Hematology and Serum Chemistry Values for Winter Flounder (*Pleuronectes americanus*)

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ABSTRACT

Clinical analysis of blood to determine hematology and plasma biochemistry values is routinely used to assess the health of wild and domestic animals. Flounder culture is a fast growing segment of the U.S. aquaculture industry and tools are needed to monitor the health of these fish. This paper reports a complete hematologic and blood biochemistry profile for normal healthy winter flounder, *Pleuronectes americanus*, maintained in recirculated artificial seawater. The following hematologic values were determined: Packed cell volume, plasma protein, erythrocyte number, hemoglobin, mean cell volume, mean cell hemoglobin, mean cell hemoglobin concentration, and leukocyte, lymphocyte, neutrophil, monocyte, and thrombocyte numbers. A description of leukocyte morphology is presented. Additionally, the following serum biochemical values were determined: Total protein, albumin, globulin, sodium, potassium, chloride, calcium, phosphorus, magnesium, glucose, blood urea nitrogen, creatinine, total bilirubin, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase,

cholesterol and triglycerides. Analysis of blood parameters can enhance flounder culture by providing a means for the early detection and identification of infectious disease and of sub-lethal conditions affecting production performance.

INTRODUCTION

Flatfish culture is one of the fastest growing segments of the U.S. aquaculture industry. With the collapse of the flounder fisheries in the north Atlantic region, raising flounder in a controlled aquaculture setting has become a necessity. Additionally, these fish are frequently maintained in public aquaria and used for research. Successful culture and maintenance of flounder can be enhanced by developing a tool to monitor the health of fish in captivity. One such tool is the standardization of blood values, as has been developed for hybrid striped bass and tilapia (Hrubec et al. 1996; 1997a,b; Hrubec and Smith 2000, Hrubec et al. 2000, 2001). Interpretation of hematologic and serum chemistry data in diseased animals requires collection of baseline information on normal healthy individuals.

Hematological and serum chemistry analysis of blood for diagnostic purposes has been used extensively for many mammalian, avian and reptilian species. The rapidly growing aquaculture industry will increasingly need to utilize information of this type in order to assess the health status of cultured fishes. Unfortunately, hematology use in aquaculture remains limited. This is mainly because reliable baseline blood values have not been determined for most fish species. To be of use, the blood values need to be determined on a sufficient number of individuals maintained under well-defined environmental conditions using standardized analytical techniques.

This paper reports baseline values for normal healthy winter flounder, *Pleuronectes americanus*, maintained in a recirculation system with artificial seawater. Previous studies have determined blood parameters for winter flounder (Levin et al. 1972; Umminger and Mahoney 1972; Fletcher 1975; Bridges et al. 1976; Mahoney and McNulty 1992). These studies, however, are limited as only a few parameters were determined or only a small number of fish were used for each determination. Additionally in some studies, fish were wild caught or were only

acclimated a few hours prior to collection of blood samples, resulting in blood values masked with a stress response. The objective of this study was to report a complete comprehensive blood profile for winter flounder acclimated to captivity using a sufficient number of fish to provide representative baseline values.

MATERIALS AND METHODS

Adult winter flounder were collected in July using an otter trawl in Niantic Bay near Niantic, CT, USA. The average mean weight of the flounder was 326 +/- 162 g and the total length was 28.8 +/- 4.4 cm. They were acclimated at Mystic Aquarium in a 6,000 L insulated fiberglass tank with an oyster shell substrate for 3 weeks prior to initiating the study. The fish were maintained at a stocking density of 3 g/L in recirculated synthetic seawater with the following composition: salinity 32-34 ppt, pH 8.1-8.3, NH_3 < 0.05 mg/L, NO_2 = 0 mg/L, NO_3 < 100 mg/L, Hardness > 2.5 mEq/L, Ca^{2+} = 400 mg/L, PO_4 < 5 mg/L, Iodine = 0.06 mg/L at 18°C. The water quality was monitored daily and maintained within the above limits by water changes with new artificial seawater (~10% exchange per day). The photoperiod was approximately 16-h light and 8-h dark to simulate natural light conditions for the season. Fish were fed three times a week to satiation (approximately 2% body weight) with a commercial pelleted diet (Ralston Purina Company, St. Louis, MO, USA) and freshly thawed chopped capelin (*Mallotus villosus*). Ten fish were necropsied at the end of the study for internal evaluation. These ten fish exhibited no gross internal or external lesions; and on internal morphological exam only showed a uniform hepatic lipidosis as is normal for flounder. No ectoparasites were observed on skin scrapes or gill biopsies of any fish.

A total of 30 fish were sampled from the stock tank in two groups of 15 fish each. The two sampling days were 15 days apart. Fish were anesthetized individually with buffered tricaine methanesulfonate (MS-222, Sigma Chemical Co., St. Louis, MO, USA) at a dose of 25 mg/L. When sedated, the fish were bled, weighed, measured, and had skin scrapings and gill biopsies performed. Blood samples of 2.5 to 3 mL were collected from the caudal vessels with a 25 gauge needle and a 3 ml syringe.

Blood was divided between an EDTA (ethylenediamine-tetraacetic acid) treated tube and a plain serum tube. Serum tubes were maintained on ice until the blood had clotted. Clotted blood was centrifuged at 2,500 x g for 5 min and the serum removed and kept frozen at –70°C until analysis. Serum was analyzed using a Cobas B10 Serum Analyzer (Roche, Switzerland) at the Pfizer Central Research Laboratory (Groton, CT, USA). The following analytes were determined: total protein, albumin, creatinine, blood urea nitrogen (BUN), total bilirubin, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), cholesterol, triglycerides, glucose, phosphorus, chloride and magnesium. A Cobas Fara Source Analyzer (Roche, Switzerland) with an ion selective electrode was used to measure sodium, potassium, and calcium. Globulin was calculated from the total protein minus the albumin.

Hematological analytes were determined from the EDTA anticoagulated blood. EDTA was superior to heparin as an anticoagulant in both preventing clot formation and preserving cellular morphology. Microhematocrit tubes filled with anticoagulated blood were centrifuged at 2,500 x g for three minutes and the hematocrit values determined. Plasma protein was determined on the microhematocrit supernatant using a temperature compensated refractometer. Total RBC count was determined manually, using an improved Neubauer hemacytomer with Natt-Herrick's solution as a diluent (Natt and Herrick, 1952; Stoskopf 1993). Blood smears, using EDTA-treated blood, were stained with Wright-Geimsa stain and were used to determine the leukocyte, thrombocyte, and differential WBC counts as follows. Relative percentages of erythrocytes and combined leukocytes-plus-thrombocytes were determined on 1,500 cells. The percentage of combined leukocytes-plus-thrombocytes was then multiplied by the total RBC determined on the hemacytometer to provide the total-leukocyte-plus-thrombocyte count. For the differential count, all leukocyte types and thrombocytes were counted until 200 leukocytes and a variable number of thrombocytes were enumerated. The percentages of each leukocyte type and of thrombocytes were multiplied by the total-leukocyte-plus-thrombocyte count to give the final cell count for each cell type. This method of determining total WBC and differential counts has been used with fish and avian blood (Hrubec et al. 1996; 1997a,b; Zinkl 1986), as automated counters are inaccurate for species with nucleated red blood

cells (Huffman and Arkoos 1997). Hemoglobin was determined using the cyanomethemoglobin method (Sigma Chemical Co, St. Louis, MO, USA). All hemoglobin test samples were centrifuged prior to determining sample absorbance in order to remove disrupted nuclear material. The red blood cell indices, mean cell volume (MCV), mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) were calculated using standard formulas (Stoskopf 1993).

RESULTS AND DISCUSSION

Although flounder culture is one of the faster growing segments of the aquaculture industry, few studies have reported information on normal hematologic values. The published literature consists of studies examining the effect of season (Umminger and Mahoney 1972; Bridges et al. 1976; Dawson 1990), capture stress (Fletcher 1975) and heavy metal toxicity (Calabrese et al. 1975; Dawson 1979) on only a few selected values. Additionally, the numbers of normal fish in these studies were often few in number, and in some instances, the fish were bled after only a short acclimation period to laboratory settings. It has been determined that a period of at least 5 days is necessary for hematologic values to return to normal after capture stress (Fletcher 1975; Bourne 1986). Therefore, although the previous studies on flounder hematology are helpful in determining the effects of environmental factors and stress, they have limited diagnostic utility.

The results of the hematologic determinations form 30 fish are listed in Table 1. Values for serum chemistry analytes are listed in Table 2. Overall, the values were similar to those reported previously for flounder and other species of fish. The wide ranges in value for the different leukocyte types, particularly lymphocytes, are frequently seen in fish held in recirculation systems (Hrubec et al. 1996; Hrubec and Smith 2000; Hrubec et al. 2000). Although individual variation for some of the blood values appears large, it is still possible to detect variation in hematologic values associated with pathological conditions (Hrubec et al. 1997b and unpublished data).

The hematocrit values determined on the flounder in the present study were similar to hematocrits reported by Umminger and Mahoney (1972), Bridges et al. (1976), and Mahoney and McNulty (1992). However,

Table 1. Hematologic values for adult winter flounder (Pleuronectes americanus) maintained in captivity.

Analyte	N	Range	Mean	Stds[1]
PCV[2] (%)	30	19-31	24.8	3.6
Plasma Protein (mg/dl)	30	3.8-7.0	4.9	0.9
Erythrocytes (x 10^6/µl)	30	1.50-3.14	2.22	0.33
Hemoglobin (g/dl)	30	5.0-7.4	6.0	0.69
MCV[3](fl)	30	86.8-227.3	116.4	25.8
MCH[4] (pg)	30	22.0-38.7	27.3	3.7
MCHC[5](g/dl)	30	10.8-28.9	24.0	3.2
Leukocytes (#/µl)	29	13,200-145,000	37,500	27,200
Lymphocytes (#/µl)				
Small	30	9,600-128,200	31,000	25,900
Large	30	0-12,300	1,800	2,500
Neutrophils (#/µl)	30	0-26,000	3,700	5,100
Monocytes (#/µl)	30	0-4,600	1,000	1,100
Thrombocytes (#/µl)	29	23,000-124,800	41,900	23,900

1. Standard deviation, 2. Packed cell volume, 3. Mean cell volume, 4. Mean cell hemoglobin, 5. Mean cell hemoglobin concentration

Calabrese et al. (1975) and Dawson (1979) reported hematocrit values that were slightly higher (35%) than the values in the present study (25%). A possible reason for the higher hematocrits reported by Calabrese et al. (1975) and Dawson (1979) is that blood was collected without anesthesia, which may have stressed the fish resulting in erythrocyte swelling. Fletcher (1975) demonstrated an increase in hematocrit post stress in flounder. The effects of stress on fish are well characterized and may consist of erythrocytosis, thrombocytosis, lymphopenia, neutrophilia, decrease in clotting time and increase in hematocrit due to erythrocyte swelling (Casillas and Smith 1977; Ellsaesser and Clem 1986, 1987; McDonald and Milligan 1992; Randall and Perry 1992). Our study determined hemoglobin values that were slightly higher than those seen by others (Bridges et al. 1976; Mahoney and McNulty 1992); with the exception of one study, which reported much higher hemoglobin values (Umminger and Mahoney 1972).

The cell types present in the blood of the flounder included erythrocytes, thrombocytes, and leukocytes. Erythrocytes were oval to round with characteristic red grey cytoplasm and a spherical and centrally located nucleus. The thrombocytes were large, approximately the size of a small erythrocyte. They had clear cytoplasm and were variable in shape, being round to oval or elongated. Nuclear shape tended to follow cytoplasmic shape, although, oval thrombocytes occasionally demonstrated bean shaped nuclei.

Leukocytes made up the remainder of the cell types seen in the blood and included small and large lymphocytes, neutrophils, heterophils and monocytes. Small lymphocytes were the smallest cell present, with just a rim of blue cytoplasm surrounding the round nucleus. However, small lymphocytes with indented "U-shaped" nuclei were observed where the lobes of the nucleus were closely situated adjacent to each other. Large lymphocytes had an abundant and bluer cytoplasm and the nucleus was larger than observed in the small lymphocyte. The nucleus of the large lymphocytes never appeared segmented.

Monocytes were the largest cell present in the blood. They had abundant dark blue cytoplasm that was frequently vacuolated and contained small cytoplasmic blebs or pseudopod projections. The round to kidney bean shaped nucleus was large with prominent chromatin clumping. Neutrophils and heterophils were present in the blood, and

Table 2. Serum biochemical values for adult winter flounder (Pleuronectes americanus) maintained in captivity.

Analytes	N	Range	Mean	Stds[1]
Total Protein (g/dl)	30	2.6-4.7	3.5	0.5
Albumin (g/dl)	30	0.8-1.5	1.1	0.2
Globulin (g/dl)	30	1.6-3.4	2.4	0.3
Sodium (mEq/L)	30	171-200	183.5	5.3
Potassium (mEq/L)	30	0.5-2.8	0.8	1.7
Chloride (mEq/L)	30	156-194	170	6
Calcium (mEq/L)	30	10.6-15.0	12.4	1.3
Phosphorus (mEq/L)	30	4.9-11.7	9.7	1.8
Magnesium (mEq/L)	30	1.5-3.9	2.3	0.7
Glucose (mg/dl)	30	17-224	49.1	13.5
BUN[2] (mg/dl)	30	1-12	7.4	2.6
Creatinine (mg/dl)	30	0.1-1.0	0.34	0.14
Total bilirubin (mg/dl)	30	0.1-0.3	0.1	0.05
ALP[3] (U/L)	30	7-27	12.8	5.5
ALT[4] (U/L)	30	0-40	5.7	10.9
AST[5] (U/L)	29	5-318	56.9	79.4
LDH[6] (U/L)	30	17-587	196	156
Cholesterol (mg/dl)	30	222->400[7]	—-	—-
Triglycerides (mg/L)	30	21-312	93	97

1. Standard deviation, 2. Blood urea nitrogen, 3. Alkaline phosphatase 4. Alanine aminotransferase (SGPT), 5. Aspartate aminotransferase (SGOT), 6. Lactate dehydrogenase, 7. Several samples were >400, the upper detection limit of the analyzer. Because of this, means and standard deviations were not calculated.

both were larger than erythrocytes. The cytoplasm of the neutrophil was a translucent grey, containing no granules and infrequent vacuoles. Nuclear shape of the neutrophil varied from round to an elongated ribbon segmented into two prominent lobes. Heterophils were similar to neutrophils except that abundant small lavender granules were observed in the cytoplasm, and the cytoplasm was often vacuolated.

The leukocytes observed in the winter flounder were typical of teleost fish (Blaxhall and Daisley 1973; Ellis 1976; Ellis 1977; Burrows and Fletcher 1986; Zinkl et al. 1991; Stoskopf 1993). The only other study to perform differential counts for winter flounder identified lymphocytes, thrombocytes and neutrophils, but not monocytes or heterophils (Bridges et al. 1976). We observed monocytes and both neutrophils and heterophils in most individuals sampled, indicating that these cells are routinely present in this species. Neutrophils and heterophils were counted together, but have a distinctly different appearance. Neutrophils and heterophils are both granulocytes, but the granules in the neutrophil do not take up dye and are neutral in color while the granules in the heterophil take up a slight color and stain a pale lavender or pale red. There is little evidence indicating a functional distinction between the two types of cells.

In general, the serum chemistry values were comparable to those from other species of finfish. The sodium and chloride values obtained in the present study were similar to those obtained in winter flounder by others (Umminger and Mahoney 1972; Fletcher 1975; Dawson 1979). However, the potassium values in the present study (0.5-2.8 mEq/L) were lower than observed by Umminger and Mahoney (1972) (4.0-5.2 mEq/L) and Dawson (1979) (4.22-6.71 mEq/L). Additionally, our study obtained calcium levels of 10.6-15.0 mEq/L, significantly higher than values reported by Dawson (1979) of 3.55-4.14 mEq/L. Several factors can account for the varied levels of electrolytes between studies. Osmoregulation and ion balance in marine fishes involves the kidneys and gills, and thus affects sodium, chloride, potassium, magnesium, phosphorus and calcium concentrations in the blood. Stress, disease or gill lesions can affect electrolytes, causing an increase in sodium and chloride values in flatfish (Fletcher 1975; Bourne 1986). Increased levels of nitrate (Hrubec et al. 1997b), mercury toxicity (Dawson 1979, 1982) and seasonal changes (Umminger and Mahoney 1972; Dawson 1990) have also been shown to affect electrolytes as well. For instance, calcium

levels can be elevated at higher temperature (Hrubec et al. 1997a), with vitellogenesis (McDonald and Milligan 1992), stress (Bourne 1986) and mercury exposure (Dawson 1979, 1982). Since albumin can act as a ligand carrier for calcium, levels of calcium often fluctuate in concordance with albumin concentrations (Stoskopf 1993).

Analysis of blood parameters can provide a wealth of information useful in analyzing the effects of disease, sub-optimal environmental conditions as well as individual variation. The standardized techniques used in this study are recommended for fish and are available around the country at veterinary and human diagnostic laboratories. This study provides baseline blood values for healthy wild caught adult winter flounder adapted to artificial seawater. Baseline values are the necessary first step in determining which specific hematologic changes can be associated with disease conditions. As the field of fish hematology develops, its usefulness to the aquaculture industry will increase. Information derived by standardized non-lethal assays will be needed for diagnostic purposes enhancing the culture of flounder and other fish species.

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