous (mg/dL)	9.0	6.7	6.6	9.2
Glucose (mg/dL)	167	136	88	111
Cholesterol (mg/dL)	204	161	164	188

Discussion

It is important to document age-related changes in blood values from growing, juvenile fish, since this is generally the age when fish are raised in aquaculture settings. Most fish are harvested before or as they reach adulthood because weight gain and feed conversion efficiency are greatest prior to sexual maturity. Changes in blood values can be expected as the hematopoietic tissues, the kidney and spleen, continue to develop and mature in juvenile fish [11]. Additionally, a fish's immune system continues to develop until adulthood [12].

A number of studies have documented changes in hematologic and biochemical values with age or size in fishes. These studies are limited by the use of comparisons between juvenile and adult fish, or by testing only wild-caught fish, in which other factors can influence blood variables. There are few controlled studies comparing blood values from fishes of known ages [13]. This lack of data makes comparison of our results with those from other fish species difficult; however, some similarities can be noted. In rainbow trout, PCV, hemoglobin concentration, MCV, MCH, and MCHC were all lower in juvenile fish compared to mature adults [14]. In the present study, although hematologic values were not determined in mature adults, higher values tended to occur in the older juvenile fish. The decrease in WBC and lymphocyte counts in older rainbow trout in this study was similar to the difference described by others between young and adult striped bass [14,15].

Previous studies have also demonstrated changes in biochemical values between juveniles and adults, and between fish of different sizes. As we observed in rainbow trout, protein levels in striped bass increased with age, both in juvenile and adult fish [13]. This increase was mainly due to an increase in the globulin fraction and to some extent the albumin fraction [13]. Glucose concentration in goldfish decreased with age [16]. This was also observed in hybrid striped bass; however in rainbow trout, both glucose and cholesterol concentrations increased as fish size increased [1]. No changes in bilirubin values were detected with age in rainbow trout [1].

Age-related changes in blood analytes of rainbow trout were surprisingly similar to those in mammals. In dogs, cows, pigs and sheep, PCV and hemoglobin values, and RBC and WBC counts increase from birth to a peak between 3 and 12 months of age; values then decrease to adult levels as the animal matures [17]. The peak may be similar to that seen in these values in rainbow trout at 9 months of age. Protein concentrations in most mammals are lower at birth and slowly increase to adult levels by 12 to 18 months of age, mainly due to increasing globulins and, to a lesser extent, albumin concentration [17]. In rainbow trout, total protein values increased between 15 and 19 months of age, mainly due to an increase in globulins. Additionally, as in mammals, ALP activity was high in younger rainbow trout compared to 19-month-old fish. It is not known whether fish have the same ALP isozymes that are found in mammals; however, the decrease in serum ALP activity with age may indicate a decrease in a bone-derived isozyme.

Water temperature differences can affect hematologic and biochemical values of rainbow trout and could be responsible for differences in results between fish of different ages. Previously we found no significant differences in hematologic results from rainbow trout maintained in water between 18 °C and 29 °C. Of the biochemical analytes, only glucose and calcium concentrations were affected by temperature, with glucose increased at low temperatures (10 - 18 °C) and calcium increased at high temperatures (24 - 29 °C). However, no significant differences attributable to temperature were seen in the present study. Water temperature varied from 22 - 26 °C. Glucose concentration was highest at 6 months of age (at 24 °C) but was not different at other ages, when the temperature ranged from 23 °C to 26 °C. Calcium values were highest at 19 months of age when the temperature was 23 °C.

This study was conducted with the goal of advancing clinical pathology as a diagnostic tool for use in fishes. Numerous studies have shown that factors such as age and sex, environmental conditions, and diet can influence fish blood values [1-8]. Few studies, however, have shown whether the differences in blood values are clinically relevant and whether they affect one's ability to diagnose medical disorders [4,5]. The first of 3 steps in developing clinical pathology as a diagnostic tool is to determine reference intervals for various blood analytes. The second step is to determine which physical or environmental factors influence blood values to the extent that they fall outside the initial reference interval; in some cases, separate reference intervals may be needed for specific conditions. The final step is to then determine how the analytes change under various infectious and non-infectious pathologic conditions.

When hematologic values at different ages were compared to reference intervals previously developed for a uniform population of 11-month-old, 124-g, original cross hybrid striped bass [2], the majority of values fell within reference limits. A number of fish had plasma protein concentrations (4 months), WBC and lymphocyte counts (19 months) and TLC counts (9 months) outside the reference interval (**Table 3**). Separate reference intervals should be generated for these analytes at these specific ages. For 3 analytes (MCHC at 15 months, plasma protein concentration at 6 months and monocyte count at 6 and 9 months), only a few individuals were slightly outside the reference range. This most likely reflected the narrow reference intervals, which were determined from a uniform population of fish. Values for PCV and hemoglobin concentration for most 9 and 15 month old fish also fell outside the reference interval. For these analytes, the authors believe the previously determined reference intervals were lower than usual for original rainbow trout. It has been our experience, since determining these reference intervals, that PCV and hemoglobin concentration for rainbow trout tend to run higher than the upper limit of these ranges. The reason for this is not known at this time.

Biochemical analytes were not compared with reference intervals because reference intervals for these analytes have not been published for rainbow trout. We can speculate that separate reference intervals would be needed for AST activity, and ammonia and glucose concentrations in fish of different ages.

In summary, there were significant differences in hematologic and biochemical values of rainbow trout as the fish aged. The changes with age were similar to those observed in rainbow trout and in mammals. Although some hematologic values were significantly different among age groups, the majority of the values fell within previously established reference intervals for hybrid striped bass. The slight differences in some blood values with age, although statistically significant, may not be clinically relevant. Thus, only slight modification of established reference intervals may be necessary to accommodate differences with age. Greater differences with age were noted for plasma protein concentration at 4 months, WBC and lymphocyte counts at 19 months and TLC count at 9 months of age, indicating that separate reference intervals should be generated for these analytes for rainbow trout at these ages.

Conclusions

The results of the present research work were very interesting. Values for PCV and RBC indices were significantly lower, and plasma protein concentration was significantly higher in younger fish. Total WBC and lymphocyte counts were significantly higher in fish at 6 and 9 months of age, while neutrophil and monocyte counts were higher at 6, 9 and 15 months. Eosinophil counts were significantly higher in 9 month old fish.

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