

THE EFFECTS OF TEMPERATURE, WATER QUALITY AND CULTURE
CONDITIONS ON THE IMMUNOLOGY, HEMATOLOGY, AND BLOOD
CHEMISTRY OF HYBRID STRIPED BASS

By

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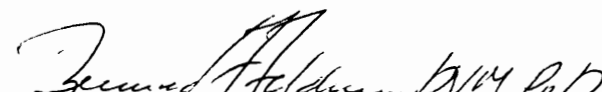
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Sunshine and palmetto bass (different crosses of hybrid striped bass) were used to determine immunologic, hematologic and serum chemistry changes under different culture conditions. The kinetics of the humoral immune response was determined for sunshine bass acclimated to 10, 18, 24, 29°C, and to elevated ammonia (0.15 mg/L NH₃) and elevated nitrate (200 mg/L). These conditions are frequently encountered in aquaculture situations. Cooler temperatures decreased both the magnitude and onset of the humoral response, being lowest at 10°C, intermediate at 18°C, the highest at 24 and 29°C. Elevated ammonia did not affect the immune response, while elevated nitrate decreased antibody production to the level of the 18°C response. Hematologic reference intervals were determined for sunshine bass in tanks and recirculating systems, and palmetto bass in tanks. Serum chemistry reference intervals were determined for sunshine bass in tanks, recirculating systems

and cage systems. Greater differences were observed in reference intervals between the culture systems, than between the two types of hybrid. To determine if environmental factors influenced the differences seen in the reference, sunshine bass were acclimated to 10, 18, 24, 29°C, elevated ammonia (0.15 mg/L NH₃) and elevated nitrate (200 mg/L). The hematology and serum chemistry profiles of these fish were compared with the reference intervals for sunshine bass in tanks. Leukocyte, lymphocyte and monocyte counts at 10°C, and glucose and calcium at 10 and 18°C deviated sufficiently to suggest generating separate reference intervals at these temperatures. In the nitrate treated fish, creatinine levels were elevated and chloride levels were lower than controls and outside the reference interval. These two responses were presumed to be pathologic changes associated with elevated nitrate levels due to the large deviation in the analytes and the mortalities seen in the nitrate treated fish. The remaining analytes for fish in the different environments were within or slightly outside the reference intervals. These slight changes were presumed to be due to individual variation as the reference intervals were determined for fish under relatively uniform conditions and may not be sufficiently broad to cover fish from more varied environments. With minor modification, the reference intervals should apply to

sunshine bass in most situations.

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DEDICATION

This thesis is dedicated to my parents Myrna and Sid Hrubec, and my husband Cliff Shaffer for their love, understanding and support throughout my education. I know you don't believe this, but this is my last PhD.

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CHAPTER 1.

REVIEW OF THE LITERATURE

INTRODUCTION

Hybrid striped bass, the fifth most common aquaculture species raised in the United States (Anonymous 1993), are raised for food and stocked into natural waters as a game fish. Virtually nothing is known about basic immune function and blood characteristics in hybrid striped bass, and knowledge on other fish species is limited. The only published report of immune function in hybrid striped bass is a short communication describing the ability of lymphocytes from a variety of fish species, including hybrid striped bass, to be stimulated by mitogens (Luft et al., 1991). The only previously published serum chemistry values for hybrid striped bass are chloride and cortisol (Tomasso et al. 1980). There has been no work describing the interaction of temperature, water quality, culture conditions and type of hybrid on the basic immunology, hematology and serum chemistry of this fish. Basic

information on these subjects is crucial to enhance the production of hybrid striped bass by providing a fundamental database for any future physiology and disease studies. Information on the kinetics of the immune response is necessary for the development of vaccines and vaccination protocols. Development of immunological assays and hematological and blood chemistry profiles are necessary before these diagnostic tools can be used to detect metabolic disorders and diseased states. The results of this research will benefit producers of hybrid striped bass and other scientists who use this species as an experimental animal. As blood cells are the effectors of immune function, and immunologic status and inflammatory changes are reflected in the blood, a basic understanding of immune function must be based on a knowledge of hematology and serum chemistry. Considering the commercial importance of this species, this research is a necessary and timely addition to the collective knowledge base for hybrid striped bass.

The designation hybrid striped bass is a collective term for the crosses and backcrosses of striped bass (*Morone saxatilis*) and white bass (*Morone chrysops*). The two most common crosses are sunshine bass - a female white bass crossed with a male striped bass, and palmetto bass - a

female striped bass crossed with a male white bass. Both hybrids are calmer and outperform striped bass in aquaculture situations, however, due to availability of the parental species, sunshine bass hybrids are far more prevalent than palmetto bass hybrids.

The volume of literature on fish hematology is quite large and covers the last 100 years (reviewed by Blaxhall 1972; Hawkins and Mawdesley-Thomas 1972; Ellis 1977; Fänge 1992). Unfortunately there is still disagreement as to the nomenclature, cellular differentiation, maturation and function of fish blood cells. The picture drawn from the literature on fish blood chemistries (reviewed by Hille 1982; McDonald and Milligan 1992) is also confusing and contradictory. The confusion and disagreement are due in part to conflicting results, out dated theories still held by some in the field, and a lack of general knowledge on fish clinical pathology, immunology and physiology.

Hematologic values may be considerably different from species to species, giving the impression of conflicting results, and making comparisons between species difficult. There is no standardized nomenclature for cell types, which makes interpretation of published literature difficult unless care is taken to carefully describe each cell type.

There is also great variation from study to study in the methods used to determine blood analytes often making comparison between studies impossible. Even though standardized hematology techniques, based on mammalian protocols, have been recommended for fish (Blaxhall and Daisley 1973) they are not always followed.

Much of the previous literature on fish hematology is based on descriptive studies conducted at the turn of the century. Cellular differentiation, function and maturation were proposed based strictly on cell morphology. Advances in mammalian hematology, and current studies demonstrating the similarity in function of fish and mammalian blood cells, make these early theories highly unlikely. The old theories, however, are slow to die, and are frequently published as fact in recent sources.

The field is also hampered by a lack of knowledge on basic fish physiology and pathology. The physiologic and pathologic responses to disease or metabolic disturbance have not been studied. This makes interpretation of the hematology and blood chemistries difficult, as direct parallels from mammals cannot always be drawn. For example, the tissues of origin for the serum enzymes are not known in fish. If the serum enzymes are elevated, the lesion may not

be located in the same tissue as in mammals.

Fishes are poikilothermic; their basic metabolism and physiologic function are controlled directly by the water temperature. It is also known that environmental water quality parameters, biologic factors (age and sex of the fish), and husbandry factors (diet and stocking density) can affect blood parameters and immune function, but the effects are not well characterized. The effects of these and other factors can only be determined by scientific investigation in the specific area where the knowledge is lacking. This is a daunting task as there are more species of fishes than all other vertebrate species combined.

This research was conducted to provide fundamental information on the immunology, hematology and blood chemistry of sunshine and palmetto bass. This work addresses the hypothesis that the immunology, hematology, and blood chemistry will be affected by different temperatures and water qualities, and by the type of hybrid bass.

Hematologic and serum chemistry reference intervals were determined for sunshine and palmetto bass under different culture conditions following the guidelines proposed by the National Committee for Clinical Laboratory Standards (NCCLS 1992). Sunshine bass were then acclimated to different

temperatures and water qualities. The kinetics of humoral antibody production (change in antibody titer with time), and hematologic and serum chemistries changes were determined. The hematology and serum chemistry of fish in the different temperatures and water qualities were compared with the reference intervals. The use of reference intervals to determine the effect of environmental and pathological conditions is a novel approach in fish hematology. The majority of the studies were conducted with sunshine bass due to their prevalence and availability from hybrid striped bass producers.

LITERATURE REVIEW

Blood Cell Morphology and Function

The blood cells present in the peripheral blood of fishes varies with the species but include the following:

erythrocytes, reticulocytes, thrombocytes, lymphocytes, monocytes, neutrophils, heterophils, eosinophils, basophils, and blast cells (Ellis 1977; Zinkl et al. 1991; Fänge 1992). The leukocytes present in some commonly raised species are as follows. Catfish have thrombocytes, lymphocytes, neutrophils, monocytes, (Williams and Warner 1976; Cannon et al. 1980; Zinkl et al. 1991) basophils (Williams and Warner

1976; Zinkl et al. 1991) and eosinophils (Williams and Warner 1976). Rainbow trout have lymphocytes, neutrophils (McCarthy et al. 1973; Yasutake and Wales 1983; Zinkl et al. 1991) and monocytes and thrombocytes (Yasutake and Wales 1983, Zinkl et al. 1991). Striped bass have thrombocytes, lymphocytes, monocytes and neutrophils (Groman 1986; Zinkl et al. 1991).

Piscine erythrocytes are nucleated and continue to produce hemoglobin throughout the life of the cell (Speckner et al. 1989). Reticulocytes are immature red cells that stain grey blue, similar to mammalian reticulocytes, and are smaller and more rounded than mature erythrocytes (Blaxhall and Daisley 1973). Due to the difference in size and shape, and the fact that they produce hemoglobin throughout their life, older red cells have higher mean cell volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC) than younger cells (Speckner et al. 1989). Erythrocytes are mainly aerobic in their metabolism, utilizing TCA cycle intermediates such as lactate, pyruvate, and glutamine rather than glucose (Tiihonen and Nikinmaa 1991; Fänge 1992). This is of interest because artifacts from slow separation of serum or plasma will be different in fish than in mammals.

Thrombocytes are the cell type responsible for coagulation of blood. They are nucleated with a pale blue to grey cytoplasm. Thrombocytes have similar morphology in all species and come in four forms: a spiked form, with an elongate tail; a spindle form, shaped like a fat cigar; an oval form; and a lone nucleus form, round with a thin rim of cytoplasm (Ellis 1977). There has been considerable difficulty distinguishing lymphocytes from the lone nuclear form of thrombocytes (Blaxhall and Daisley 1973; Ellis 1977). This has led to conflicts in the literature, including one report of lymphocytes and thrombocytes respectively comprising 82 % and 13% of all leukocytes while another report on the same species found 70 % of the leukocytes were thrombocytes (Ellis 1977). It has also been proposed (and still held to be true by many in the field) that thrombocytes are derived from lymphocytes (Gardner and Yevich 1969). It is difficult to accept this view point in light of mammalian blood cellular differentiation, and lymphocyte specialization. Ultrastructurally, thrombocytes are similar to mammalian platelets with an interconnecting canalicular system and cytoplasmic granules (Daimon et al. 1979) quite distinct from lymphocyte structure (Ferguson 1976). Additionally, thrombocytes are phagocytic, unlike lymphocytes (Gardner and Yevich 1969; Ferguson 1976). Blood coagulation in fishes is not well studied, but appears

similar to mammalian coagulation with intrinsic and extrinsic pathways (van Vliet et al. 1985a; Fänge 1992). Stress can cause a thrombocytosis and a decrease in clotting time (Casillas and Smith, 1977; van Vliet et al. 1985b).

Lymphocytes are small leukocytes that function in immunological defenses. As in mammals, there are T, B and nonspecific cytotoxic lymphocytes (Ellis 1988; Clem et. al 1991; Evans and Jaso-Friedmann 1992; Vallejo 1992a, 1992b). Lymphocytes with surface immunoglobulin (sIg+) are B cells and sIg- lymphocytes are T cells (Clem et. al 1991). The population of sIg+ cells exhibit hapten specificity, differentiate into antibody secreting cells, respond to lipopolysaccharide (LPS) but not concanavalin A (ConA) and do not respond in the mixed leukocyte response (MLR). The sIg- cells exhibit carrier specificity, provide help for antibody production to T-dependent antigens, are not required for the antibody response of T-independent antigens, respond well to ConA but poorly to LPS, and will respond in the MLR in the presence of Interleukin-2. Stress or cortisol administration causes a lymphopenia (Ellsaesser and Clem 1986, 1987) which has been characterized by the use of monoclonal antibodies, to be a decrease B cell percentages and an increase in T cell percentages (Ainsworth et al 1991).

Neutrophils (often called heterophils) are one of the largest circulating cell with a pale grey cytoplasm. In most species the nucleus is oval to bilobed, but in salmonids and cyprinids, the nucleus is lobulated and polymorphic (Zinkl et al. 1991; Ainsworth 1992; Fänge 1992). Their primary function is phagocytosis (O'Neill 1986; Fänge 1992; Secombes and Fletcher 1992), although older sources state that granulocytes are nonphagocytic or weakly phagocytic (Ellis 1977). Neutrophils in fishes, as in mammals, destroy pathogens and foreign material through the respiratory burst by the production of superoxide anions, hydrogen peroxide, and hydroxyl radicles forming antibacterial compounds (O'Neill 1986; Ainsworth 1992; Fänge 1992; Secombes and Fletcher 1992). Neutrophils respond with increased numbers in early inflammation (Secombes and Fletcher 1992), but the neutrophilia may be short lived (Ellis 1977). Neutrophils respond with chemotaxis to a chemostimulant (Ainsworth 1992) and increase in number with stress or cortisol administration (Ellsaessar and Clem 1986, 1987; Ainsworth et al. 1991). Stress or cortisol administration did not affect phagocytic ability until repeated doses of cortisol were given (Ainsworth et al. 1991). Phagocytosis by neutrophils, however, is reduced at cooler temperatures (O'Neill 1986).

Eosinophils and basophils are present in some fish species; however, their function is not well understood (Ellis 1977; Fänge 1992). In fish that do not have neutrophils, eosinophils function as a phagocytic cell (Ainsworth 1992). Eosinophils also function in parasite killing, neutralization of parasite products, and attraction of leukocytes to the area (Ainsworth 1992).

Monocytes are large cells with basophilic staining cytoplasm and irregular shaped nuclei (Ellis 1977; Zinkl et al. 1991). They circulate through the blood, migrate into the tissues and become macrophages (Ellis 1977). In the past, there was disagreement on the terminology used for monocytes. One group argued that there were no true monocytes in fishes, and the cells should be called small and large hemoblasts (Ellis 1977). Monocytes are still occasionally called hemoblasts by some investigators. Monocytes and macrophages are the primary phagocytic cell in fishes (Ellis 1977; Blazer 1991; Secombes and Fletcher 1992). Macrophages use the respiratory burst to kill engulfed bacteria (Secombes and Fletcher 1992). The phagocytic and killing ability is dependent on a number of factors such temperature, dietary vitamin levels and dietary fatty acid composition (Scott et al. 1985; Blazer 1991). Macrophages also act as antigen processing and presenting cells (Vallejo 1992a, 1992b),

however, antigen processing and presenting is not affected by low temperatures (Vallejo et al. 1992a). Macrophages in fishes, produce a lymphokine similar to interleukin-1 (Secombes and Fletcher 1992) and respond to a substance similar to mammalian macrophage activating factor (Secombes 1987).

Hematology

The following hematological analytes are frequently determined, PCV, hemoglobin, and erythrocyte number to monitor physiologic changes and responses to stress (Fänge 1992). Only occasionally are erythrocyte indices (MCV, MCH, and MCHC), leukocyte number and differential calculated. When the white cell differential determined, the results are usually expressed in percentages, making absolute changes in cell number difficult to determine. Almost no attempt is made in the published literature to interpret the hemogram, other than to point out which analytes are affected. The main reason for this is the lack of knowledge on underlying physiologic processes which influence the hemogram. Hematological changes with stress are fairly well characterized, but other responses are not fully characterized. This is partly because the main focus of piscine hematology has been on environmental or

toxicological changes. These effects are never correlated with reference intervals and only rarely correlated with documented pathological changes.

The PCV ranges from around 30% to 50% in a number of fish species (Burton and Murray 1979; Murray and Burton 1979; Sandnes et al. 1988; LeaMaster et al. 1990; Hunn et al. 1992). Changes in the PCV have been noted with different seasons. Striped bass (Lochmiller et al. 1989) and winter flounder (Mahoney and McNulty 1992) have a higher PCV during fall and winter. Rainbow trout on the other hand, had lower PCV in winter (Lane 1979), and carp had no change in the PCV with the season (Fourie and Hattingh 1976). PCV increases due to splenic contraction in stressed fish (Randall and Perry 1992) and due to increased erythrocyte swelling from accumulation of intracellular sodium and chloride (Ling and Wells 1985; Chiocchia and Motaïs 1989; Salama and Nikinmaa 1990). Catecholamines inhibit the sodium potassium exchange on the erythrocyte cell membrane causing an increase of intracellular sodium. Water moves in to the cell osmotically and increases the size of the cell up to 7% (Salama and Nikinmaa, 1990). Erythrocytes also swell with time after collection, producing an artifactual increase in PCV and MCV, and a decrease the MCHC (Heath 1986). Diploid and triploid grass carp and bighead carp hybrids had higher

PCV and erythrocyte counts than either parent (Barker et al. 1983).

Erythrocyte numbers in fishes usually range from $0.8 - 3 \times 10^6$ cells / μ L between different species (Blaxhall 1972; Burton and Murray 1979; Murray and Burton 1979; Sandnes et al. 1988; LeaMaster et al. 1990; Hunn et al. 1992).

Erythrocyte counts vary with the season, being highest in the winter and lowest in the summer (Fourie and Hattingh 1976; Lochmiller et al. 1989; Mahoney et al. 1992). Rainbow trout, however, had lower erythrocyte counts in the winter (Lane, 1979). Erythrocyte counts can decrease with disease (Iwama et al. 1986; Mahoney et al. 1992), but is unclear if the decrease is due to changes in red cell production and destruction or simply differences in hydration state of the fish.

Although the hemoglobin and erythrocyte indices have been determined in a number of studies, not enough is known about anemia in fishes to utilize the data fully. The erythrocyte indices are more often used to characterize the size of erythrocytes, than to describe anemias. Seasonal changes have been documented in a number of species. In striped bass, hemoglobin, and MCHC were highest and the MCV and MCH

were lowest during winter (Lochmiller et al. 1989). In carp, greater hemoglobin was seen in the winter, however, no changes were seen in the MCV, MCH, and MCHC (Fourie and Hattingh 1976). Rainbow trout had lower hemoglobin, and higher MCV, MCH, MCHC in the colder months (Lane 1979).

Leukocyte counts and differentials are determined infrequently. This is probably because identification of leukocyte type is difficult and requires specialized training. In general, lymphocytes are the most abundant leukocyte present, followed by monocytes or neutrophils (Ellis 1977). Stress causes a leukopenia characterized by a lymphopenia and a neutrophilia (Ellsaesser and Clem 1986, 1987). Changes with disease are not well documented. However, in chinook salmon with bacterial kidney disease, leukocyte counts were initially lower than controls, but increased to levels above controls after 16 days (Iwama et al. 1986).

Hematology in Striped Bass

Although there have not been any published values for sunshine or palmetto bass, values have been published for striped bass. The PCV may range from 31 - 57 % (Courtois 1975, 1976; Westin 1978; Tisa and Strange 1983; Lochmiller 1989), plasma protein ranged from 3.8 - 10.3 g/dL (Courtois

1975, 1976; Hunn and Greer 1990), and hemoglobin ranged from 6.2 - 10.9 g/dL (Courtois 1975, 1976; Westin 1978; Tisa and Strange 1983; Lochmiller 1989). Erythrocyte counts ranged from $2.0 - 4.2 \times 10^6/\mu\text{L}$ (Westin 1978; Lochmiller 1989), and the erythrocyte indices were MCV 155 fL, MCH 31.3 pg, MCHC 20.5 g/dL (Lochmiller 1989). Studies on striped bass blood have noted the following leukocytes: small and large lymphocytes, monocytes, neutrophils, and thrombocytes (Groman 1982; Zinkl et al. 1991). Peritoneal exudate from striped bass contained neutrophils and eosinophils which were both phagocytic (Bodammer 1986).

Serum Chemistry

Serum chemistry analytes are determined more frequently than hematological determinations in fishes. Serum chemistry analytes can be measured using standardized and automated equipment designed for human samples. The automated analyzers have been shown to be accurate for fish blood, even when operated at mammalian temperatures of 37°C (Warner et al. 1978, 1979; Smith and Ramos 1980). The majority of studies monitor serum chemistry changes associated with exposure to a toxin. However, correlated histopathologic lesions are not always documented, making interpretation of the data difficult. As with hematological data, the serum

chemistry changes have not been compared with reference intervals. The information gained from the toxicologic experiments, though helpful in determining the mechanism of action for a toxin, cannot be used for diagnostic purposes. Unless the change in an analyte is large enough to fall outside the reference interval, one cannot tell if the change is due to disease or individual variation for a given fish.

A number of proteins are present in the plasma of fishes including albumin, globulins, calcium binding proteins (ceruloplasmin and vitellogenin), blood clotting proteins (fibrinogen and prothrombin) transferrin, and lipoproteins (McDonald and Milligan 1992). Total plasma protein in fishes ranges from 2-8 g/dL (McDonald and Milligan 1992). Changes in protein levels are mainly from changes in the hydration state of the fish, stress and strenuous exercise (McDonald and Milligan 1992). Lower plasma protein levels were associated with bacterial and protozoal infections in rainbow trout (Hille 1982), bacterial infections in winter flounder (Mahoney and McNulty 1992), and bacterial kidney disease in chinook salmon (Iwama et al. 1986). Elevated plasma protein levels are seen at warmer temperatures (Woo 1990) and also with stress (McDonald and Milligan 1992).

Blood urea nitrogen, routinely measured in mammals to monitor kidney function, is of questionable diagnostic value in fishes as their main excretory product is ammonia and not urea. Most teleosts, including bass, lack the enzymes needed to synthesize urea (Mommsen and Walsh 1989). Some fishes can synthesize urea by the ornithine-urea cycle, however, their main excretory product is still ammonia (Saha and Ratha 1989). Fishes without ornithine-urea cycle enzymes can produce urea by uricolysis - hydrolysis of arginine by arginase, but the levels of urea produced are low (Mommsen and Walsh 1989). It is not known if other mammalian indicators of renal function such as creatinine and phosphorus are applicable to fish.

Creatinine is produced from creatine and is excreted by the kidneys in fishes (McDonald and Milligan 1992). Creatinine is rarely determined in fishes, but levels in catfish were between 0 and 3.7 mg/dL (Warner and Williams 1977; Bentinck-Smith et al. 1987) and in trout were 0.2 to 2.17 (Hille 1982).

Bilirubin is present in fishes and forms from the breakdown of heme, however, the clinical significance of elevated blood bilirubin is not known (McDonald and Milligan 1992). Levels of bilirubin varied in catfish from 0 to 10.29 mg/dL

(Warner and Williams 1977; Bentinck-Smith et al. 1987) and in trout from 0.03 to 2.1 mg/dL (Hille, 1982).

Very few studies have investigated the tissue origin of serum enzymes present in the blood of fish. Serum enzyme distribution in tissues has been determined for a number of enzymes (Bucher 1990; McDonald and Milligan 1992) but it is not known how tissue distribution relates to amounts detected in the serum. Alkaline phosphatase is found in liver, kidney, small intestine (McDonald and Milligan 1992), and erythrocytes (Gaudet et al. 1975; McDonald and Milligan 1992). Aspartate aminotransferase is found in the heart (Gaudet et al. 1975; McDonald and Milligan 1992) and in the liver (Casillas et al. 1982). There are a number of studies which have correlated increases in serum enzymes with tissue pathology (Asztalos 1986; Michael et al. 1987; Lemaire et al. 1991; Grizzle and Kiryu 1993). While others (Bucher and Hofer 1990), have not show increases in serum enzymes with tissue pathology. Further work is needed before serum enzymes can be used for diagnostic purposes in fishes. Alkaline phosphatase in catfish varied from 0 to 99 mU/mL (Warner and Williams 1977; Bentinck-Smith et al. 1987) and from 83 to 330 mU/mL in trout (Hille 1982).

Of all the serum components in fishes, the electrolytes are

the most studied. Electrolyte changes and osmolality give an indication of the fishes ability to osmoregulate. This ability is often compromised with disease, stress or gill pathology due to an increased permeability of the gills to sodium and chloride (McDonald and Milligan 1992). Stress did not affect the serum electrolytes of channel catfish (Ellsaesser and Clem 1987) indicating possible species differences in the response to stress. In catfish, sodium ranged from 102 to 180 mEq/L, and chloride was 72 to 122 mEq/L (Warner and Williams 1977; Bentinck-Smith et al. 1987). In trout, sodium ranged from 123 - 163 mEq/L and chloride 84 to 160 mEq/L (Hille 1982).

Changes in the other electrolytes - calcium, phosphorus, potassium and magnesium, due to pathology or stress are not as well understood in fishes. Calcium levels are controlled by the hormones calcitonin and stanniocalcin (McDonald and Milligan 1992). A percentage of the calcium in the blood is bound to protein carriers; and as the protein concentration increases calcium also increases (McDonald and Milligan 1992). In catfish calcium ranged from 0.8 to 24.4 mg/dL (Warner and Williams 1977; Bentinck-Smith et al. 1987), and in rainbow trout from 2.6 - 12.8 mEq/L (Hille 1992).

Potassium levels are unaffected by stress conditions which

increase gill electrolyte permeability, but stress conditions causing an intracellular acidosis (strenuous exercise) will elevate potassium levels (McDonald and Milligan 1992). Potassium in catfish ranged from 0 to 13 mEq/L in catfish (Warner and Williams 1977; Bentinck-Smith et al. 1987), and in rainbow trout from 1 to 11 mEq/L (Hille 1982).

Cortisol and glucose have been used to monitor primary and secondary stress responses, although, cortisol is a more sensitive indicator of stress (Silbergeld 1974; McDonald and Milligan 1992). Both glucose and cortisol levels increase with stressful events such as handling, confinement, transport, angling, disease, and unsuitable water conditions (McDonald and Milligan 1992). The intensity and duration of the stress affects the degree and duration of the stress response. If the stress is sufficiently prolonged or severe, a state of exhaustion is reached characterized by decreased levels of glucose and cortisol, and atrophy of the pituitary gland (Hontela et al. 1992).

Cortisol has both annual and daily variations, with the highest levels occurring just before dawn and during the warmer summer months (Kühn et al. 1986; Planas et al. 1990; McDonald and Milligan 1992). Cortisol levels vary from

species to species. The following are resting levels reported for the different species: plaice, 169 ± 32 ng/mL (White and Fletcher 1989); catfish, 30 to 50 ng/mL (Strange 1980); Atlantic salmon, 237 ± 60 ng/mL (Kjartansson et al. 1988) and sea bass, 100 to 600 ng/mL (Planas et al. 1990).

Serum Chemistry in Sunshine Bass and Striped Bass

Very little is known about the blood chemistry profile of sunshine bass, with chloride (133 mEq/L) and cortisol (0.8 μ g/dL) the only published analytes (Tomasso et al. 1980). Striped bass have been studied more extensively. The most comprehensive study on striped bass, conducted by Tisa and Strange (1983), determined reference intervals for osmolality, chloride, glucose, cortisol, total protein, and cholesterol. Most studies involving striped bass are limited in value due to the number of analytes measured (Westin 1978; Davis et al. 1982; Hunn and Greer 1990), or by the small number of fish sampled (Courtois 1975, 1976; Brown et al. 1987).

Immunology

The immune system in fishes is basically similar to the immune system in mammals. It is composed of specific and

non-specific immune defense mechanisms, and a localized immunity in the skin (Ingram 80) and gut (Hart 88). Teleost lymphoid organs include the thymus, spleen and kidney. As in mammals, fishes have functionally distinct T, B and nonspecific cytotoxic lymphocytes (Sigel 84; Ellis 1988; Clem et. al 1991; Evans and Jaso-Friedmann 1992; Vallejo 1992a, 1992b), but only have one class of immunoglobulin, an IgM-like molecule (Sigel 84; Ellis 88).

It is well known that temperature plays an important role in modulating immune function in fishes. Cooler temperatures decrease antibody production in carp (Rijkers et al. 1981; Wishkovsky and Avtalion 1982); in summer flounder (Stolen et al. 1984; Bureson and Frizzell 1986); in rainbow trout (van Ginkel et al. 1985); and catfish (Klesius 1990). Non-specific immune functions such as phagocytosis and chemiluminescent response are also inhibited (Scott et al. 1985; O'Neill 1986). The effects of warm temperature stress on antibody production have not been studied as thoroughly. Warm temperatures have been shown to suppress antibody production in rainbow trout (van Ginkel 1985).

Suboptimal water temperatures seem to inhibit antibody production in one of two patterns. Coho salmon (Paterson and Fryer 1974) and three species of flounder (Stolen et al.

1982), demonstrated both a decrease in the magnitude and a delay in the time of antibody response at colder temperatures. Carp (Rijkers et al. 1981), summer flounder (Stolen et al. 1984) and plaque-forming cells from rainbow trout (van Ginkel et al. 1985) delayed antibody production at cooler temperatures, but the magnitude of the response did not change. Miller and Clem (1984) determined that the magnitude of response was dependent of the antigen. The response to T-independent antigens was not affected by temperature, but the magnitude of the response to T-dependent antigens was decreased at lower temperatures. This is because only T cells are sensitive to lower temperatures (Clem et al. 1991). Although T and B cells have the same overall membrane viscosity and homeoviscous adaptation to colder temperatures, T cells apparently have small areas on the cell surface that do not undergo appropriate homeoviscous adaptation. Supplementation with unsaturated fatty acids in a mitogen response assay "rescued" the decreased response at colder temperatures (Clem et al. 1991).

Immune Responses of Sunshine Bass and Striped Bass

The only published report of immune function in hybrid striped bass is a short communication describing the ability of lymphocytes from a variety of fish species, including

hybrid striped bass, to be stimulated by mitogens (Luft et al. 1991). The reports of immune function in pure striped bass are also limited. Robohm (1986) described the stimulation of antibody production by sublethal doses of cadmium, while Wechsler (1986) described inhibition of antibody production by corticosteroids. Stave et al. (1983, 1985, 1986) described the chemiluminescent response of striped bass phagocytes.

CONCLUSION OF LITERATURE REVIEW

The quantity of information covering fish immunology, hematology and serum chemistry is vast, however there are still areas where almost nothing is known. The immunology and hematology of sunshine bass, one of the more popular aquaculture species, has only been investigated in two studies. Immunologic, hematologic and serum chemistry changes with disease or metabolic disturbances are just beginning to be elucidated in all species. Recent advances concerning the immunologic function of the blood cells, will hopefully shed some light on the hematological changes seen in diseased fish. Additionally, further studies on the physiologic and pathophysiologic responses of fishes should advance the use of hematology and serum chemistry profiles

for diagnostic purposes.

LITERATURE CITED

- Ainsworth, A.J., C. Dexiang, and P.R. Waterstrat, 1991.
Changes in peripheral blood leukocyte percentages and
function of neutrophils in stressed channel catfish.
J. Aquat. An. Health 3:41-47.
- Ainsworth, A.J., 1992. Fish granulocytes: morphology,
distribution, and function. Ann. Rev. Fish Dis. 2:123-
148.
- Anonymous, 1992. The status of world aquaculture 1991.
Aquaculture Magazine Jan. 1992 pp. 8-28.
- Asztalos, B., 1986. LDH enzyme as a tool on monitoring
tissue necrosis in fishes caused by pesticides. Acta
Biol. Hung. 37:59-65.
- Barker, C.J., M.L. Beck, and C.J. Biggers, 1983.
Hematologic and enzymatic analysis of *Ctenopharyngodon*
idella x *Hypophthalmichthys nobilis* F1 hybrids. Comp.
Biochem. Physiol. 74A:915-918.
- Bentinck-Smith, J., H.M. Bealeau, P. Waterstart, C.S. Tucker,
F. Stiles, R.P. Bowser, and L.A. Brown, 1987.
Biochemical reference ranges for commercially reared
channel catfish. Prog. Fish-Cult. 49:108-114.
- Blaxhall, P.C., and K.W. Daisley, 1973. Routine
haematological methods for use with fish blood. J.
Fish Biol. 5:771-781.
- Blaxhall, P.C., 1972. The haematological assessment of the
health of freshwater fish - A review of selected
literature. J. Fish Biol. 4:593-604.
- Blazer, V.S., 1991. Piscine macrophage function and
nutritional influences: A review. J. Aquat. Animal
Health, 3:77-86.

- Bodammer, J.E., 1986. Ultrastructural observations on peritoneal exudate cells from the striped bass. *Vet. Immunol. Immunopathol.* 12:127-140.
- Brown, C.L., G. Young, R.S. Nishioka, and H.A. Bern, 1987. Preliminary report on the physiological status of striped bass in the Carginez Strait die-off. *Fish. Res* 6:5-16.
- Bucher, F., 1990. Organ patterns and natural fluctuations of blood enzymes of rainbow trout (*Salmo gairdneri* Rich.). *Comp. Biochem. Physiol.* 96B:795-799.
- Bucher, F., and R. Hofer, 1990. Effects of domestic waste water on the serum enzyme activities of brown trout (*Salmo trutta*). *Comp. Biochem. Physiol.* 97C:381-385.
- Burreson, E.M., and L.J. Frizzell, 1986. The seasonal antibody response in juvenile summer flounder (*Paralichthys dentatus*) to the hemoflagellate *Trypanoplasma bullocki*. *Vet. Immunol. Immunopathol.* 12:395-402.
- Burton, C.B., and S.A. Murray, 1979. Effects of density on goldfish blood - I Hematology. *Comp. Biochem. Physiol.* 62A:555-558.
- Cannon, M.S., H.H. Mollenhauer, T.E. Eurell, D.H. Lewis, A.M. Cannon, C. Tompkins, 1980. An ultrastructural study of the leukocytes of the channel catfish, *Ictalurus punctatus*. *J. Morph.* 164:1-23.
- Casillas, E., and L.S. Smith, 1977. Effect of stress on blood coagulation and hematology in rainbow trout (*Salmo gairdneri*). *J. Fish Biol.* 10:481-491.
- Casillas, E., J. Sundquist, and W.E. Ames, 1982. Optimization of assay conditions for, and the selected tissue distribution of, alanine aminotransferase and aspartate aminotransferase of English sole, *Parophrys vetulus* Girard. *J. Fish Biol.* 21:197-204.

- Chiocchia, G., and R. Motaïs, 1989. Effect of catecholamines on the deform of red cells from trout: relative roles of cyclic AMP and cell volume. J. Physiol. 412:321-332.
- Clem, L.W., N.W. Miller, J.E. Bly, 1991. Evolution of lymphocyte subpopulations, their interactions, and temperature sensitivities. In: The phylogeny of immune functions, Cohen, N. and G.W. Warr Eds., CRC Press Inc., Boca Raton, FL. Pages 191-213.
- Courtois, L.A., 1975. Blood and serum analysis of adult striped bass, *Morone saxatilis*, captured in the Sacramento River. Calif. Fish and Game 61:245-246.
- Courtois, L.A., 1976. Hematology of juvenile striped bass, *Morone saxatilis* (Walbaum), acclimated to different environmental conditions. Comp. Biochem. Physiol. 54A:221-223.
- Daimon, T., V. Mizuhira, I. Takahashi, and K. Uchida, 1979. The surface connected canalicular system of carp (*Cyprinus carpio*) thrombocytes: Its fine structure and three-dimensional architecture. Cell Tissue Res. 203:355-365.
- Davis, K.B., N.C. Parker, and M.A. Suttle, 1982. Plasma corticosteroids and chlorides in striped bass exposed to tricaine methanesulfonate, quinaldine, etomidate, and salt. Prog. Fish-Cult. 44:205-207.
- Ellis, A.E., 1977. The leukocytes of fish: a review. J. Fish Biol. 11:453-491.
- Ellis, A.E., 1988. Ontogeny of the immune system in the teleost fish. In: A.E. Ellis (Editor), Fish Vaccination. Academic Press, New York, NY, pp. 20-31.
- Ellsaesser, C.F., and L.W. Clem, 1986. Hematological and immunological changes in channel catfish stressed by handling and transport. J. Fish Biol. 28:511-521.

- Ellsaesser, C.F., and L.W. Clem, 1987. Cortisol-induced hematologic and immunologic changes in channel catfish (*Ictalurus punctatus*). *Comp. Biochem. Physiol.* 87A: 405-408.
- Evans, D.L., and L. Jaso-Friedmann, 1992. Nonspecific cytotoxic cells as effectors of immunity in fish. *Ann Rev. Fish Dis.* 2:109-121.
- Ferguson, H.W., 1976. The ultrastructure of plaice (*Pleuronectes platessa*) leucocytes. *J. Fish Biol.* 8:139-142.
- Fourie, F.L.R., and J. Hattingh, 1976. A seasonal study of the hematology of carp (*Cyprinus carpio*) from a locality in the transvaal, South Africa. *Zool. Afric.* 11:75-80.
- Fänge, R., 1992. Fish Blood Cells. In: W.S. Hoar, D.J. Randall, and A.P. Farrell, editors, *Fish Physiology* volume 12 Part B, *The Cardiovascular System*. Academic Press Inc., San Diego California. Pages 1-54.
- Gardner, G.R., and P.P. Yevich, 1969. Studies on the blood morphology of three estuarine cyprinidontiform fishes. *J. Fish. Res. Board Can.* 26:433-447.
- Gaudet, M., J.G. Racicot, and C. Leray, 1975. Enzyme activities of plasma and selected tissues in rainbow trout *Salmo gairdneri* Richardson. *J. Fish Biol.* 7:505-512.
- Grizzle, J.M., and Y. Kiryu, 1993. Histopathology of gill, liver, and pancreas, and serum enzyme levels of channel catfish infected with *Aeromonas hydrophila* complex. *J. Aquat. Animal Health* 5:36-50.
- Groman, D.B., 1982. *The Histology of the Striped Bass*. American Fisheries Society, Monograph 3. American Fisheries Society, Bethesda, Maryland. Pages 18-20.

- Hart, S., A.B. Wrathmell, J.E. Harris, T.H. Grayson, 1988. Gut Immunology in Fish: A Review. Dev. Comp. Immunol. 12:453-480.
- Hawkins, R.I., and L.E. Mawdesley-Thomas, 1972. Fish hematology a bibliography. J. Fish Biol. 4:193-232.
- Heath, A.G., 1987. Water Pollution and Fish Physiology. CRC Press, Boca Raton, FL.
- Hille, S., 1982. A literature review of the blood chemistry of rainbow trout, *Salmo gairdneri* Rich. J. Fish Biol. 20:535-569.
- Hontela A., J.B. Rasmussen, C. Audet, and G. Chevalier, 1992. Impaired cortisol stress response in fish from environments polluted by PAHs, PCBs, and mercury. Arch. Environ. Contam. Toxicol. 22:278-283.
- Hunn, J.B., and I.E. Greer, 1990. Colorimetric and refractometer estimates of total plasma protein in striped bass, *Morone saxatilis* (Walbaum). J. Fish Biol. 36:617-618.
- Hunn, J.B., R.H. Wiedmeyer, I.E. Greer, and A.W. Grady, 1992. Blood chemistry of laboratory-reared golden trout. J. Aquat. An. Health 4:218-221.
- Ingram, G.A., 1980. Substances Involved in the Natural Resistance of Fish to Infection - A Review. J. Fish Biol. 16:23-60.
- Iwama, G.K., G.L. Greer, D.J. Randall, 1986. Changes in selected haematological parameters in juvenile chinook salmon subjected to a bacterial challenge and a toxicant. J. Fish Biol. 28:563-572.

- Kjartansson, H., S. Fivelstad, J.M. Thomassen, and M.J. Smith. 1988. Effects of different stocking densities on physiological parameters and growth of adult Atlantic salmon (*Salmo salar* L.) reared in circular tanks. *Aquaculture* 73:261-274.
- Klesius, P.H., 1990. Effect of size and temperature on the quantity of immunoglobulin in channel catfish, *Ictalurus punctatus*. *Vet. Immunol. Immunopathol.* 24:187-195.
- Kühn, E.R., S. Corneillie, and F. Ollevier, 1986. Circadian variations in plasma osmolality, electrolytes, glucose, and cortisol in carp (*Cyprinus carpio*). *Gen. Comp. Endocrinol.* 61:459-468.
- Lane, H.C., 1979. Progressive changes in hematology and tissue water of sexually mature trout, *Salmo gairdneri* Richardson during the autumn and winter. *J. Fish Biol.* 15:425-436.
- LeaMaster, B.R., J.A. Brock, R.S. Fugioka, and R.M. Nakanura, 1990. Hematologic and blood chemistry values for *Sarotherodon melanotheron* and a red hybrid tilapia in fresh water and seawater. *Comp. Biochem. Physiol.* 97A:523-529.
- Lemaire, P., P. Draï, A. Mathieu, S. Lemaire, S. Carrière, J. Guidicelli, and M. Lafaurie, 1991. Changes with different diets in plasma enzymes (GOT, GPT, LDH, ALP) and plasma lipids (Cholesterol, triglycerides) of sea-bass (*Dicentrarchus labrax*). *Aquaculture* 93:63-75.
- Ling, N, and R.G.M. Wells, 1985. Plasma catecholamines and erythrocyte swelling following capture stress in a marine teleost fish. *Comp. Biochem. Physiol.* 82C 231-234.

- Lochmiller, R.L., Weichman J.D., and Zale A.V., 1989
Hematological assessment of temperature and oxygen stress in a reservoir population of striped bass (*Morone saxatilis*). *Comp. Biochem. Physiol.* 93A:535-541.
- Luft, J.C., L.W. Clem, and J.E. Bly, 1991. A serum-free medium for bony fish leucocyte in vitro mitogen-induced proliferation. *Fish and Shellfish Immunol.* 1:233-235.
- Mañoney, J.B., and J.K. McNulty, 1992. Disease-associated blood changes and normal seasonal hematological variation in winter flounder in the Hudson-Raritan estuary. *Trans. Amer. Fish. Soc.* 121:261-268.
- McCarthy, D.H., J.P. Stevenson, and M.S. Roberts, 1973. Some blood parameters for rainbow trout (*Salmo gairdneri* Richardson). I. The kamloops variety. *J. Fish Biol.* 5:1-8.
- McDonald, D.G., and C.L. Milligan, 1992. Chemical Properties of Blood. In: W.S. Hoar, D.J. Randall, and A.P. Farrell, editors. *Fish Physiology Vol. 12 Part B, The Cardiovascular System.* Academic Press, New York. Pages 56-135.
- Michael, M.I., A.M. Hilmy, N.A. El-Domiaty, and K. Wershana, 1987. Serum transaminase activity and histopathological changes in *Clarias lazera* chronically exposed to nitrite. *Comp. Biochem. Physiol.* 86C:255-262.
- Miller, N.W., and L.W. Clem, 1984. Temperature mediated processes in teleost immunity: Differential effects of temperature on catfish in vitro antibody responses to thymus-dependent and thymus-independent antigens. *J. Immunol.* 133:2356-2359.
- Mommsen, T.P., and P.J. Walsh, 1989. Evolution of urea synthesis in vertebrates: the piscine connection. *Science* 243:72-75.

- Murray, S.A., and C.B. Burton, 1979. Effects of density on goldfish blood - II Cell morphology. *Comp. Biochem. Physiol.* 62A:559-562.
- NCCLS (National Committee for Clinical Laboratory Standards), 1992. How to define, determine, and utilize reference intervals in the clinical laboratory; proposed guidelines. NCCLS Document C28-P (ISBN 1-56238-143-1). NCCLS, Villanova, Pennsylvania.
- O'Neill, J.G., 1986. An in vitro study of polymorphonuclear phagocytosis and the effect of temperature. In: J.S Stolen, D.P. Anderson, and W.B. van Muiswinkel (Editors), *Fish Immunology*, Elsevier Science Pub., London. Pages 47-55.
- Paterson, W.P., and J.L. Fryer, 1974. Effect of temperature and antigen dose on the antibody response of juvenile coho salmon (*Oncorhynchus kisutch*) to *Aeromonas salmonicida* endotoxin. *J. Fish. Res. Board Can.* 31:1734.
- Planes, J., J. Gutierrez, J. Fernandez, M. Carrillo, and P. Canals, 1990. Annual and daily variation of plasma cortisol in seabass, *Dicentrarchus labrax* L. *Aquaculture* 91:171-178.
- Randall, D.J., and S.F. Perry, 1992. Catecholamines. In: Hoar W.S., D.J. Randall, and A.P. Farrell (Editors), *Fish Physiology* Vol. 12 Part B, Academic Press, New York. Pages 255-300.
- Robohm, R.A., 1986. Paradoxical effect of cadmium exposure on antibacterial antibody response in two fish species: Inhibition in cunners (*Tautogolabrus adspersus*) and enhancement in striped bass (*Morone saxatilis*). *Vet. Immunol. Immunopathol.* 12:251-262.

- Rijkers, G.T., J.A. Wiegerinck, R. van Oosterom, and W.B. van Muiswinkel, 1981. Temperature dependence of humoral immunity in carp (*Cyprinus carpio*). In: Solomon, B.J. (Editor), Aspects of Developmental and Comparative Immunology. Proc. of the 1st Congress of Developmental and Comparative Immunology, 1980, Aberdeen, Pergamon Press, Oxford, Pages 477-482.
- Saha, N., and B.K. Ratha, 1989. Comparative study of ureogenesis in freshwater, air-breathing teleosts. J. Exp. Zool. 252:1-8.
- Salama, A., and M. Nikinmaa, 1990. Effect of oxygen tension on catecholamine-induced formation of cAMP and on swelling of carp red blood cells. J. Amer. Phys. Soc. C723-C726.
- Sandnes, K., O. Lie, and R. Waagbo, 1988. Normal ranges of some blood chemistry parameters in adult farmed Atlantic salmon *Salmo salar*. J. Fish Biol. 32:129-136.
- Scott, A.L., W.A. Rogers, and P.H. Klesius, 1985. Chemiluminescence by peripheral blood phagocytes from channel catfish: Function of opsonin and temperature. Dev. Comp. Immunol. 9:241-250.
- Secombes, C.J., 1987. Lymphokine release from rainbow trout leucocytes stimulated with concanavalin A. Effects upon macrophage spreading and adherence. Dev. Comp. Immunol. 11:512-520.
- Secombes, C.J., and T.C. Fletcher, 1992. The role of phagocytes in the protective mechanisms of fish. An. Rev. Fish Dis. 2:52-71.
- Sigel, M.M., 1984. Overview of Selected Immunological Components of the Immune System in Food Animals With Consideration of Immune Modulation. In: M. Kende, J. Gainer, M. Chirigos, (Editors). Chemical Regulation of Immunity in Veterinary Medicine. Alan R. Liss New York.

- Silbergeld, E.K., 1974. Blood glucose: a sensitive indicator of environmental stress in fish. Bull. Environ. Contam. Toxicol. 11:20.
- Speckner, W., J.F. Schindler, C. Albers, 1989. Age-dependent changes in volume and hemoglobin content of erythrocytes in the carp (*Cyprinus carpio* L.). J. exp. Biol. 141:133-149.
- Smith, A.C., and F. Ramos, 1980. Automated chemical analysis in fish health assessment. J. Fish Biol. 17:445-450.
- Stave, J.W., and B.S. Roberson, 1985. Hydrocortisone suppresses the chemiluminescent response of striped bass phagocytes. Dev. Comp. Immunol. 9:77-84.
- Stave, J.W., B.S. Roberson, and F.M. Hetrick, 1983. The chemiluminescence of phagocytic cells isolated from the pronephros of striped bass. Dev. Comp. Immunol. 7:269-276.
- Stave, J.W., T.M. Cook, and B.S. Roberson, 1987. Chemiluminescent responses of striped bass, *Morone saxatilis* (Walbaum), phagocytes to strains of *Yersinia ruckeri*. J. Fish Dis. 10:1-10.
- Stolen, J.S., T. Gahn, and J. Nagle J., 1982. The humoral antibody formation to erythrocyte antigens in three species of flatfish. Dev. Comp. Immunol, suppl. 2:101-106.
- Stolen, J.S., T. Gahn, V. Kasper, and J.J. Nagle, 1984. The effect of environmental temperature on the immune response of a marine teleost (*Paralichthys dentatus*). Dev. Comp. Immunol. 8:89-98.
- Strange, R.J., 1980. Acclimation temperature influences cortisol and glucose concentrations in stressed channel catfish. Trans. Amer. Fish. Soc. 109:298-303.

- Tiihonen, K., and M. Nikinmaa, 1991. Substrate utilization by carp (*Cyprinus carpio*) erythrocytes. J. Exp. Biol. 161:509-514.
- Tisa, M.S., and R.J. Strange, 1983. Hematology of striped bass in fresh water. Prog. Fish-Cult. 45(1):41-44.
- Tomasso, J.R., K.B. Davis, and N.C. Parker, 1980. Plasma corticosteroid and electrolyte dynamics of hybrid striped bass (white bass X striped bass) during netting and hauling. Proc. World Maricult. Soc. 11:303-310.
- Vallejo, A.N., N.W. Miller and L.W. Clem, 1992a. Cellular pathway(s) of antigen processing in fish APC: effect of varying in vitro temperatures on antigen catabolism. Dev. Comp. Immunol. 16:367-381.
- Vallejo, A. N., N.W. Miller, and L.W. Clem, 1992b. Antigen processing and presentation in teleost immune responses. Ann. Rev. Fish Dis. 2:73-89.
- van Ginkel, F.W., W.B. van Muiswinkel, B. Merchant, E.F. Lizzio, O.W. Dixon, and D.P. Anderson, 1985. Temperature comparisons for antibody production in vitro by plaque-forming cells from the trout, *Salmo gairdneri* (Richardson), and mice. J. Fish Biol. 27:265-272.
- van Vliet, K.J., G.L. Smit, J.J. Pieterse, H.J. Schoonbee, and J.H.J. van Vuren, 1985a. Thrombelastographic diagnosis of blood coagulation in two freshwater fish species. Comp. Biochem. Physiol. 82A:19-21.
- van Vliet, K.J., G.L. Smit, J.J. Pieterse, H.J. Schoonbee, and J.H.J. van Vuren, 1985b. A thrombelastographic study of the effect of stress on the blood coagulation in *Cyprinus carpio* (Cyprinidae) and *Oreochromis mossambicus* (Cichlidae). Comp. Biochem. Physiol. 82A:23-27.

- Warner, M.C., R.W. Williams, 1977. Comparison between serum values of pond and intensive raceway cultured channel catfish *Ictalurus punctatus* (Rafinesque). J. Fish Biol. 11:385-391.
- Warner M.C., S.A. Diehl, and A.M. Tomb, 1978. Effects of dilution and temperature of analysis on blood serum values in rainbow trout *Salmo gairdneri*. J. Fish Biol. 13:257-263.
- Warner, M.C., A.M. Tomb, and S.A. Diehl, 1979. Variability and stability of selected components in rainbow trout *Salmo gairdneri* serum and the precision of automated analysis in measuring these components. J. Fish Biol. 15:141-151.
- Wechsler, S.J., P.E. McAllister, Hetrick, F.M., and D.P. Anderson, 1986. Effect of exogenous corticosteroids on circulating virus and neutralizing antibodies in striped bass (*Morone saxatilis*) infected with infectious pancreatic necrosis virus. Vet. Immunol. Immunopathol. 12:305-311.
- Westin, D.T., 1978. Serum and blood from adult striped bass, *Morone saxatilis*. Estuaries 1:126-128.
- Williams, R.W., and M.C. Warner, 1976. Some observations on the stained blood cellular elements of catfish, *Ictalurus punctatus*. J. Fish Biol. 9:491-497.
- Wishkovsky, A., and R.R. Avtalion, 1982. Induction of helper and suppressor functions in the carp (*Cyprinus carpio*) and their possible implication in seasonal disease in fish. Dev. Comp. Immunol., suppl. 2:83-91.
- White, A., and T.C. Fletcher, 1989. The effect of physical disturbance, hypoxia and stress hormones on serum components of the plaice, *Pleuronectes platessa* L. Comp. Biochem. Physiol. 91A: 455-461.

Woo, N.Y.S., 1990. Metabolic and osmoregulatory changes during temperature acclimation in the red sea bream, *Chrysophrys major*: Implications for its culture in the subtropics. *Aquaculture* 87:197-208.

Yasutake, W.T., J.H. Wales, 1983. Microscopic anatomy of salmonids: An atlas. U.S. Dept. of the Interior, Fish and Wildlife Service, Resource Publication 150, Washington D.C. Pages 82-91.

Zinkl, J.G., W.T. Cox, and C.S. Kono, 1991. Morphology and cytochemistry of leukocytes and thrombocytes of six species of fish. *Comp. Hematol. Int.* 1:87-195.

CHAPTER 2

The Effect of Temperature and Water Quality on Antibody Response to *Aeromonas salmonicida* in Sunshine Bass (*Morone chrysops* X *Morone saxatilis*).

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ABSTRACT

The kinetics of the antibody response (change in antibody titer with time) to a formalin killed *Aeromonas salmonicida* bacterin were determined in sunshine bass (female *Morone chrysops* X male *Morone saxatilis*) at different temperatures and water quality parameters. Groups of fish were acclimated to water at four different temperatures (10, 18, 24, and 29°C), and to elevated ammonia and nitrate levels for one month. Fish were then immunized by intraperitoneal injection of bacterin and the antibody response monitored with an ELISA for 75 days. All groups of immunized fish had significantly higher antibody responses to the bacterin than sham immunized controls. The greatest antibody production was seen in fish held at 24°C and 29°C, and was significantly higher than fish held at 18°C or 10°C. The fish at 18°C had significantly higher responses than the fish at 10°C. In the water quality study, the control fish (at 24°C) and fish in the elevated ammonia tank had antibody responses comparable to the 24°C and 29°C fish from the temperature study. The antibody response of fish held at elevated nitrate levels was depressed and similar to that of fish held at 18°C. From these experiments, we conclude that the humoral immune response of sunshine bass to an A.

salmonicida bacterin was affected by temperature and water quality. At cooler temperatures, the immune response was slower and of less magnitude than the response at warmer temperatures. Temperatures warmer than 24°C, and elevated ammonia concentrations did not affect the immune response, while elevated nitrate levels reduced antibody production.

INTRODUCTION

Sunshine bass (hybrids of female *Morone chrysops* X male *Morone saxatilis*) are an important aquaculture species in this country with annual production outpacing wild harvest of striped bass. Little is known about the immune system and its function in hybrid striped bass. The only published report of immune function in hybrid striped bass is a short communication describing the ability of lymphocytes from a variety of fish species, including hybrid striped bass, to be stimulated by mitogens (Luft et al., 1991). There are also few studies that describe the immune response of pure striped bass. Robohm (1986) described the stimulation of antibody production by cadmium, while Wechsler (1986) described inhibition of antibody production by corticosteroids, and Stave et al. (1983, 1985, 1986)

described the chemiluminescent response of striped bass phagocytes. The lack of information on the structure and function of the immune system in hybrid striped bass is surprising considering the importance of this fish in commercial aquaculture.

It is well known that temperature plays an important role in modulating immune function in fishes. Cooler temperatures decrease antibody production (Rijkers et al. 1981, Wishkovsky and Avtalion 1982, Stolen et al. 1984, van Ginkel et al. 1985, Bureson and Frizzell 1986, Klesius 1990) and also inhibit non-specific immune functions (Scott et al. 1985, O'Neill 1986). The effects of warm temperature stress on antibody production have not been studied as thoroughly. Warm temperatures have been shown to suppress antibody production in rainbow trout (van Ginkel et al. 1985). It was not known if temperatures higher than that preferred by sunshine bass would affect the humoral immune response.

The effect of other water quality parameters on the humoral immune response is also not known. Adverse water quality is known to affect the survival, growth and physical condition of cultured fishes. Fishes frequently develop diseases 5 to 10 days after exposure to poor water quality. In particular, Hazel et al. (1971) found that elevated ammonia

reduced the resistance to disease in striped bass. It was not known if this lowered resistance to disease was accompanied by a reduced immune response or whether it was caused by a multiplicity of factors.

This study was conducted to determine if specific water quality stressors influenced the ability of sunshine bass to mount a humoral immune response. Specifically, the effect of low and high temperatures, and elevated ammonia and nitrate levels on the antibody response in sunshine bass immunized with an *A. salmonicida* bacterin was determined. Antibody production was quantified by an enzyme-linked immunosorbent assay (ELISA). This procedure has been used previously to evaluate humoral immunity in fishes (Hamilton et al. 1986, Arkoosh and Kaattari 1990, Smith et al. 1992b).

MATERIALS AND METHODS

Fish and Experimental Conditions

For the study on the effects of temperature, sunshine bass were purchased from a commercial producer (Day Spring Farm, Etlan, VA) when the fish were 13 months old. Fish were divided into four groups of seventy fish each and placed into separate 2000 L circular tanks with undergravel

filters. Fish were fed a commercial pelleted fish feed (Zeigler Brothers Inc., Gardner, PA) daily at 1% body weight per day. Fish were allowed to acclimate to their laboratory setting for one week. Temperatures were then adjusted over the course of one week and held constant for three weeks before the fish were immunized. The four tanks were maintained at 10°C, 18°C, 24°C and 29°C for the duration of the experiment.

To study the effects of water quality, 16 month old sunshine bass were obtained from the Aquaculture Center at Virginia Polytechnic Institute and State University. These fish were divided into three groups of fifty fish each and were placed in 2000 L tanks with undergravel filters. Fish were fed a commercial pelleted fish feed (Zeigler Brothers Inc., Gardner, PA) daily at 1% body weight per day. All water quality tanks were maintained at 24°C. The fish were allowed to acclimate to their laboratory setting for one week before adjusting the water quality. Experimental conditions for the water quality study consisted of a control tank with "normal water", a tank with elevated ammonia, and a tank with elevated nitrate. Ammonia and nitrate levels were gradually increased over a two week acclimation period and then maintained at elevated levels

for the duration of the experiment. In the elevated ammonia tank, ammonium chloride (Sigma Chemical, Saint Louis, MO) was added daily to bring the un-ionized ammonia concentration within a range of 0.05 to 0.15 mg/L. Ammonia levels dropped from nitrification to a mean of 0.005 mg/L after 10 hours. In the elevated nitrate tank, sodium nitrate (Sigma Chemical) was added to bring the levels to 200 mg/L $\text{NO}_3\text{-N}$. Additional sodium nitrate was added after routine water changes to keep nitrate levels at 200 mg/L. The fish were maintained at these experimental conditions for 4 weeks prior to immunization.

Water Quality

Water quality was monitored as follows: pH, temperature, and ammonia were measured daily; alkalinity, hardness, nitrites, nitrates and dissolved oxygen were measured twice weekly. In the elevated nitrate tank for the water quality experiment, nitrate levels were measured daily. The pH was measured using a pocket pH meter (Hanna Instruments, Woonsocket, RI). The other parameters were measured with a commercial kit (Hach Chemical, Loveland, CO). Ranges for water quality over the duration of the study are shown in Table 2-1.

Bacterial Antigen Production

A stock culture of *Aeromonas salmonicida* was obtained from the National Fish Health Research Laboratory (Kearneysville, WV). The cultures were grown on TSA agar in Kohle flasks at 20°C. Prior to harvesting, a subculture was grown on Coomassie brilliant blue agar (an indicator agar for *A. salmonicida*) to verify purity and presence of A-layer in the culture. Bacteria were harvested in sterile phosphate buffered saline (PBS), added to 2 L of PBS with 1% formalin and incubated 12 hours at 4°C. The bacteria were then resuspended by inverting the flask and incubated at 4°C until the bacteria had settled (approximately 24 hours). The PBS-formalin was decanted and the bacteria resuspended in 2 L PBS. When the bacteria had settled, the PBS was decanted and the concentrated bacteria from different lots pooled to form a stock solution of bacterin. The stock bacterin had a protein concentration of 11.8 mg/mL as determined by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL).

Immunization procedure

For immunization, the fish were netted and placed into a 400 L holding tank filled with water from the experimental tank. Buffered methane-tricainesulfonate (MS-222 at 0.25g/L),

(Sigma Chemical) was added to the water in the holding tank to sedate the fish. Fish were able to swim and maintain normal body position but did not struggle when netted. Fifty fish from each temperature group and 35 fish from each water quality group were immunized by intraperitoneal injection of 200 μ g bacterial protein. Twenty fish from each temperature and 15 from each water quality group were sham immunized with sterile PBS and tagged through the base of the dorsal fin with a fingerling tag (Floy Tags Inc., Seattle, WA) for identification.

Levels of antibody to *A. salmonicida* were determined from blood samples collected every four days until the mean antibody levels peaked, and then were monitored every two weeks. In all tanks, two sham immunized fish and four bacterin immunized fish were sampled to determine the mean antibody response. The fish were netted quickly, anesthetized in buffered MS-222 (0.25g/L) and bled from the caudal vessels with a 21 gauge needle and a 1 mL plastic syringe. The blood was placed into a pediatric serum tube and clotted for 1 hour at room temperature. The blood was centrifuged at 14,000 x g for 5 minutes in a microcentrifuge (Fisher Scientific, Pittsburgh, PA), and the serum removed and immediately frozen at -10°C.

Positive control

Eight 7 month old sunshine bass were maintained separately in a 200 L tank. They were immunized by intraperitoneal injection with 200 μ g protein of the *A. salmonicida* bacterin; two weeks later the immunization was repeated. The fish were bled ten days after the second immunization and then every other week for a total of 4 times. The blood clotted for 1 hour at room temperature and then was centrifuged for 5 minutes at 14,000 x g in a microcentrifuge. Serum from these fish was collected and pooled to form a standard positive control for use in the ELISA assay. Aliquots were stored frozen at -10°C.

Assay Procedure

Specific anti-*A. salmonicida* antibody levels were measured using the following ELISA procedure. All incubations, unless otherwise noted, were at room temperature in a moist chamber. All wells were rinsed three times between incubations with 300 μ L PBS-Tween 20 wash (8g NaCl, 0.2g KH_2PO_4 , 0.9g Na_2HPO_4 , 0.2g KCl, 0.5 mL Tween 20 to 1 L).

- 1) Plates (Linebro-Titertek, ICN Biomedicals Inc., Horsham, PA) were coated with 50 μ L *A. salmonicida* bacterin (3.5 μ g bacterial protein per well) in coating buffer (0.32g Na_2CO_3 , 0.58g NaHCO_3 to 200

mL, pH 9.6). The plates were incubated overnight at 4°C.

2) Wells were blocked with 100 μ L of blocking/diluting buffer (3.0g Tris-HCl pH 7.4, 0.18g EDTA, 4.3g NaCl, 1.5g bovine serum albumin, and 0.25 mL Tween-20 to 500 mL) for one hour.

3) Fifty μ L of blocking/diluting buffer and 2 μ L test serum or positive control was added to each well. All unknown samples were run in duplicate. Positive controls were run in triplicate. The plates were mixed after the addition of the serum and incubated for 1 hour.

4) Fifty μ L rabbit anti-hybrid striped bass immunoglobulin at 60 mg/mL protein (Smith 1992a) diluted 1:1000 in diluting/blocking buffer was placed in each well and incubated for 1 hour.

5) Fifty μ L of a commercial goat anti-rabbit IgG labeled with alkaline phosphatase (American Qualex, LaMirada, CA) diluted 1:5000 in diluting/blocking buffer was added to each well and incubated for 1 hour.

6) Fifty μ L of diethanolamine phosphate substrate (Pierce Chemical, Rockford, IL) was added to each well, and incubated for 1 hour.

7) Color production was determined in each well by measuring the absorbance at 402 nm by an automatic ELISA reader (Molecular Devices Corp, Menlo Park, CA). The specific antibody level of the test samples was calculated as a percentage of the positive control.

Statistical analysis

Statistical evaluation of the data was conducted using a commercial statistical program for the PC (Statistix Version 4. Analytical Software, Tallahassee, FL). One-way analysis of variance (ANOVA) was used to determine if the antibody levels of immunized fish were different from those of sham immunized fish for each treatment; and to determine if antibody levels of sham immunized fish in the different treatments were different from each other. A one-way ANOVA was also used to determine if the response of the immunized fish at 24°C in the temperature study was different from the immunized control fish at 24°C in the water quality experiment. A randomized control block ANOVA was used to determine differences with respect to time and treatment for the immunized fish from all experimental groups. In order to balance the randomized control block ANOVA, the sampling days were blocked into 7 time segments covering up to day 47

post immunization. The antibody response was measured up to day 97 post immunization for the temperature study and day 73 for the water quality study, although, there was no significant change in response past day 24 in any of the treatments. For all ANOVAs, differences between means was determined by a Tukey's pairwise comparisons of means.

RESULTS

The background antibody levels in the sham immunized fish for all treatment groups averaged 22 % of the positive control, and did not change significantly (p values were all greater than 0.05) over the time course of the experiment (Table 2-2, Fig. 2-1 & 2-2). This background activity was most likely due to cross reaction of *A. hydrophila* antibodies with the *A. salmonicida* in the ELISA, since serum from sunshine bass immunized with *A. hydrophila* did react with a low positive response on the *A. salmonicida* coated plate. It is possible, since *A. hydrophila* is present in most water sources, that fish are continually exposed to this antigen and have a constant background titer. ANOVA analysis of the sham immunized fish demonstrated minor differences ($p < 0.001$) in background titers between fish in the temperature group and fish in the water quality group.

To compensate for the differences in background titer, the mean response of the sham immunized fish for each tank was subtracted from the response of the immunized fish.

Antibody production in all treatment groups of immunized fish averaged 72 % of the positive control. Significant differences ($p < 0.0001$) were evident between immunized fish and sham immunized fish for all treatments (Table 2-2, Figs. 2-1 & 2-2). Immunized fish maintained at cooler temperatures and in the elevated nitrate tank had a lower antibody response compared to the other groups of immunized fish (Figs. 2-1 and 2-2, Table 2-3). When corrected for the differences in background, the plateau maximum response at 10°C and 18°C was 32.6 % and 56 % of the positive control, respectively, and 48 % of the positive control in the elevated nitrate tank. The antibody responses in the 10°C and 18°C tanks were also slower to reach maximum levels (Figs. 2-1 & 2-2). Maximum antibody levels were reached in 8 days in the 24°C, 29°C and all water quality tanks, while it took 12 days at 18°C, and 30 days at 10°C.

When corrected for the differences in background antibody levels, there was no difference ($p = 0.0886$) between the immunized fish held in the 24°C temperature tank and the

fish in the control water quality tank at 24°C. This allowed for comparison of antibody responses between fish in the temperature group and fish in the water quality group. A randomized control block ANOVA of the immunized fish from all groups was used to determine differences between treatment groups. The time component was used as a block effect to test different treatments as there was an expected difference due to time. Analysis showed significant treatment ($p < 0.0001$) and time ($p < 0.0001$) components between the different experimental groups (Table 2-3). The means comparison test for immunized fish from the 7 experimental groups (Table 2-3) indicated no difference in the antibody response of fish between the temperature tanks at 24°C, 29°C, the water quality control tank and the elevated ammonia tank. The antibody response was similar for fish in the 18°C tank and the elevated nitrate tank; but was of a lower magnitude than the response of the fish in the treatments listed above. Fish in the 10°C tank had a significantly lower antibody response than fish in any of the other treatments. Trend analysis of the four temperature tanks indicated a significant linear ($P < 0.0001$) trend showing an increase in antibody response with increasing temperature.

The fish in the nitrate tank appeared to become blind three weeks into the experiment, one week after the nitrate concentration reached 200 mg/L. The fish would often swim into objects and the walls of the tank. The skin color became darker, but there were no obvious gross lesions or changes to the eyes. When the first four fish were netted and moved to the holding tank for immunization, they immediately became agitated and then rolled upside down and died. This response was not due to a rapid change in water quality, as the holding tank contained water direct from the experimental tank. Adding MS-222 (0.25g/L) to the water in the holding tank prior to addition of the fish calmed them down sufficiently to immunize the majority of the fish without high mortality. The fish responded to the handling in a similar manner, but only 5 of 48 fish died. Three weeks after the fish were immunized (7 weeks at elevated nitrate) the fish began to die. Each day approximately 3 % of the fish were found moribund. About 50 % of the moribund fish were icteric, with a yellow color around the operculum, gills, ventral abdomen and in the viscera and fat. The other moribund fish showed no clinical signs. The icteric fish were anemic with a packed cell volume (PCV) of 20 - 25 %, while the other moribund fish had a PCV of 30 - 45 %. All the fish in the tank died by day 50 post immunization. There was no analytical evidence of contaminants in the

sodium nitrate added to the tank.

DISCUSSION

Antibody production is one way in which the immune system responds to protect an animal from disease. Antibody production in ectothermic vertebrates is influenced by temperature, specifically antibody response is diminished or delayed at cooler temperatures. This decreased response at cooler temperatures may play a role in the increased incidence of fish diseases in the spring; when water temperatures have warmed sufficiently to allow pathogens to multiply, yet not enough to allow a full antibody response from the fish. Other water quality parameters are known to decrease a fish's resistance to disease (Nicholson et al. 1990); however, the mechanism for this lowered resistance to disease is not well understood.

Sunshine bass are a warm water fish, and although they tolerate overwintering in temperate climates, most of their growth is in the warmer months of the year. The suggested range of temperatures for culturing sunshine bass is 18-32°C (Nicholson et al. 1990). Proposed water quality conditions

for rearing striped and hybrid bass are as follows: pH 6.7-8.6, un-ionized ammonia < 0.02 mg/L, nitrite < 0.01 mg/L, nitrate < 1.0 mg/L, alkalinity > 20 mg/L, calcium hardness > 52 mg/L and dissolved oxygen > 5 mg/L (Warren et al. 1990). With the exception of some of the experimental conditions purposefully outside these ranges, our water quality was within the ranges determined to be conducive to sunshine bass culture.

Optimum temperature for sunshine bass is difficult to define as there are numerous factors that can be optimized such as growth, feed conversion, disease resistance, physiologic homeostasis and many others. Early work demonstrated that the optimum temperature for growth of young adult hybrid striped bass was 26°C (Nicholson et al. 1990). A later study indicated that hybrid striped bass demonstrated maximal growth at 21°C, while growth declined above this temperature (Woiwode and Adelman 1991). If it is assumed that maximal growth is a reflection of efficient physiologic function, and that growth will decrease if the fish is not functioning under optimal conditions, then sunshine bass function more efficiently at temperatures in the low to mid 20s. In support of this temperature range as optimal, are the results of a study conducted by (Douglas and Jahn 1987).

They found that hybrid striped bass congregated at the cooler temperatures of 20-25°C during the warmer months, and even tolerated reduced oxygen concentrations of 2 mg/L to avoid water temperatures above 27°C. It has also been shown that hybrid striped bass and other fish will congregate in the heated water outlets of industrial plants in the colder months (Glass and Maughan 1985). In these instances, the fish are moving to temperatures which allows more efficient physiologic function.

More information is available on striped bass than on hybrid striped bass. Striped bass when given a choice of temperatures will congregate at temperatures less than 23°C (van den Avyle and Evans 1990) during the summer months. Lochmiller et al. (1989) postulated that striped bass in the summer were stressed at temperatures ranging from 26 to 29 °C. Davis and Parker (1990) showed that striped bass were stressed at 30°C resulting in higher cortisol levels and slower recovery times in response to netting stress. Sunshine bass maintained at elevated temperatures also have higher cortisol levels than fish at 18 and 10°C (Hrubec et al.). This all suggests that sunshine bass are stressed at the higher temperatures and do not function at optimal physiologic levels.

It is possible that stress and the resulting elevated cortisol levels at higher temperatures could depress antibody production. The effect of temperatures above optimal on antibody production has only been addressed by van Ginkel et al. (1985) who found antibody production suppressed at higher temperatures in rainbow trout. We did not find suppression of antibody production in sunshine bass at 29°C, even though this is above the suggested optimal range of low to mid 20s. Other studies have not shown a decrease in antibody production at elevated temperatures, although, the optimal temperatures for the given species were not discussed (Rijkers et al. 1981, Stolen et al. 1984).

Sunshine bass maintained at suboptimal water temperatures respond immunologically in a manner similar to coho salmon (Paterson and Fryer 1974) and three species of flounder (Stolen et al. 1982), demonstrating both a decrease in the magnitude and a delay in the time of antibody response. Other studies have found that although the response is delayed at cooler temperatures, the magnitude of the response is unchanged. This was demonstrated in carp immunized with sheep red blood cells (Rijkers et al. 1981), summer flounder immunized with horse erythrocytes (Stolen et

al. 1984) and in rainbow trout plaque-forming cells *in vitro* (van Ginkel et al. 1985). An explanation for the difference in the magnitude of antibody response at cooler temperatures may be the type of antigen. Miller and Clem (1984) found *in vitro* that the magnitude of response by channel catfish plaque-forming cells to T-independent antigens was not affected by temperature, but the magnitude of the response to T-dependent antigens was decreased at lower temperatures.

Aeromonas salmonicida has been shown to act as a T-independent antigen (Ellis 1988). Miller and Clem's work indicates that the antibody response to *A. salmonicida* will not be affected by cooler temperatures. Our work with *A. salmonicida* in sunshine bass, and Paterson and Fryer's (1974) work with *A. salmonicida* in coho salmon, do not follow this pattern. Instead both species demonstrate a delay in the time and suppression of maximum response at cooler temperatures. These results demonstrate a response similar to that of a T-dependent antigen as described by Miller and Clem (1984). The discrepancy may be due to the presence of both T-independent and T-dependent antigens present on *A. salmonicida*, or a species difference in the *in vivo* response to this particular antigen.

Ammonia is the major excretory product of fishes and is also

formed from the breakdown of uneaten feed. Ammonia is in an equilibrium between NH_3 and NH_4^+ depending mainly on the temperature and pH, although other factors influence the equilibrium. Un-ionized ammonia is usually regarded as the toxic component, however, ionized ammonia may also be toxic to a lesser degree. Signs of ammonia toxicity in fishes generally include damage to the respiratory epithelia, and neurologic signs. These clinical signs are similar to those exhibited by terrestrial animals. In mammals, damage to respiratory epithelium occurs from elevated environmental ammonia, while elevated blood ammonia levels cause an hepatic encephalopathy. In fishes, plasma ammonia levels parallel environmental ammonia concentrations (McDonald and Milligan 1992), allowing for environmental ammonia to induce systemic signs of toxicity.

Ammonia toxicity in fishes has been reviewed by Meade (1985). There is disagreement about safe levels of ammonia for fishes. Growth is reduced at levels of 0.05 - 0.2 mg/L un-ionized ammonia, and gill damage occurred at levels as low as 0.005 mg/L; however, other studies have shown that NH_3 levels from 0.0125 to 0.3 mg/L did not cause gill hyperplasia (Meade, 1985). In striped bass and hybrid striped bass, the total ammonia (un-ionized plus ionized

forms) 96 hour LC_{50} is 1.5-2.8 mg/L (Nicholson et al. 1990). Total ammonia concentrations of > 0.6 mg/L reduced feeding, slowed growth, caused histologic changes to the gill filaments and reduced resistance to disease (Nicholson et al. 1990).

Total ammonia concentration in the elevated ammonia tank of this study fluctuated daily between 0.2 mg/L before addition of the ammonium chloride to 2.6 mg/L after the addition of ammonium chloride, giving an un-ionized range of 0.001 to 0.15 mg/L. This chronic exposure to ammonia, caused no change in the antibody response to *A. salmonicida*. The reduced resistance to disease seen with elevated ammonia levels is therefore probably not caused by direct suppression of antibody production. Elevated cortisol levels induced by chronic environmental stress could suppress antibody production and reduce disease resistance. It is possible that ammonia levels in this experiment were too low to affect antibody production via a cortisol induced stress response. It is also possible that ammonia toxicity and possible immune suppression are dependent on interacting environmental factors as demonstrated by the conflicting published data on toxic levels of ammonia.

Elevated nitrate levels affected the immune response to the same extent as the suboptimum 18°C temperature. Nitrate is generally considered non-toxic to fishes. In most culture systems, nitrate levels do not exceed 50 mg/L, but in intensive recirculation systems nitrate levels often are above 100 mg/L. Adult striped bass can tolerate nitrate levels up to 800 mg/L, but fry show signs of stress at 200 mg/L (Bonn et al. 1976). However, both adult and fry feed better and have a greater growth rate at nitrate concentrations below 38 mg/L (Bonn et al. 1976). There is no information available on the tolerance levels for striped bass hybrids such as sunshine or palmetto bass. The cause of the clinical signs and mortality seen in the nitrate treated fish may be from the inability of sunshine bass to tolerate nitrate levels to the same extent as striped bass. In addition, the sunshine bass were maintained at elevated levels for 7 weeks; this extended time of exposure may have enhanced any pathophysiology associated with nitrates.

The cause of the clinical signs and mortality seen in the nitrate treated fish is unclear. Some fish suffered from a hemolytic crisis, becoming anemic and icteric prior to death, but whether this was a direct result of exposure to nitrate is not known. There is a report of jaundice in

cultured eels associated with a hemolytic crisis, although, excessive destruction of erythrocytes was not the primary cause of jaundice as the bilirubin was mainly conjugated (Endo et al. 1992). The cause of the cholestasis in eels was not determined, but based on histologic changes the authors felt there was an intrahepatic disorder. The observed suppression of the immune response may be a direct effect of nitrate on the immune system, or may be due to the fish being physiologically compromised and unable to mount an adequate response. Further work to elucidate the effect of nitrate on sunshine bass is needed before further conclusions can be made.

CONCLUSIONS

Temperature and water quality factors affected the immune response of sunshine bass. Temperatures of 10°C and 18°C decreased the magnitude and delayed the time of the antibody response to *A. salmonicida*. The antibody response was not affected at 29°C, which is above the optimal temperature for sunshine bass. Elevated un-ionized ammonia concentrations of 0.15 mg/L also did not affect the antibody response. Elevated nitrate levels of 200 mg/L, however, decreased the

antibody response to the same extent as 18°C water.

REFERENCES

- Arkoosh, M.R., Kaattari, S.L., 1990. Quantification of fish antibodies to a specific antigen by an enzyme-linked immunosorbent assay (ELISA). In: J.S. Stolen, T.C. Fletcher, D.P. Anderson, B.S. Roberson, and W.B. van Muiswinkel (Editors), *Techniques in Fish Immunology*, Vol. 1, SOS Publications, Fair Haven, NJ, pp. 15-24.
- Bonn, E.W., Bailey, W.M., Bayless, J.D., Erickson, K.E., and Stevens, R.E., 1976. Guidelines for striped bass culture. Striped Bass Committee, Southern Division, American Fisheries Society, Bethesda, Maryland, p. 69.
- Burreson, E.M., and Frizzell, L.J., 1986. The seasonal antibody response in juvenile summer flounder (Paralichthys dentatus) to the hemoflagellate Trypanoplasma bullocki. *Vet. Immunol. Immunopathol.* 12:395-402.
- Davis, K.B., and Parker, N.C., 1990. Physiological stress in striped bass: effect of acclimation temperature. *Aquaculture* 91:349-358.
- Douglas, D.R., and Jahn, L.A., 1987. Radiotracking hybrid striped bass in Spring Lake, Illinois, to determine temperature and oxygen preferences. *N. Amer. J. Fish. Manag.* 7:531-534.
- Ellis, A.E., 1988. Ontogeny of the immune system in the teleost fish. In: A.E. Ellis (Editor), *Fish Vaccination*. Academic Press, New York, NY, pp. 20-31.
- Endo, M., Sakai, T., Yamaguchi, T., and Nakajima, H., 1992. Pathology of jaundice in the cultured eel Anguilla japonica. *Aquaculture* 103:1-7.
- Glass, R.D., and Maughan, O.E., 1985. Concentrated harvest of Striped Bass X White bass near a heated water outlet. *N. Amer. J. Fish. Manag.* 5:105-107.

- Hamilton, A.J., Fallon, M.J.M., Alexander, J., and Canning E.U., 1986. A modified enzyme-linked immunosorbent assay (ELISA) for monitoring antibody production during experimental Aeromonas salmonicida infection in rainbow trout Salmo gairdneri). Dev. Comp. Immunol. 10:443-448.
- Hazel, C.R., Thomsen, W., and Meith, S.J., 1971. Sensitivity of striped bass and stickleback to ammonia in relation to temperature and salinity. Calif. Fish and Game 57:138-153.
- Hrubec, T.C., Robertson, J.L., and Smith, S.A., The effects of temperature on the hematology and serum chemistry of sunshine bass (Morone chrysops X Morone saxatilis). Submitted to Comparative Biochemistry and Physiology 1994.
- Klesius, P.H., 1990. Effect of size and temperature on the quantity of immunoglobulin in channel catfish, Ictalurus punctatus. Vet. Immunol. Immunopathol. 24:187-195.
- Lochmiller R.L., Weichman, D.J., and Zale, A.V., 1989. Hematological assessment of temperature and oxygen stress in a reservoir population of striped bass (Morone saxatilis). Comp. Biochem. and Physiol. 93A:535-541.
- Luft, J.C., Clem, L.W., and Bly, J.E., 1991. A serum-free medium for bony fish leucocyte in vitro mitogen-induced proliferation. Fish and Shellfish Immunol 1:233-235.
- McDonald, D.G., and Milligan, C.L., 1992. Chemical Properties of Blood. In: W.S. Hoar, D.J. Randall, and A.P. Farrell (Editors), Fish Physiology, Vol. 12 Part B, The Cardiovascular System. Academic Press, New York, NY, pp. 56-135.
- Meade, J.W., 1985. Allowable ammonia for fish culture. Prog. Fish. Cult. 47:135-145.

- Miller N.W., and Clem, L.W., 1984. Temperature mediated processes in teleost immunity: Differential effects of temperature on catfish *in vitro* antibody responses to thymus-dependent and thymus-independent antigens. J. Immunol. 133:2356-2359.
- Nicholson, L.C., Woods, L.C., and Woiwode, J.G., 1990. Intensive culture techniques for the striped bass and its hybrids. In: R.M. Harrell, J.H. Kerby, and R.V. Minton (Editors), Culture and Propagation of Striped Bass and its Hybrids, American Fisheries Society, Bethesda, MD, pp. 141-158.
- O'Neill, J.G., 1986. An in vitro study of polymorphonuclear phagocytosis and the effect of temperature. In: J.S. Stolen, D.P. Anderson, and W.B. van Muiswinkel (Editors), Fish Immunology, Elsevier Science Pub., London, pp. 47-55.
- Paterson, W.P., and Fryer, J.L., 1974. Effect of temperature and antigen dose on the antibody response of juvenile coho salmon (Oncorhynchus kisutch) to Aeromonas salmonicida endotoxin. J. Fish. Res. Board Can. 31:1734.
- Rijkers, G.T., Wiegerinck, J.A., van Oosterom, R., and van Muiswinkel, W.B., 1981. Temperature dependence of humoral immunity in carp (Cyprinus carpio). In: Solomon, B.J. (Editor), Aspects of Developmental and Comparative Immunology. Proc. of the 1st Congress of Developmental and Comparative Immunology, 1980, Aberdeen, Pergamon Press, Oxford, pp. 477-482.
- Robohm, R.A. 1986. Paradoxical effect of cadmium exposure on antibacterial antibody response in two fish species: Inhibition in cunners (Tautogolabrus adspersus) and enhancement in striped bass (Morone saxatilis). Vet. Immunol. Immunopathol. 12:251-262.

- Scott, A.L., Rogers, W.A., and Klesius, P.H., 1985. Chemiluminescence by peripheral blood phagocytes from channel catfish: Function of opsonin and temperature. Dev. Comp. Immunol. 9:241-250.
- Smith, S.A. 1992a. Affinity purification of serum immunoglobulin from fish. In: J.S. Stolen, T.C. Fletcher, D.P. Anderson, B.S. Roberson, and W.B. van Muiswinkel (Editors), Techniques in Fish Immunology, Vol. 2, SOS Publications, Fair Haven, NJ, pp. 125-130.
- Smith S.A., Levy M.G., and Noga E.J., 1992b. Development of an enzyme-linked immunosorbent assay (ELISA) for the detection of antibody to the parasitic dinoflagellate Amyloodinium ocellatum in Oreochromis aureus. Vet Parasit 42:145-155.
- Stave, J.W., and Roberson, B.S., 1985. Hydrocortisone suppresses the chemiluminescent response of striped bass phagocytes. Dev. Comp. Immunol. 9:77-84.
- Stave, J.W., Roberson, B.S. and Hetrick, F.M., 1983. The chemiluminescence of phagocytic cells isolated from the pronephros of striped bass. Dev. Comp. Immunol. 7:269-276.
- Stave, J.W., Cook, T.M., and Roberson, B.S., 1987. Chemiluminescent responses of striped bass, Morone saxatilis (Walbaum), phagocytes to strains of Yersinia ruckeri. J. Fish Dis. 10:1-10.
- Stolen J.S., Gahn, T., and Nagle J., 1982. The humoral antibody formation to erythrocyte antigens in three species of flatfish. Dev. Comp Immunol, suppl. 2:101-106.
- Stolen, J.S., Gahn, T., Kasper, V., and Nagle, J.J., 1984. The effect of environmental temperature on the immune response of a marine teleost (Paralichthys dentatus). Dev. Comp. Immunol. 8:89-98.

- van den Avyle, M.J., and Evans, J.W. 1990. Temperature selection by striped bass in a Gulf of Mexico coastal river. N. Amer. J. Fish. Manag. 10:58-66.
- van Ginkel F.W., van Muiswinkel, W.B., Merchant, B., Lizzio, E.F., Dixon, O.W., and Anderson, D.P., 1985. Temperature comparisons for antibody production in vitro by plaque-forming cells from the trout, Salmo gairdneri (Richardson), and mice. J. Fish Biol. 27:265-272.
- Warren, H.J., Harrell, R.M., Geiger, J.G., and Rees, R.A., 1990. Design of rearing facilities for striped bass and hybrid striped bass. In: R.M. Harrell, J.H. Kerby, and R.V. Minton (Editors), Culture and Propagation of Striped Bass and its Hybrids, American Fisheries Society, Bethesda, MD, pp. 17-28.
- Wechsler, S.J., McAllister, P.E., Hetrick, F.M., and Anderson, D.P., 1986. Effect of exogenous corticosteroids on circulating virus and neutralizing antibodies in striped bass (Morone saxatilis) infected with infectious pancreatic necrosis virus. Vet. Immunol. Immunopathol. 12:305-311.
- Wishkovsky, A., and Avtalion, R.R., 1982. Induction of helper and suppressor functions in the carp (Cyprinus carpio) and their possible implication in seasonal disease in fish. Dev. Comp Immunol, suppl. 2:83-91.
- Woiwode, J.G., and Adelman, I.R., 1991. Effects of temperature, photoperiod, and ration size on growth of hybrid striped bass X white bass. Trans. Amer. Fish. Soc. 120:217-229.

Table 2-1. Ranges of the water quality parameters over the course of the experiment for both temperature and water quality studies.

TEMPERATURE TANKS ¹				
PARAMETER	10°C	18°C	24°C	29°C
Temperature (C)	9-11	17-18	23-25	27-30
pH	7.4-8.3	7.4-8.1	7.1-8.1	7.3-8.1
Ammonia (NH ₃ mg/L)	0-0.005	0-0.006	0-0.009	0-0.009
Nitrite (NO ₂ -N mg/L)	0-0.06	0-0.05	0-0.15	0-0.05
Nitrate (NO ₃ -N mg/L)	2-6	3-7	3-6	3-6
Alkalinity (mg/L)	86-103	86-120	86-137	86-137
Hardness (mg/L)	86-154	103-154	103-154	86-137
Dissolved Oxygen (mg/L)	8-9	7-9	5-6	5-6

WATER QUALITY TANKS ²			
PARAMETER	CONTROL	AMMONIA	NITRATE
Temperature (C)	24-25	24-25	24-25
pH	7.4-7.9	7.5-7.9	7.5-8.0
Ammonia (NH ₃ mg/L)	0-0.011	0-0.150	0-0.011
Nitrite (NO ₂ -N mg/L)	0-0.03	0-0.06	0-0.20
Nitrate (NO ₃ -N mg/L)	2-8	4-16	180-220
Alkalinity (mg/L)	68-86	68-86	68-86
Hardness (mg/L)	86-171	103-171	103-188
Dissolved Oxygen (mg/L)	6-7	6-7	7-8

1) The temperature designation used for each group was determined by the mean temperature over the course of the experiment.

2) Ammonium chloride and sodium nitrate were added to the respective tanks to elevate these water quality parameters.

Table 2-2. Analysis of variance results, A) comparing the change in antibody response of sham immunized fish with time over the course of the experiment, and B) comparing differences in the antibody response between immunized and sham immunized fish.

A) Sham-immunized fish vs. time within each treatment

TREATMENT	P ¹	MIN ²	MAX ²
10°C	0.4197	15.9	27.9
18°C	0.1094	12.6	36.0
24°C	0.4203	9.2	17.6
29°C	0.1070	9.3	26.2
Control water	0.1172	19.6	39.3
Ammonia tank	0.2189	25.6	50.1
Nitrate tank	0.0680	20.0	45.1

B) Immunized vs. sham-immunized fish within each treatment

TREATMENT	P ³	MEAN IMMUNIZED ⁴	MEAN SHAM ⁴
10°C	<0.0001	38.4	21.3
18°C	<0.0001	63.0	23.2
24°C	<0.0001	75.9	12.9
29°C	<0.0001	79.8	19.0
Control water	<0.0001	86.0	29.3
Ammonia tank	<0.0001	92.4	38.2
Nitrate tank	<0.0001	68.2	26.5

1) A p-value < 0.05 indicates a significant change in antibody level over the time course of the experiment.

2) Minimum and maximum percentages of the ELISA positive control over the time course of the experiment for the treatment group.

3) A p-value < 0.05 indicates a significant difference in the antibody levels of immunized and sham immunized fish for each treatment.

4) Mean percentages of the ELISA positive control for immunized and sham immunized fish in each group.

Table 2-3. A randomized control block ANOVA used to determine differences with respect to time and treatment for the immunized fish corrected for differences in background titers.

Randomized Control Block ANOVA Table

SOURCE	DF	SS	MS	F	P
Treatment (A)	6	39167.4	6527.9	48.08	0.0000
Time (B)	5	25940.0	5188.1	38.21	0.0000
Fish (C)					
A*B*C	156	21182.1	135.78		
TOTAL	167	86289.9			

Tukey's Means Comparison Results for Immunized Fish by Treatment

TREATMENT	MEAN	HOMOGENOUS GROUPS
Ammonia	60.10	I
29°C	56.91	I
24°C	53.14	I
Control	51.67	I
18°C	41.60	..I
Nitrate	38.50	..I
10°C	11.87	I

Critical q value 4.168, Rejection level 0.05

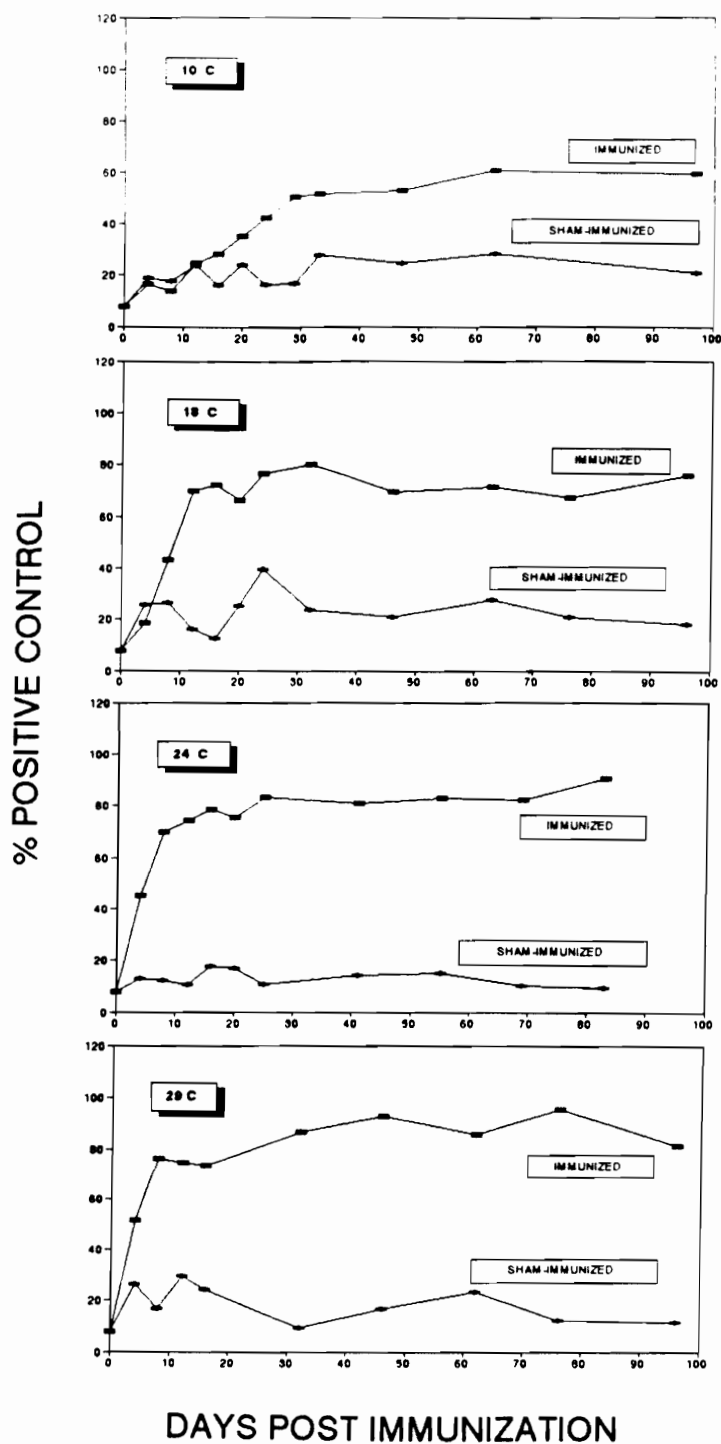


Figure 2-1. The antibody response of immunized and sham immunized sunshine bass to an *Aeromonas salmonicida* bacterin at different water temperatures. Antibody levels were monitored with an ELISA, using the percentage of a positive control to determine the response.

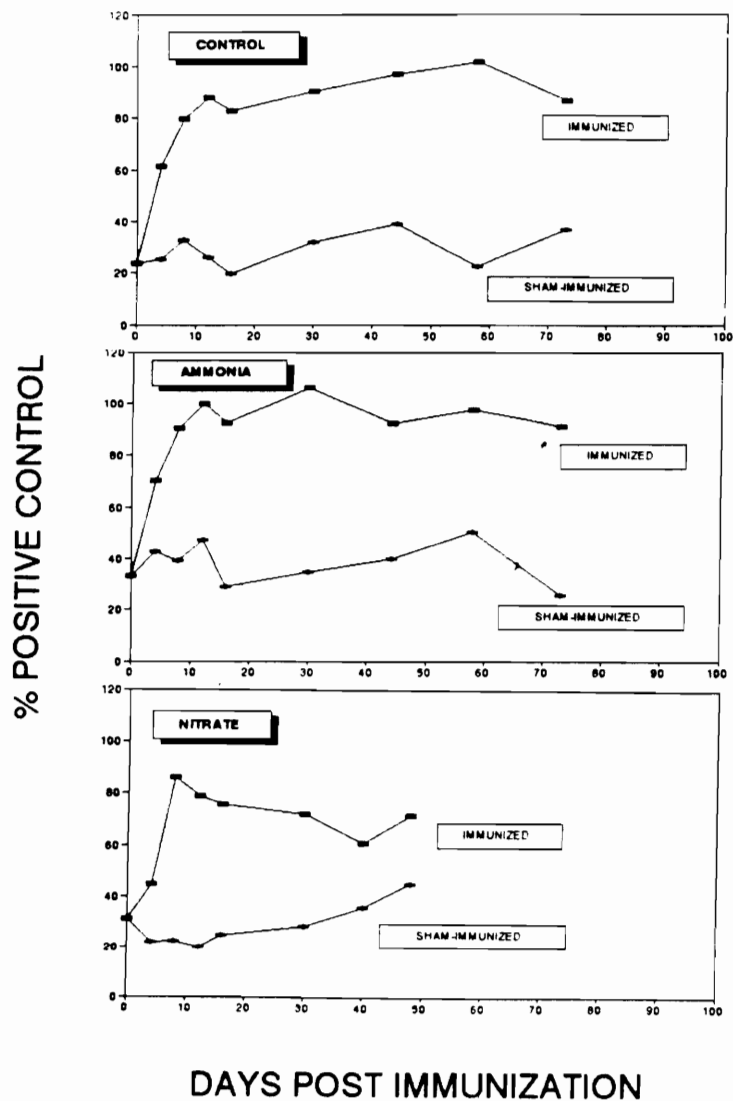


Figure 2-2. The antibody response of immunized and sham immunized sunshine bass to an *Aeromonas salmonicida* bacterin in control, elevated ammonia and elevated nitrate tanks. Antibody levels were monitored with an ELISA, using the percentage of a positive control to determine the response.

CHAPTER 3

Hematologic Reference Intervals for Hybrid Striped Bass:
Comparisons Between Culture System and Type of Hybrid.

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ABSTRACT

Hematologic reference intervals were determined for clinically healthy sunshine bass (female *Morone chrysops* X male *Morone saxatilis*) raised in high density recirculating systems, sunshine bass raised in tanks, and palmetto bass (female *Morone saxatilis* X male *Morone chrysops*) raised in tanks. The reference intervals were compared for fish in both types of culture systems and between the types of hybrids. The reference intervals for the sunshine bass in recirculating systems, sunshine bass in tanks, and palmetto bass in tanks respectively were:

packed cell volume 23-44, 34-44, 20-34 %

plasma protein 4.8-7.4, 5.1-6.5, 4.1-6.2 g/dL

red cell count 2.10-4.32, 3.66-4.96, 2.42-4.72 $\times 10^6/\mu\text{L}$

leukocyte count 58000-211000, 33000-115000 43000-118000 $/\mu\text{L}$

small lymphocytes 24000-116000, 10000-54000, 11000-78000 $/\mu\text{L}$

large lymphocytes 700-18800, 1300-6900, 500-6000 $/\mu\text{L}$

neutrophils 500-8200, 200-2100, 0-4000 $/\mu\text{L}$

monocytes 1200-10900, 800-5200, 0-1900 $/\mu\text{L}$

eosinophils 0-1000, 0-200, 0-1800 $/\mu\text{L}$

thrombocyte-like-cell 0-1300, 6-3700, 0-4000 $/\mu\text{L}$

thrombocytes 47000-113000, 31000-74000, 21000-104000 $/\mu\text{L}$

The reference intervals were significantly different for all three groups of fish for packed cell volume, plasma protein, erythrocyte count, small lymphocytes, monocytes and the thrombocyte-like-cell. For the other analytes, two of the groups were not significantly different while the third group was different. The analytes which were not statistically different for sunshine bass in tanks and palmetto bass in tanks were leukocyte count, large lymphocytes, neutrophils and thrombocytes. The only analyte which was not significantly different for the two groups of sunshine bass under different culture systems was eosinophil count. Two previously undescribed cell types were observed, an eosinophil and a thrombocyte-like-cell, although the latter cell may be a subset of thrombocyte.

INTRODUCTION

Sunshine bass (female *Morone chrysops* X male *Morone saxatilis*) and palmetto bass (female *Morone saxatilis* X male *Morone chrysops*) are important aquaculture species raised for both food and sport release, yet little is known about their basic physiology. Many changes in physiologic state are reflected in the blood, affecting both hematological and blood chemistry parameters. Hematological parameters can reflect acute and chronic physiologic changes due to nutrition, water quality, toxic insults and disease processes. A large body of literature has been published relating to fish hematology (Hawkins and Mawdesley-Thomas 1972; Blaxhall 1972; Blaxhall and Daisley 1973; Ellis 1977; Fänge 1992), however, the results are often conflicting and difficult to interpret. First, hematologic values may be considerably different in different fish species. Second, there is no standardized nomenclature for cell types, in part due to the lack of knowledge of the function of piscine blood cells. Even though standardized techniques have been recommended (Blaxhall and Daisley 1973) they are not always used. Third, environmental and biologic factors, culture conditions, and capture and sampling techniques affect hematologic parameters (Lane 1979; Ellsaesser and Clem 1986; Kjartansson et al. 1988; Bhaskar and Rao 1989; Hunn et al.

1992).

Hematologic analytes include the complete blood counts and cell morphology, the erythrocyte indices, packed cell volume (PCV) and plasma proteins. These analytes are fundamental tools in human and veterinary medicine; they are used in the diagnosis and prognosis of disease, and to monitor the effect of therapeutic, nutritional or environmental management. Hematology has not been used extensively in fish medicine due to the lack of reference intervals for various fish species, and because hematological changes with disease are not well documented. Blood analytes have been used to assess the physiologic effects of various toxins and diseases (Brown et al. 1987; Prasad et al. 1991; Haney et al. 1992; Salah El Deen and Rogers 1992), but, reference intervals were not determined in any of these studies. The information gained from these experiments, though helpful in determining the mechanism of action for a toxin or disease, cannot be used for diagnostic purposes. Unless the change in an analyte is large enough to fall outside the reference interval, one cannot tell if the change is due to disease or individual variation for a given fish.

Most hematologic studies only measure a few selected parameters, and there are only a couple comprehensive

studies for other species (Bhaskar and Rao 1989; LeaMaster et al. 1990). There are no previously published hematologic values for sunshine or palmetto bass, although some serum chemistry analytes have been measured (Tomasso et al 1980). Hematological studies of striped bass are more numerous; but most are limited by the small number of analytes measured (Westin 1978; Davis et al. 1982; Hunn and Greer 1990), or the small number of fish sampled (Courtois 1975, 1976; Brown et al. 1987). Reference intervals have been determined for PCV and hemoglobin in striped bass (Tisa and Strange 1983), and blood cell morphology has been described for striped bass (Groman 1982, Zinkl et al. 1991) but not for either of the hybrid bass. This is a comprehensive study to determine and publish a complete set of reference intervals, including blood cell counts, for sunshine and palmetto bass. It also is a comparison of hematological values between the type of hybrid bass and between the culture conditions under which the fish are raised.

MATERIALS AND METHODS

Experimental Animals and Their Maintenance:

Sunshine Bass in High Density Recirculating Systems

Sunshine bass fingerlings had been stocked the previous year

and grown indoors in 10,219 L recirculating systems (Nunley 1992). At the beginning of our study, the fish were 15 months old with an average weight of 454 g and a stock density of 80 g/L. The fish were fed a commercial pelleted diet (Bass Grower Formulation "Biosponge" Sheridan Extrusion Co, Sheridan, NY) at a rate of 3 % body weight per day. Two separate tanks with the same stock density were used in the study. In one tank, the water was treated with ozone at a rate of 135 g/day from a corona discharge generator (American Ozone, USA). All fish appeared clinically healthy; they had good body condition and no gross external or internal lesions. Skin scrapings and gill biopsies were negative for parasites, and bacterial cultures of the kidney (the main blood filtering organ in fishes) were negative.

Palmetto Bass in Tanks

Palmetto bass were spawned at Virginia Tech's Aquaculture Center and raised indoors until 4 months of age. The fish were brought to the VA-MD Regional College of Veterinary Medicine's Aquatic Medicine Laboratory where they were maintained indoors in 2000 L tanks with undergravel filters for an additional 5 months. The fish were stocked at approximately 1 g/L and fed daily a commercial pelleted diet (Zeigler Brothers Inc. Gardner PA). Fish from two tanks were sampled when the fish were 11 months old. All fish

appeared clinically healthy; they had good body condition and no gross external or internal lesions. Parasites were not seen on skin scrapings.

Sunshine Bass in Tanks

Sunshine bass were obtained from the Aquaculture Center when the fish were 16 months old and transported to the Aquatic Medicine Laboratory. The fish were maintained indoors in a 2000 L tank for an additional 2 months and then divided into four 600 L tanks to give a stock density of 4 - 6 g/L. The fish were moved to the smaller tanks to facilitate catching them with minimal stress. They were allowed to acclimate to the 600 L tanks for two weeks before sampling. The fish were fed daily a commercial pelleted diet (Floating Fish Nuggets, Zeigler Brothers Inc. Gardner, PA). All fish appeared healthy and in good body condition. There were no gross external or internal lesions, and no parasites were seen on skin scrapings.

Water Quality

Water quality was monitored as follows: pH, temperature and ammonia were measured daily; alkalinity, hardness, nitrites, and nitrates were measured twice weekly. Dissolved oxygen (DO) was measured daily in the recirculating production system, and twice a week in the tank systems. Temperature

and DO in the recirculating production system were measured using an oxygen meter (YSI, Yellow Springs, OH). The pH was measured using a pocket pH meter. The remaining analytes including DO in the tank systems were determined with a Hach Water Analysis Kit (Hach Chemical, Loveland, CO). Ranges for water quality over the duration of the study are shown in Table 3-1.

Sampling Schedule

Sunshine bass in the high density recirculating system, were sampled four times at 2 - 3 week intervals. At each sampling time, 10 fish from each recirculating system were quickly netted, sedated with aerated tricaine methanesulfonate (MS-222 at 0.25g/L) (Sigma Chemical Co., Saint Louis, MO). For both the sunshine and palmetto bass in tanks, the water was lowered to facilitate catching the fish. They were quickly netted and anesthetized in aerated, buffered MS-222 (0.25g/L). The palmetto bass in tanks were sampled over a 2 week period and at each sampling time 6 to 10 fish were bled. The sunshine bass in tanks were sampled on 4 consecutive days, one tank per day. All fish in a tank were caught and bled within 10 minutes. Fish from all three systems were fasted for 24 hours prior to sampling.

Blood Handling

Fish were bled using plastic syringes fitted with 21 gauge needles and the blood put into ethylenediamine tetra-acetic acid (EDTA) blood tubes. Blood smears for all fish were made using the EDTA treated blood. A direct smear on glass (without anticoagulant) was made with blood from the sunshine bass in the recirculating production system before putting the blood in the EDTA tube. The direct smears were used for cell morphology and size determination to prevent artifacts from the anticoagulant. The packed cell volume (PCV) and plasma protein (total solids) were determined in microhematocrit tubes by centrifugation (Clay Adams Autocrit-ultra 3, Parsippany, NJ) for 5 min. Plasma protein was determined with a refractometer (Reichert-Jung, Leica, Buffalo, NY). Total red and white cell counts were determined manually with a Neubauer hemacytometer using Natt-Herrick's solution as a diluent (Natt and Herrick 1952). This diluent has been recommended for use with fish blood (Campbell and Murru 1990; Stoskoph 1993). Thrombocytes could not be distinguished from leukocytes on the hemocytometer and were counted as a combined leukocyte/thrombocyte population. Blood smears stained with Wright's Geimsa stain (Hematek 1000 automatic stainer, Miles Inc., Elkhart, IN) were used for the differential white cell count and thrombocyte count. Leukocytes and thrombocytes

were easily distinguished on the smears and were counted until 200 leukocytes were enumerated. The number of thrombocytes was then subtracted from the leukocyte/thrombocyte count to give the total leukocyte count. Hemoglobin was determined using the cyanomethemoglobin method (Stanbio Laboratory Inc., San Antonio TX). Blood cell sizes were measured from the direct smears with length measured on the long axis of the cell and width measured at the widest part of the cell perpendicular to the long axis. For each cell type, 10 cells from each of eight fish were measured; fewer eosinophils (a total of 53) were measured.

Blood samples from all fish were analyzed for PCV and plasma protein. Total red and white counts, a differential white count and hemoglobin were determined on all fish from tanks. Twelve fish at each of the four sampling times were used for the cell counts of fish in the recirculating system. All clotted samples were discarded before analysis.

Statistical Analysis

Statistical evaluation of the hematological values was conducted using a commercial statistical program for the PC (Statistix Version 4, Analytical Software Tallahassee, FL). The reference intervals were determined according to the

guidelines proposed by the National Committee for Clinical Laboratory Standards for determining reference intervals in clinical laboratories (1991). Non-parametric methods were used to determine the reference intervals. The values were ranked, and the low and high 2.5 % discarded, with the range of the remaining values determining the reference interval. A Kruskal-Wallis test (for non-parametrically distributed analytes) and analysis of variance (ANOVA - for parametrically distributed analytes) was used to test for differences between reference intervals for the three groups of fish. If there was a significant difference ($P < 0.05$), a Tukey means comparison test was used to identify which group was different.

RESULTS

The sunshine bass had three groups of circulating cells: erythrocytes, leukocytes and thrombocytes. The leukocytes were further sub-divided into small and large lymphocytes, monocytes, neutrophils, eosinophils and thrombocyte-like-cells. Reference intervals for the hematological parameters are listed in Table 3-2. Erythrocytes were the most numerous cell in the blood, followed by thrombocytes and then small lymphocytes.

Cell Morphology

Erythrocytes were round to oval in shape with an average size in smears of $9.5 \times 6.8 \mu\text{m}$. The nuclei were oval and condensed in the mature cell. Reticulocytes, immature red cells, were observed frequently in blood smears. They had a characteristic blue-purple color to their cytoplasm similar to reticulocytes in mammalian blood. Reticulocytes were smaller and rounder ($8.8 \times 7.8 \mu\text{m}$) than mature erythrocytes, and their nuclei were more open in appearance with prominent chromatin clumping.

Small lymphocytes (Fig. 1, Plate 3-A) were the smallest cell type in the blood, averaging $5.3 \times 4.3 \mu\text{m}$. They had a condensed nucleus with a scanty rim of blue cytoplasm encircling the nucleus. Large lymphocytes (Plate 3-A and 3-D) were larger ($7.3 \times 6.2 \mu\text{m}$) than small lymphocytes with a more abundant and darker blue cytoplasm, and a more open nucleus. Even though the cytoplasm was abundant, the nucleus still took up more than 50 % of the cell volume. Both large and small lymphocytes often formed cytoplasm projections giving the lymphocyte a rough outline. Azurophilic granules were occasionally seen in both large and small lymphocytes.

Monocytes (Fig. 1, Plate 3-B) were about the same size (9.2

x 8.2 μm) as erythrocytes but had a dark cobalt blue cytoplasm which was often vacuolated. The cytoplasm was more abundant than that of the large lymphocyte with greater than 50 % of the cell consisting of cytoplasm. The nucleus was open in texture and variable in shape, being oval, round or horseshoe shaped. Neutrophils were the largest cell type in the blood averaging 10.3 x 9.1 μm (Fig. 1, Plate 3-B and 3-D). Abundant pale grey cytoplasm gave this cell an oval to round shape. There were no distinct cytoplasmic granules visible in the neutrophil, although the cytoplasm had a slight granular appearance. The nucleus was open and chromatin clumping was present. The nucleus was usually oval and occasionally indented (bean to horseshoe shaped), but was never segmented. Eosinophils (Fig. 2, Plate 3-C) were seen infrequently. They were small cells (8.1 x 6.9 μm) with clear grey cytoplasm containing eosinophilic granules. The nucleus was round to oval and eccentric with condensed chromatin. The cytoplasmic granules were usually distinct, but in some cells only gave a reddish cast to the cytoplasm. Occasionally eosinophils were as large as neutrophils.

The spindle form of thrombocyte seen on direct smears was an elongate oval (8.7 x 3.8 μm) with a light cerulean blue cytoplasm and a condensed oval nucleus (Fig. 1). Often, the

cells appeared in a spiked form with an elongated cytoplasmic tail. As the thrombocytes began the hemostatic process, their morphology changed dramatically; becoming round, with a round nucleus, and a rim of cytoplasm surrounding the nucleus. At this point, thrombocytes began to aggregate and appeared to decrease in size (Fig. 3). The cells were probably not shrinking but rounding up, thus providing a smaller cross sectional area when viewed on the smear. In the anticoagulated blood, the thrombocytes took on another appearance becoming round, oval or angular with a fair amount of cytoplasm (Fig. 4, Plate 3-D and 3-E). The nucleus became segmented and lobulated, but occasionally remained elongated or oval. The cytoplasm retained its light blue color, and in some cells became vacuolated. Other cell types did not change morphologically with the EDTA.

An additional cell type, a thrombocyte-like-cell was seen (Plate 3-D). The cytoplasm was pale grey and slightly granular in appearance. The nucleus was eccentric and always round to slightly oval and condensed. The cytoplasm was slightly more abundant and greyer than thrombocytes. These cells may be a subset of thrombocytes. In most blood smears, there were cells which had phagocytosed portions of erythrocytes (Fig. 5, Plate 3-E). The cell was always small

with a blue-grey cytoplasm, although, the cytoplasm was difficult to see because it was obscured by the large phagosome. The nucleus was pushed to the side of the cell due to the phagosome and was moderately condensed and round to oval. Positive identification of the cell type was not possible. Intensely blue staining cells were also observed infrequently and were interpreted to be immature blast cells (Plate 3-F). Due to their immaturity, it was impossible to determine their cell line morphologically.

Hematology

The reference intervals for the three groups of fish are given in Table 3-2. The groups were significantly different for all analytes measured based on the Kruskal-Wallis test or ANOVA with a $P < 0.0029$. These tests only determine if there is a difference between one or more groups; a means comparison test is then used to determine which groups are different. The means comparison test (Table 3-3) showed differences for all three groups of fish for PCV, plasma protein, erythrocyte count, small lymphocytes, monocytes, and thrombocytes. The sunshine bass raised in the recirculating system were different from the sunshine bass in tanks and the palmetto bass in tanks for the leukocyte count, large lymphocytes, neutrophils, and thrombocytes. The only analyte for which the two groups of sunshine bass

were not different was eosinophils. There were no significant differences in the hematologic values based on the sex of the fish.

DISCUSSION

The cell types described here are similar to the ones described by Groman (1982) and Zinkl et al. (1991). The same nomenclature has been adopted except that lymphocytes were further divided into large and small; and additional cell types, the eosinophil and the thrombocyte-like-cell were seen. Ellis (1977) clearly argues the case for not adopting mammalian nomenclature for use in fishes without prior demonstration of functional, ontogenetic and morphological similarities between the cell types. Our paper represents an entry into the systematized nomenclature and examination for which Ellis argues. However, the cells need to be identified, and adopting the mammalian and preexisting fish terminology is probably the most useful solution until more is known about fish blood cells. With this in mind, we have retained Groman's nomenclature for the previously described cell types, and have called the cell with eosinophilic granules an eosinophil. This does not imply that this cell is homologous with the mammalian

eosinophil.

Eosinophils have not been described previously in the blood of striped bass but have been described in other species of fishes (Gardner and Yevich 1969; Zinkl et al. 1991). Since eosinophils were seen in sunshine and palmetto bass, both hybrid fish, it may mean that eosinophils are present in white bass and their offspring, but not striped bass. Alternatively, eosinophils may only be present under certain conditions, so that they were not seen or recognized in the previous studies on striped bass hematology. In a study of the ultrastructure of the hematopoietic tissue of the striped bass, Bodammer et al. (1990) identified cells as eosinophils. It is unclear from their description whether these are "blood" eosinophils as we have seen, or if they are "tissue" eosinophils similar to the mast cell described by Ellis (1985). If Bodammer et al.'s cell is a circulating eosinophil, this is evidence that these cells exist in striped bass.

Morphologically, the thrombocyte-like-cell looked similar to a thrombocyte. Particularly in the EDTA treated blood where the thrombocytes appeared to have more cytoplasm. In the direct smears, however, the thrombocyte-like-cells observed were distinctly different from any of the forms of

thrombocytes. The color of the cytoplasm was greyer, and more abundant than in the activated thrombocyte. The thrombocyte-like-cells also were never involved in the clumped thrombocyte rafts which often formed in the direct smears. In the smears made from EDTA treated blood, the nuclei of the thrombocytes were usually segmented or indented and condensed, while the thrombocyte-like-cell nuclei were always round and more open.

Thrombocyte morphology in fishes has been characterized previously, and different classification systems have been proposed for different shapes of thrombocytes (Gardner and Yevich 1969; Ellis 1976). Much of the early work on thrombocytes, reviewed by Gardner and Yevich (1969), described developmental stages of thrombocytes based solely on morphologic appearance. The proposed early developmental stages, prothrombocytes, are similar to lymphocytes; and later developmental stages, definitive thrombocytes, are elongated or spindle shaped. They also suggest that small lymphocytes are precursors of thrombocytes. This view of the development of thrombocytes is not held by Ellis (1976) and Ferguson (1976) as thrombocytes are ultrastructurally and functionally distinct from lymphocytes. Ellis divides thrombocytes into four categories: spiked, spindle, ovoid and lone nucleus forms. The ovoid and lone nuclear forms

were found in fish where the blood was clotting more rapidly. In this study, we adhered to the classification given by Ellis (1976). All four forms of thrombocytes were seen on the direct smears. The thrombocytes in the lone nuclear form were difficult to distinguish from lymphocytes and for this reason all differential counts were done on smears of anticoagulated blood. In EDTA anticoagulated blood, the thrombocytes were variable shape, but were easily distinguished from lymphocytes and thrombocyte-like-cells.

The thrombocyte-like-cell may be a subset of thrombocyte which is involved in phagocytosis as opposed to hemostasis. Thrombocytes have been found to be phagocytic in other fish species (Gardner and Yevich 1969; Ferguson 1976). In birds, it has been shown that thrombocytes are phagocytic and are equally important as traditional phagocytes for removal of bacteria and other foreign particles from the blood (Chang and Hamilton 1979). The cells in this study which were seen phagocytosing erythrocytic debris may be the thrombocyte-like-cell acting as a phagocytic thrombocyte. The cytoplasm color of the phagocyte was darker than the thrombocyte-like-cell, but this may be due to the cytoplasmic contents which take up the stain being concentrated in a smaller area due to displacement by the phagocytosed red cell. There is no proof for this scenario other than morphologic observation,

however, electron microscopy, and functional tests on isolated cells might reveal ultrastructural and functional characteristics which would help determine the identity of the cell.

Macrophages are known to phagocytose erythrocytes in the spleen, although there have been no reports of monocytes phagocytosing erythrocytes intravascularly. Neutrophils are also phagocytic, but the phagocyte seen did not resemble either a monocyte or a neutrophil. The phagocyte was too small and the cytoplasm was not abundant enough. The color was too grey to be a monocyte and not the same shade as a neutrophil. We cannot preclude the possibility that these phagocytes are immature monocytes or neutrophils with a different appearance, however, no erythrocyte fragments were ever seen inside mature clearly identifiable monocytes or neutrophils.

Hematological values for other species, including striped bass, are listed in Table 3-4. Although mean values not reference intervals are listed, the means can be used as a basis for comparison. In general, the published values for striped bass are similar to those determined for the sunshine and palmetto bass in this study. The PCVs for striped bass tend to be higher but this may be caused by

erythrocyte swelling due to the stress of capture of wild fish on gill nets (the most-used method of capture in the papers listed). The hemoglobin measurements and the erythrocyte counts for striped bass are similar to those determined in this study for the hybrid bass. The erythrocyte counts for trout, salmon and goldfish are lower than we saw in the hybrid bass. This is most likely a species difference.

There are no previously published values for leukocyte counts in striped bass or its hybrids. The values presented here are higher than those reported for trout and goldfish. Some of the increase may be due to the fact that sunshine bass are hybrids. Barker et al. (1983) found hybrid *Ctenopharyngodon idella* X *Hypophthalmichthys nobilis* to have higher white cell numbers than either parent species. However, hybridization is unlikely to be the sole cause for the high count. The white count may be influenced by the culture system, as the sunshine bass in the high density recirculating system had a higher level of leukocytes than either hybrid bass maintained in tanks. Heavily stocked recirculating systems characteristically have a high organic load and high bacterial count in the water which could be inducing chronic leukocyte production in the fish.

For clinical diagnosis, sample means are not useful and are never used. Reference intervals are determined for a select population and used to determine whether an individual's analyte is above or below what is expected for the population. To be useful, the reference interval must be broad enough to accommodate slight differences in values due to daily fluctuations of environmental conditions, yet narrow enough to be able to detect a change due to a diseased state. Clinical reference intervals have been determined for rainbow trout PCV and hemoglobin (Wedemeyer and Nelson 1975); striped bass PCV and hemoglobin (Tisa and Strange 1983); and milkfish (*Chanos chanos*) PCV, hemoglobin, RBC, MCV, MCH, MCHC, leukocyte count and differential white count (Bhaskar and Rao 1989). In these studies, the data collected at different sampling times were combined to determine the reference intervals. In determining the reference intervals for this study, we also combined data collected on different days and tanks from the same population of fish. Before combining the data from the fish in the high density recirculating system, we did determine that there were no differences (ANOVA $p < 0.05$) for any of the analytes of fish from the ozone treated water and the fish from untreated water. The data collected on different days were combined provide a reference interval broad enough to accommodate variations in the analytes due to slight

changes in environmental conditions.

This study has shown that reference intervals are significantly different between sunshine bass and palmetto bass, and between sunshine bass raised in tanks and sunshine bass raised in recirculating systems. The differences are sufficient to recommend that separate sets of reference intervals be used for the different groups of fish.

However, as hematology is used in the future as a clinical tool, the reference intervals may need to be adjusted to either accommodate a wider selection of fish from different environments, or narrowed to be even more specific. It is important to note that the sunshine bass and palmetto bass in tanks had a greater number of analytes with similar reference intervals than did the sunshine bass under the two culture conditions. This implies that culture conditions under which fish are raised influences the hematology to a greater extent than the sex of the parents used to produce the striped bass / white bass crosses.

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LITERATURE CITED

- Barker, C. J., M. L. Beck, and C. J. Biggers. 1983. Hematologic and enzymatic analysis of Ctenopharyngodon idella x Hypophthalmichthys nobilis F1 hybrids. Comparative Biochemistry and Physiology 74A:915-918.
- Bhaskar, B. R., and K. S. Rao. 1989. Influence of environmental variables on hematology, and compendium of normal hematological ranges of milkfish, Chanos chanos (Forsk.) in brackish culture. Aquaculture 83:123-136.
- Blaxhall, P. C. 1972. The haematological assessment of the health of freshwater fish - A review of selected literature. Journal of Fish Biology 4:593-604.
- Blaxhall, P. C., and K. W. Daisley. 1973. Routine haematological methods for use with fish blood. Journal of Fish Biology 5:771-781.
- Bodammer, J. E., D. P. Anderson, and O. M. Dixon. 1990. Ultrastructure of the spleen and head kidney of striped bass. Journal of Aquatic Animal Health 2:182-193.
- Brown, C. L., G. Young, R. S. Nishioka, and H. A. Bern. 1987. Preliminary report on the physiological status of striped bass in the Carginez Strait die-off. Fisheries Research 6:5-16.
- Burton C. B., and S. A. Murray. 1979. Effects of density on goldfish blood - I Hematology. Comparative Biochemistry and Physiology 62A:555-558.
- Campbell, T., and F. Murru. 1990. An introduction to fish hematology. The Compendium on Continuing Education for the Practicing Veterinarian 12:525-532.
- Chang, C. F., and P. B. Hamilton. 1979. The thrombocyte as the primary phagocyte in chickens. Journal of the Reticuloendothelial Society 25:585-590.

- Courtois, L. A. 1975. Blood and serum analysis of adult striped bass, Morone saxatilis, captured in the Sacramento River. California Fish and Game 61:245-246.
- Courtois, L. A. 1976. Hematology of juvenile striped bass, Morone saxatilis (Walbaum), acclimated to different environmental conditions. Comparative Biochemistry and Physiology 54A:221-223.
- Davis, K. B., N. C. Parker, and M. A. Suttle. 1982. Plasma corticosteroids and chlorides in striped bass exposed to tricaine methanesulfonate, quinaldine, etomidate, and salt. Progressive Fish-Culturist 44:205-207.
- Ellis, A. E. 1976. Leucocytes and related cells in the plaice Pleuronectes platessa. Journal of Fish Biology 8:143-156.
- Ellis, A. E. 1977. The leukocytes of fish: a review. Journal of Fish Biology 11:453-491.
- Ellis A. E. 1985. Eosinophilic granular cells (EGC) and histamine responses to Aeromonas salmonicida toxins in the rainbow trout. Developmental and Comparative Immunology 9:251-260.
- Ellsaesser, C. F., and L. W. Clem. 1986. Hematological and immunological changes in channel catfish stressed by handling and transport. Journal of Fish Biology 28:511-521.
- Fänge, R. 1992. Fish Blood Cells. Pages 1-54 in W. S. Hoar, D. J. Randall, and A. P. Farrell editors. Fish Physiology volume 12 Part B, The Cardiovascular System. Academic Press Inc., San Diego California.
- Ferguson, H. W. 1976. The ultrastructure of plaice (Pleuronectes platessa) leucocytes. Journal of Fish Biology 8:139-142.

- Gardner, G. R., and P. P. Yevich. 1969. Studies on the blood morphology of three estuarine cyprinidontiform fishes. *Journal of the Fisheries Research Board of Canada* 26:433-447.
- Groman, D. B. 1982. The Histology of the Striped Bass. Pages 18-20 *American Fisheries Society, Monograph 3*. American Fisheries Society, Bethesda, Maryland.
- Haney, D. C., D. A. Hursh, M. C. Mix, and J. R. Winton. 1992. Physiological and hematological changes in chum salmon artificially infected with erythrocytic necrosis virus. *Journal of Aquatic Animal Health* 4:48-57.
- Hawkins, R. I., and L. E. Mawdesley-Thomas. 1972. Fish hematology a bibliography. *Journal of Fish Biology* 4:193-232.
- Hunn, J. B., and I. E. Greer. 1990. Colorimetric and refractometer estimates of total plasma protein in striped bass, Morone saxatilis (Walbaum). *Journal of Fish Biology* 36:617-618.
- Hunn, J. B., R. H. Wiedmeyer, I. E. Greer, and A. W. Grady. 1992. Blood chemistry of laboratory-reared golden trout. *Journal of Aquatic Animal Health* 4:218-221.
- Kjartansson, H., S. Fivelstad, J. M. Thomassen, and M. J. Smith. 1988. Effects of different stocking densities on physiological parameters and growth of adult atlantic salmon (Salmo salar L.) reared in circular tanks. *Aquaculture* 73:261-275.
- Lane, H. C. 1979. Progressive changes in hematology and tissue water of sexually mature trout, Salmo gairdneri Richardson during the autumn and winter. *Journal of Fish Biology* 15:425-436.

- LeaMaster, B. R., J. A. Brock, R. S. Fugioka, R. M. Nakanura. 1990. Hematologic and blood chemistry values for Sarotherodon melanotheron and a red hybrid tilapia in fresh water and seawater. *Comparative Biochemistry and Physiology* 97A:523-529.
- Lochmiller, R. L., J. D. Weichman, and A. V. Zale. 1989. Hematological assessment of temperature and oxygen stress in a reservoir population of striped bass (Morone saxatilis). *Comparative Biochemistry and Physiology* 93A:535-541.
- Murray, S. A., and C. B. Burton. 1979. Effects of density on goldfish blood - II Cell morphology. *Comparative Biochemistry and Physiology* 62A:559-562.
- NCCLS (National Committee for Clinical Laboratory Standards). 1992. How to define, determine, and utilize reference intervals in the clinical laboratory; proposed guidelines. NCCLS Document C28-P (ISBN 1-56238-143-1). NCCLS, Villanova, Pennsylvania.
- Natt, M. P., and C. A. Herrick. 1952. A new blood diluent for counting erythrocytes and leukocytes of the chicken. *Poultry Science* 31:735-738.
- Nunley, C. E. 1992. Production of hybrid striped bass (Morone chrysops X Morone saxatilis) in a recirculating aquaculture system. M.S. Thesis, Virginia Polytechnic Institute and State University, Blacksburg, Virginia.
- Prasad, T. A. V., T. Srinivas, G. M. Rafi, and D. C. Reddy. 1991. Effect in vivo of atrazine on hematology and O₂ consumption in fish Tilapia mossambica. *Biochemistry International* 23:157-161.
- Salah El Deen, M., W. A. Rogers. 1992. Acute toxicity and some hematological changes in grass carp exposed to Diquat. *Journal of Aquatic Animal Health* 4:277-280.

- Sandnes, K., O. Lie, and R. Waagbo. 1988. Normal ranges of some blood chemistry parameters in adult farmed Atlantic salmon Salmo salar. Journal of Fish Biology 32:129-136.
- Stoskopf, M. K. 1993. Clinical Pathology. Pages 113-131 in M. K. Stoskopf editor. Fish Medicine. W. B. Saunders Co. Philadelphia, Pennsylvania.
- Tisa, M. S., and R. J. Strange. 1983. Hematology of striped bass in fresh water. Progressive Fish-Culturist 45:41-44.
- Tomasso, J. R., K. B. Davis, and N. C. Parker. 1980. Plasma corticosteroid and electrolyte dynamics of hybrid striped bass (white bass X striped bass) during netting and hauling. Proceedings of the World Mariculture Society 11:303-310.
- Wedemeyer, G. A., N. C. Nelson. 1975. Statistical methods for estimating normal blood chemistry ranges and variance in rainbow trout (Salmo gairdneri), Shasta strain. Journal of the Fisheries Research Board of Canada 32:551-554.
- Westin, D. T. 1978. Serum and blood from adult striped bass, Morone saxatilis. Estuaries 1:126-128.
- Zinkl, J. G., W. T. Cox, and C. S. Kono. 1991. Morphology and cytochemistry of leukocytes and thrombocytes of six species of fish. Comparative Hematology International 1:87-195.

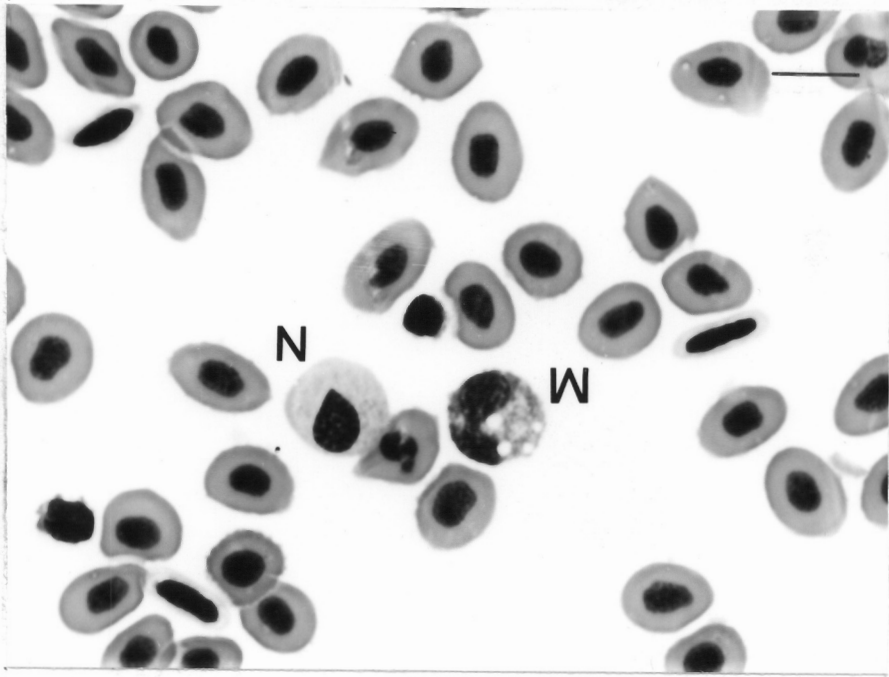


Figure 3-1. Neutrophil and Monocyte. - The neutrophil has pale grey slightly granular cytoplasm, the monocyte has vacuolated dark blue cytoplasm and an irregular nucleus. Between these cells is an erythrocyte with an irregular nucleus. Also present are spindle shaped thrombocytes and a lymphocyte. Direct smear. Bar is 10 μm .

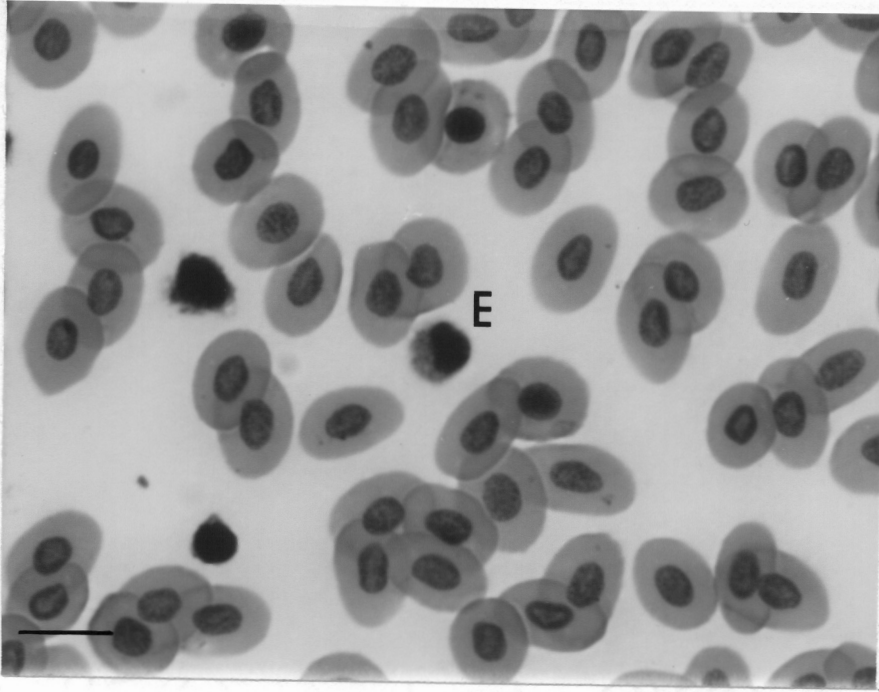


Figure 3-2. Eosinophil - A typical eosinophil with an eccentric nucleus and eosinophilic granules in the cytoplasm. EDTA smear. Bar is 10 μm .

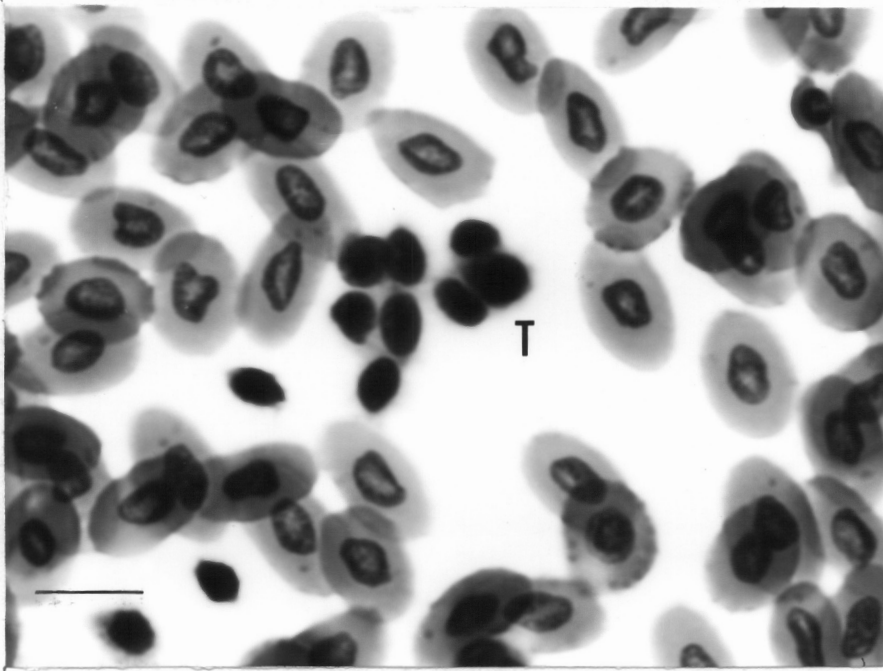


Figure 3-3. Thrombocytes - When activated during clotting, thrombocytes lose the oval shape, become round and clump into "rafts". Direct smear. Bar is 10 μm .

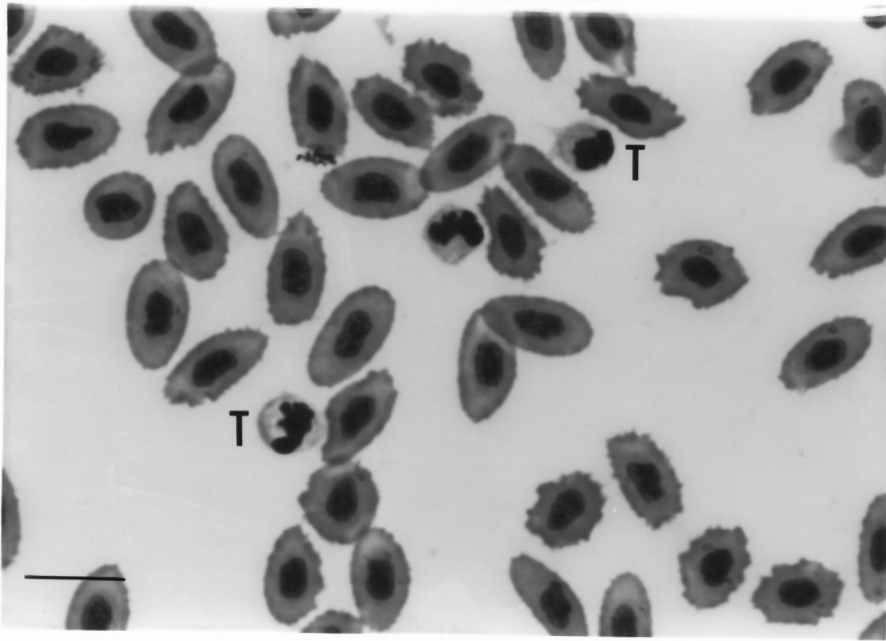


Figure 3-4. Thrombocytes - In EDTA anticoagulated blood, thrombocytes become round with a lobulated irregular nucleus. The cytoplasm remains a pale blue color. EDTA smear. Bar is 10 μm .

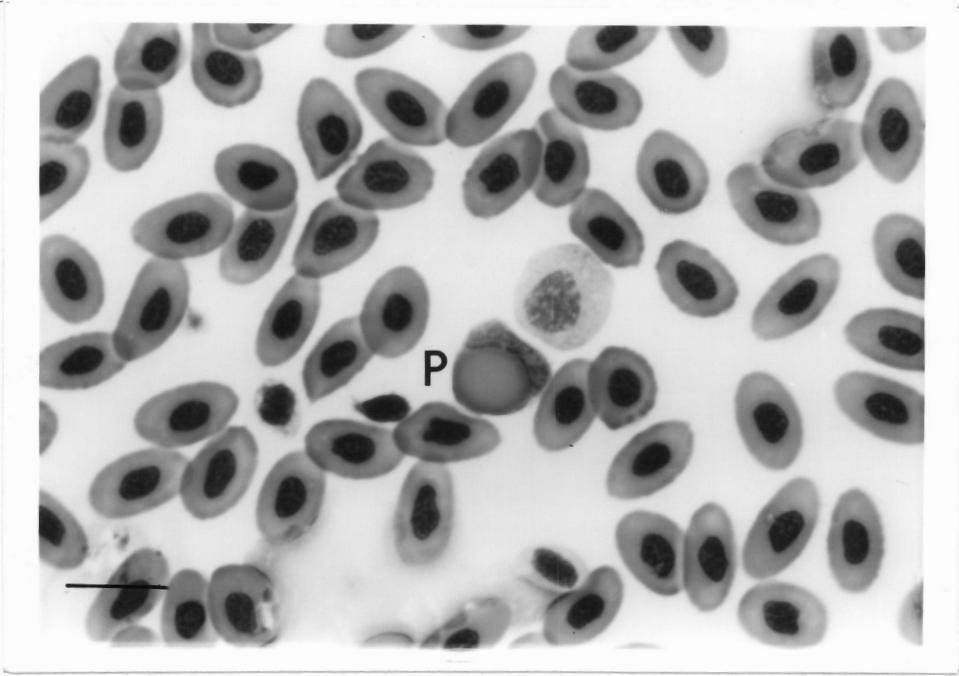


Figure 3-5. Phagocytic cell - The large phagosome distorts the cell and obscures the cytoplasm. This cell may be a phagocytic thrombocyte, or thrombocyte-like-cell. The pale cell to the right of the phagocytic cell is a disintegrating cell. EDTA smear. Bar is 10 μ m.

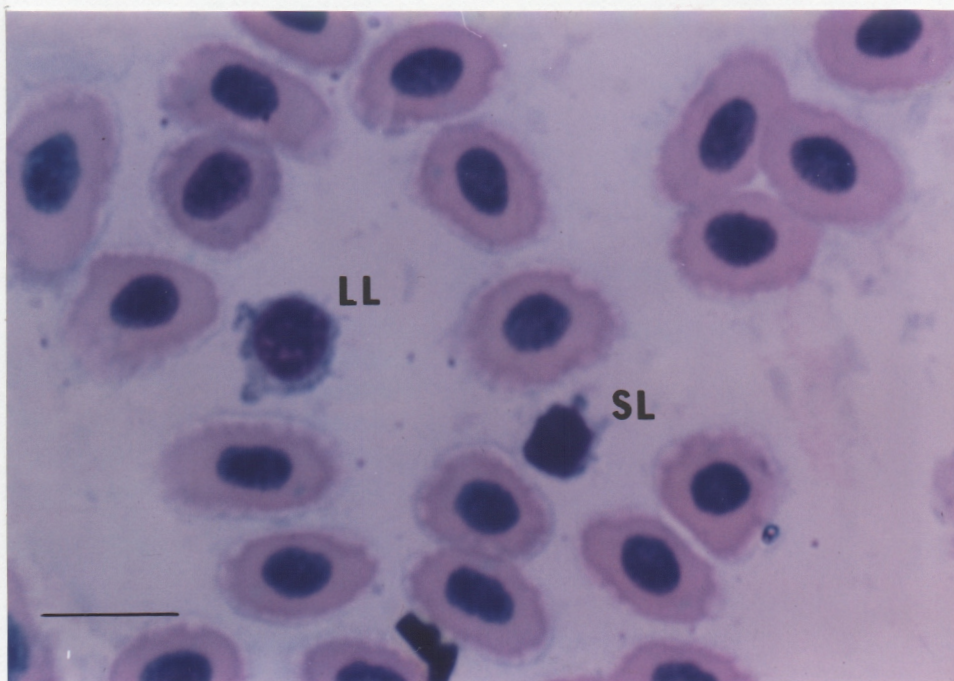


Plate 3-A) Small and Large Lymphocytes. EDTA smear. Bar is 10 μm .

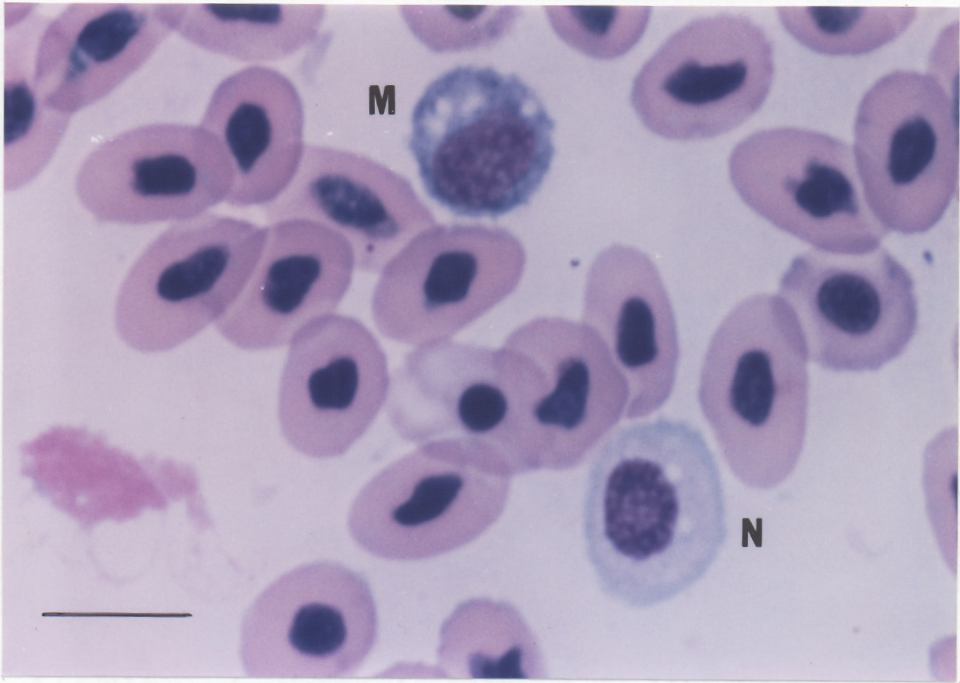


Plate 3-B) Neutrophil and Monocyte. Direct smear. Bar is 10 μm .

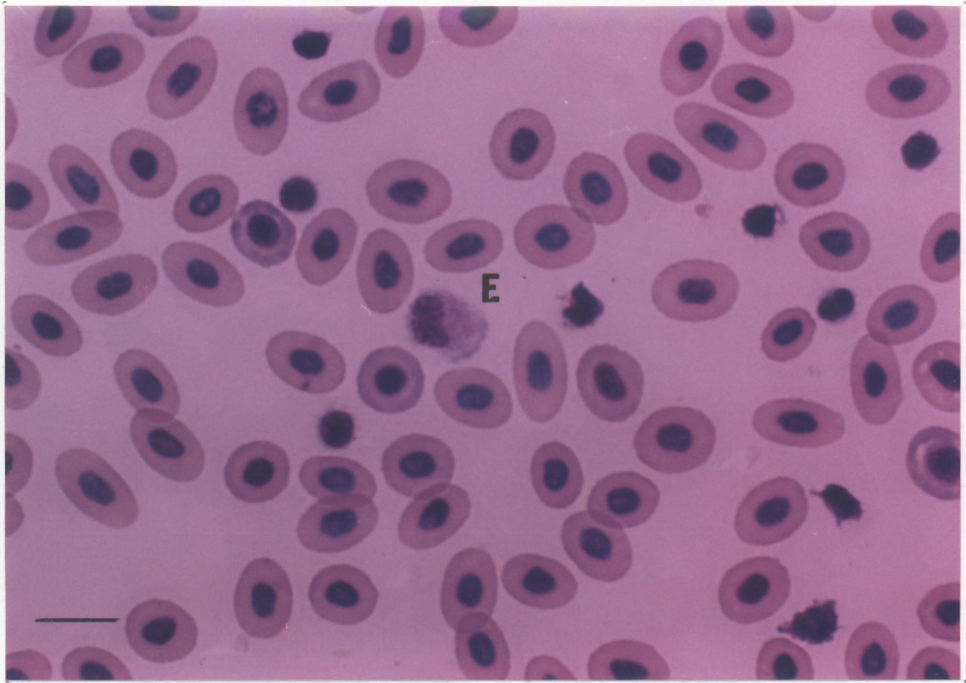


Plate 3-C) Large eosinophil. Direct smear. Bar is 10 μm .

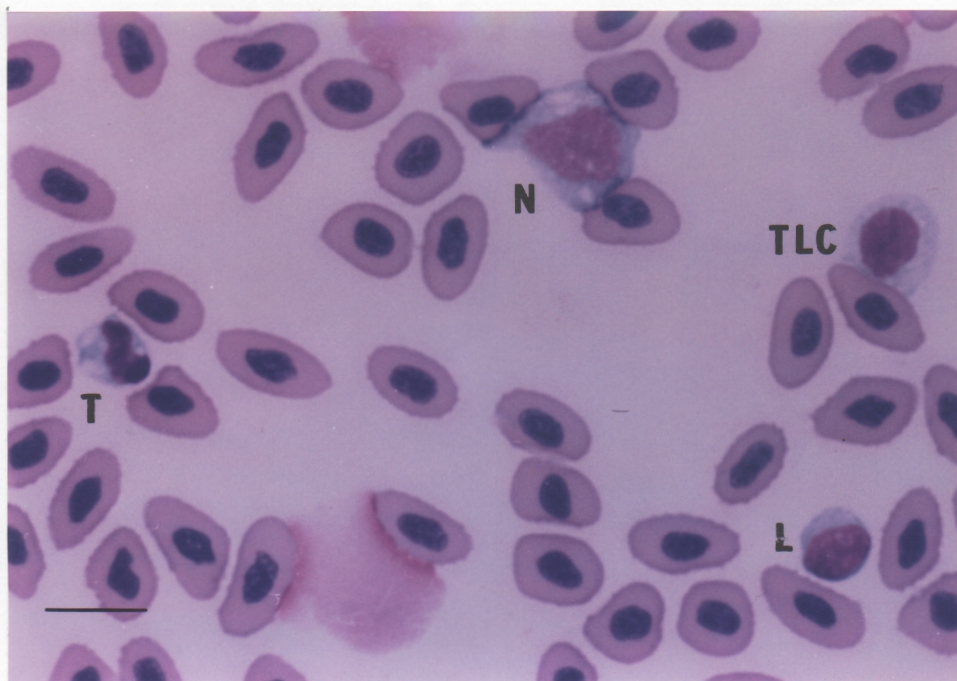


Plate 3-D) Thrombocyte-like-cell, thrombocyte, lymphocyte and a neutrophil. EDTA smear. Bar is 10 μm .

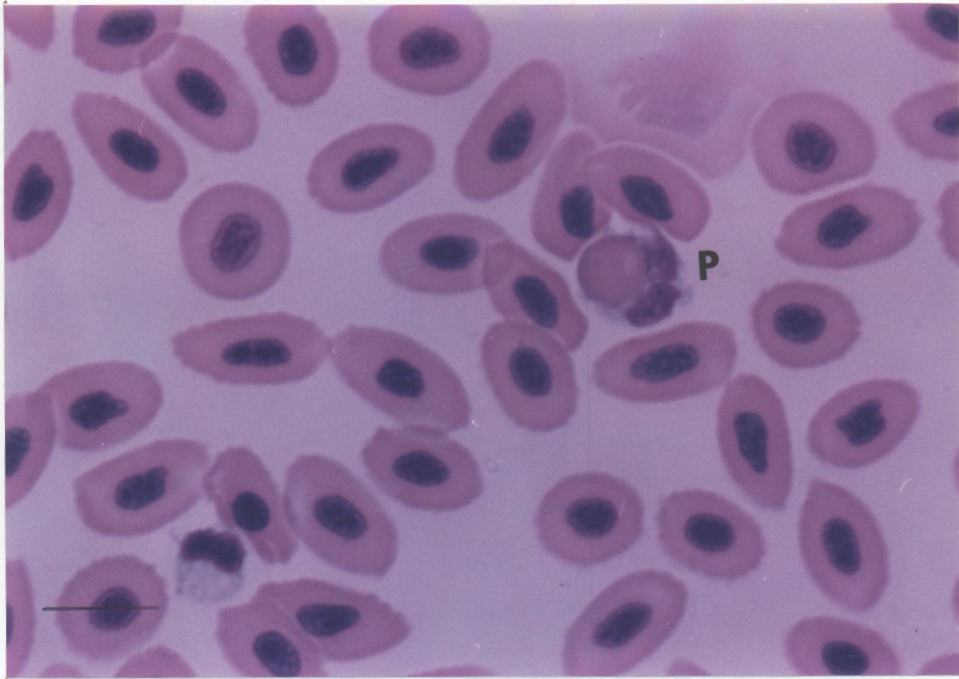


Plate 3-E) Phagocytic cell and thrombocyte. EDTA smear.
Bar is 10 μ m.

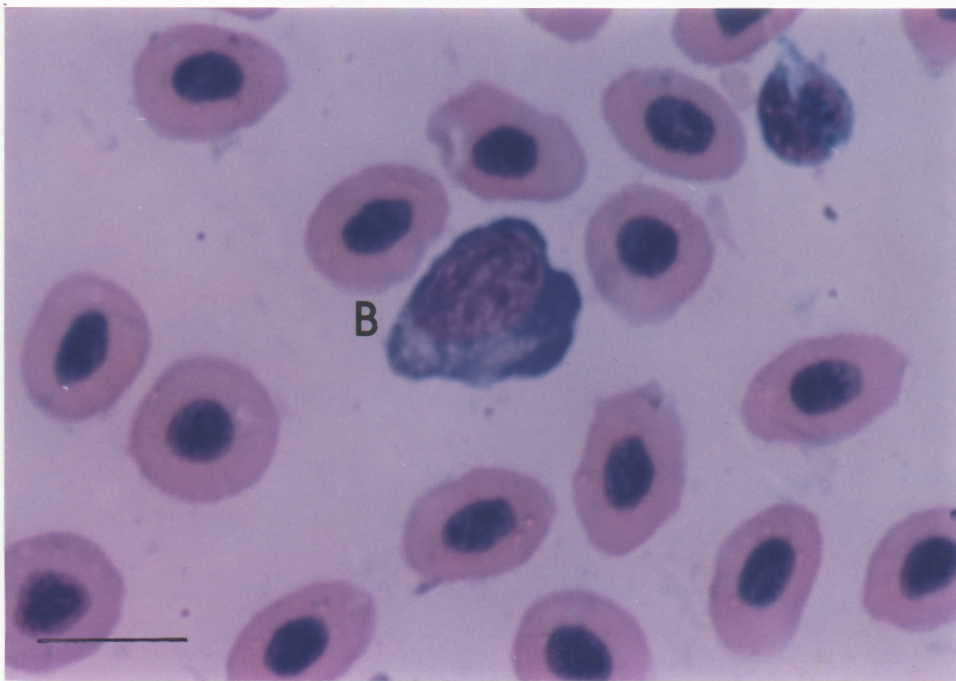


Plate 3-F) Blast cell. EDTA smear. Bar is 10 μm .

Table 3-1. Ranges for the water quality at the sampling times for sunshine and palmetto bass under different culture conditions.

Parameter	Sunshine Bass		Palmetto Bass	
	Recirculating	Tanks	Tanks	Tanks
Temperature (C)	24 - 26	25 - 26	20 - 25	
pH	6.8 - 7.2	7.7 - 7.8	7.6 - 8.1	
Ammonia ¹ (mg/L)	0.006 - 0.01	0.002 - 0.011	0.000 - 0.040	
Nitrite (NO ₂ -N mg/L)	0.05 - 0.40	0.02 - 0.06	0.01 - 0.10	
Nitrate (NO ₃ -N mg/L)	95 - 202	4 - 11	---	
Alkalinity (mg/L)	57 - 150	68 - 103	34 - 120	
Hardness (mg/L)	318 - 521	86 - 154	68 - 137	
Dissolved Oxygen (mg/L)	7.4 - 12.5 ²	6.0 - 6.0	7.0 - 8.0	

1. Unionized ammonia
2. Supplemental oxygen gas was used to maintain high oxygen levels in these systems.

Table 3-2. Hematologic reference intervals for sunshine bass in high density recirculating system, sunshine bass in tanks, and palmetto bass in tanks.

Analyte	N ¹	Sunshine Bass		Palmetto Bass	
		Recirculating	Tanks	Tanks	Tanks
PCV ³ %	(78/50/42)	23 - 44	34 - 47	20 - 34	
Plasma protein (g/dL)	(78/50/42)	4.8 - 7.4	5.1 - 6.5	4.1 - 6.2	
Hemoglobin (g/dL)	(-/50/42)	ND ²	8 - 12	4 - 8	
MCV ⁴ (fL)	(-/49/40)	ND	81 - 106	65 - 117	
MCH ⁵ (pg)	(-/49/39)	ND	19.6 - 26.4	16.2 - 24.8	
MCHC ⁶ (g/dL)	(-/50/42)	ND	22 - 30	19 - 26	
Erythrocytes (x10 ⁶ /μL)	(39/49/41)	2.10 - 4.29	3.66 - 4.96	2.42 - 4.72	
Leukocytes (x103/μL)	(40/50/42)	57.5 - 211.4	32.6 - 115.1	42.8 - 118.2	
Lymphocytes (x10 ³ /μL)					
Small	(40/50/42)	24.4 - 115.5	9.8 - 54.1	11.3 - 77.8	
Large	(40/50/42)	0.7 - 18.8	1.3 - 6.9	0.5 - 6.0	
Neutrophils (x10 ³ /μL)	(39/48/42)	0.5 - 8.2	0.2 - 2.1	0.0 - 4.0	
Monocytes (x10 ³ /μL)	(39/49/42)	1.2 - 10.9	0.8 - 5.2	0.0 - 1.9	
Eosinophils (x10 ³ /μL)	(39/50/42)	0.0 - 1.3	0.0 - 0.2	0.0 - 1.8	
TLC ⁷ (x10 ³ /μL)	(40/49/42)	0.0 - 1.3	0.6 - 3.7	0.0 - 4.0	
Thrombocytes (x10 ³ /μL)	(39/50/42)	47.4 - 113.2	30.7 - 74.1	21.3 - 104.3	

1) Number of fish used in the respective groups to determine the reference interval
2) Not determined. 3) Packed cell volume. 4) Mean cell volume. 5) Mean cell hemoglobin. 6) Mean cell hemoglobin concentration. 7) Thrombocyte-like-cell

Table 3-3. Differences between the means of hematological analytes for sunshine bass in high density recirculating systems, sunshine bass in tanks and palmetto bass in tanks. P values are for ANOVA or Kruskal-Wallis test results as appropriate for the distribution of the data. A Tukey Means Comparison Test was used to determine which groups were different ($\alpha = 0.05$) as indicated by different letters.

Analyte	P	Sunshine Bass		Palmetto Bass	
		Recirculating	Tanks		Tanks
PCV ¹	<0.0001	36	A	39	B
Plasma protein	<0.0001	6.1	A	5.8	B
Hemoglobin	<0.0001	ND		10.0	A
MCV	0.0029	ND		90.8	A
MCH	<0.0001	ND		22.9	A
MCHC	0.0020	ND		25	A
Erythrocytes	<0.0001	3.07	A	4.36	B
Leukocytes	<0.0001	127000	A	63800	B
Lymphocytes					
Small	<0.0001	65700	A	26900	B
Large	<0.0001	6742	A	3205	B
Neutrophils	<0.0001	2702	A	829	B
Monocytes	<0.0001	3661	A	2252	B
Eosinophil	<0.0001	352	A	28	A
TLC	<0.0001	306	A	1932	B
Thrombocytes	<0.0001	75500	A	51600	B
				40700	C
				2580	B
				767	B
				540	C
				540	B
				1004	C
				55400	B

1) Units and abbreviations are the same as in Table 3-2.

Table 3-4. Comparison of hematological values determined for striped bass and other species.

Species	Analyte	Value ¹	N ²	Reference
<i>Morone saxatilis</i>	PCV ³	45 - 57	4 - 8	Courtois 1976
	Plasma Protein	3.8 - 10.3	4 - 8	"
	Hemoglobin	8.4 - 8.9	4 - 8	"
	PCV	53	5	Courtois 1975
	Hemoglobin	9.4	5	"
	Plasma Protein	7.6	12	Hunn and Greer 1990
	PCV	48	31	Westin 1978
	Plasma Protein	9.4	20	"
	Hemoglobin	9.1	31	"
	Erythrocytes	3.79	15	"
	PCV	34 - 55	2 - 18	Lochmiller 1989
	Hemoglobin	6.2 - 10.9	2 - 18	"
	Erythrocytes	2.0 - 4.2	2 - 18	"
	MCV	155		"
	MCH	31.3		"
<i>Carassius auratus</i>	MCHC	20.5		"
	PCV	31 - 38	6 - 16	Tisa and Strange 1983
	Hemoglobin	6.8 - 10.6	6 - 16	"
	PCV	38 - 40	~60	Burton and Murray 1979
	Hemoglobin	9.7 - 10.6	~60	"
	MCV	241 - 245	~60	"
	MCH	63 - 66	~60	"
	MCHC	26	~60	"
	Erythrocytes	1.60 - 1.80	55 - 56	Murray and Burton 1979
	Leukocytes	10100 - 14700	55 - 56	"
	Lymphocytes	9540 - 13660	55 - 56	"
	Thrombocytes	30000 - 46100	55 - 56	"

Species	Analyte	Value ¹	N ²	Reference
<i>Salmo salar</i>	PCV	44 - 49	20	Sandnes et al. 1988
	Hemoglobin	8.9 - 10.4	20	"
	Erythrocytes	0.85 - 1.1	20	"
	MCV	441 - 553	20	"
	MCH	94 - 106	20	"
	MCHC	19.4 - 21.7	20	"
<i>Oncorhynchus aguabonita</i>	PCV	44 - 52	3 - 7	Hunn et al. 1992
	Erythrocytes	1.197	7	"
	Leukocytes	21000	5	"
	Lymphocytes	18799	5	"
	Granulocytes	1582	5	"
	Monocytes	588	5	"
	Thrombocytes	135000-310000	1 - 4	"
<i>Sarotherodon melanotheron</i>	PCV	31 - 34	40	LeaMaster et al. 1990
	Hemoglobin	7.3 - 9.0	40	"
	Erythrocytes	1.37 - 1.69	40	"
	MCV	203 - 228	40	"
	MCH	54	40	"
	MCHC	24 - 26	40	"
	Leukocytes	61900 - 62900	40	"
	Lymphocytes (%)	11.6 - 10.9	40	"
	Neutrophils (%)	3.1 - 3.8	40	"
	Monocytes (%)	3.6 - 4.2	40	"
	Thrombocytes (%)	80.5 - 82.3	40	"

- 1) The values listed are means. A range indicates that means were determined for groups based on sex, blood collection method, time of the year etc.
- 2) The number of fish used to generate each mean.
- 3) The abbreviations and units are the same as listed in Table 3-2.

Table 3-4. cont.

CHAPTER 4

Serum Chemistry Reference Intervals for Sunshine Bass
(*Morone chrysops* X *Morone saxatilis*): A Comparison of
Different Culture Systems.

Submitted for publication to
Journal of Aquatic Animal Health as the second of two
companion papers.

Hrubec T.C., S.A. Smith, B. Feldman, H.P. Veit, G.S. Libey,
M.K. Tinker, J.L. Robertson. Serum chemistry reference
intervals for sunshine bass (*Morone chrysops* X *Morone*
saxatilis): A comparison of different culture systems.
Submitted to *J. Aquat. Animal Health* 1994.

ABSTRACT

Reference intervals were determined and compared for selected serum chemistry analytes of sunshine bass raised in high density recirculating systems, low density tanks, and cages in a fresh water pond. Serum chemistry reference intervals for fish in the recirculating system, cages and tanks respectively were:

total protein 4.0-6.6, 3.6-4.7, 3.2-3.9 g/dL
albumin 1.7-2.4, 1.4-1.8, 1.2-1.5 g/dL
globulin 2.4-4.2, 2.2-2.9, 2.0-2.5 g/dL
creatinine 0.2-1.3, 0.1-0.2, 0.1-0.3 mg/dL
total bilirubin 0.1-0.7, 0.0-0.3, 0.0-0.4 mg/dL
alkaline phosphatase 36-105, 49-73, 46-66 mU/mL
aspartate aminotransferase 11-123, 0-113, 0-72 mU/mL
glucose 69-162, 89-163, 72-171 mg/dL
cholesterol 164-285, 152-222, 172-247 mg/dL
osmolality 327-421, 329-378, 286-363 mOsm/kg
sodium 150-188, 159-173, 153-165 mEq/L
potassium 1.4-8.3, 2.4-4.5, 2.4-3.7 mEq/L
chloride 91-123, 136-157, 137-148 mEq/L
calcium 11.8-15.5, 11.4-13.1, 10.4-12.1 mg/dL
phosphorus 10.6-22.3, 6.5-9.4, 6.3-8.1 mg/dL.

Ammonia, magnesium and cortisol were not determined for fish from the recirculating systems. Reference intervals for fish in cages and tanks respectively were 235-493, 173-417 $\mu\text{g/dL}$ for ammonia, 1.7-2.4, 1.9-2.4 mg/dL for magnesium, and 5.5-53.8, 4.3-43.7 for cortisol. Reference intervals were significantly different for all three groups of fish for all the analytes except the following. Creatinine, alkaline phosphatase, and cortisol were not significantly different for sunshine bass in tanks and cages. Cholesterol was not significantly different for fish in tanks and recirculating systems, and potassium was not different for fish in recirculating systems and cages. Glucose intervals were not significantly different for any group. Ammonia and magnesium, only measured in tanks and cages, were significantly different.

INTRODUCTION

In mammals, many changes in physiologic state are reflected in the blood, affecting both hematology and serum chemistry values. Clinical chemistries are a fundamental tool in human and veterinary medicine to diagnose and prognose disease; and to monitor the effect of therapeutic, nutritional or environmental management. In fishes, blood

analytes have been used to assess the physiologic effects of various toxins and diseases (Hille 1982; Sharma et al. 1982; Brown et al. 1987; Michael et al. 1987; Bucher and Hofer 1990; Haney et al. 1992). Serum chemistry analytes can also be affected by capture and sampling technique (Ellsaesser and Clem 1987; McDonald and Milligan 1992), environmental factors, culture conditions, and the age and sex of the fish (Warner and Williams 1977; Lane 1979; Hille 1982; Bentinck-Smith et al. 1987; Bhaskar and Rao 1989; Bucher 1990; Hunn et al. 1992). The information gained from these experiments cannot be used for diagnostic purposes though they are helpful in determining the physiologic response or mechanism of action of a toxin or disease. Unless the deviation in a measured value is large enough to fall outside a reference interval that indicates normal variation in a population, one can not tell if the deviation is due to disease or individual variation for a given fish. Reference intervals for the serum analytes measured were not determined in the majority of the studies listed above. Serum chemistry values are not commonly used as a diagnostic tool in fish medicine in part due to the lack of reference intervals for the various fish species. Additionally, characteristic changes in blood analytes with specific diseases are only just being determined.

Sunshine bass (reciprocal cross: female *Morone chrysops* X male *Morone saxatilis*) are an important aquaculture species raised for both food and sport release. Sunshine bass are raised under a variety of culture conditions such as in ponds, confined in cages, and in low and high density tanks with flow through or recirculated water. Very little is known about the blood chemistry profile of sunshine bass, with chloride and cortisol the only previous published analytes (Tomasso et al. 1980). Striped bass have been studied more extensively. The most comprehensive study on striped bass, conducted by Tisa and Strange (1983), determined reference intervals for osmolality, chloride, glucose, cortisol, total protein, and cholesterol. Most studies involving striped bass are limited in value due to the number of analytes measured (Westin 1978; Davis et al. 1982; Hunn and Greer 1990), or by the small number of fish sampled (Courtois 1975, 1976; Brown et al. 1987).

This study had two goals; 1) determine the blood chemistry reference intervals for sunshine bass, and 2) determine if these intervals are the same for fish under different culture conditions. Presented here are reference intervals for young adult sunshine bass maintained in high density recirculating systems, low density tanks, and a cage culture system in a fresh water pond. To the best of our knowledge,

this is the first comprehensive study on the clinical serum chemistry of the sunshine bass.

MATERIALS AND METHODS

Experimental Animals and Their Maintenance:

High Density Recirculating Systems

Sunshine bass fingerlings had been stocked the previous year and grown in 10,219 L recirculating systems (Nunley 1992). At the beginning of our experiment, the fish were 15 months old with an average weight of 454 g and a stock density of 80 g/L. The fish were fed daily a commercial pelleted diet (Bass Grower Formulation, Sheridan Extrusion Co, Sheridan, NY) at a rate of 3% body weight per day. The fish were maintained indoors with a photoperiod of 11 hours light and 13 hours dark. Two separate tanks with the same stock density were used in the study; in one, the filtered water was treated with ozone at a rate of 135 g/day from a corona discharge generator (American Ozone, USA). All fish used appeared clinically healthy with no gross external or internal lesions. External parasites were not seen on skin scrapings, and bacterial cultures of the posterior kidney were negative.

Tank Systems

Sunshine bass were obtained from Virginia Tech's Aquaculture Center when the fish were 16 months old. The fish were maintained indoors in a 2000 L tank for 2 months with a photoperiod of 13 hours light and 11 hours dark. They were fed daily a commercial pelleted diet (Floating Fish Nuggets, Zeigler Brothers Inc., Gardner, PA) at a rate of 1% body weight per day. The fish were then divided into four 600 L tanks equipped with power filters with 12 to 13 fish in each tank and a stock density of 4 to 6 g/L. The fish were moved to the smaller tanks to facilitate catching the fish with minimal stress. The fish were acclimated to the smaller tanks for two weeks before sampling. All sampled fish appeared clinically healthy and no external parasites were seen on skin scrapes.

Cages Culture System

The sunshine bass in cages were raised on a commercial fish farm (Day Spring Farm, Etlan, VA). The fish had been stocked into the cages as fingerlings the previous spring. At the time of sampling, the fish were stocked at 360 kg/4x4x12' cage in a 4 acre pond with an average day length of 14 hours light. There were five other cages containing sunshine bass stocked at 400 kg/cage in the pond. Fish were fed a commercial pelleted feed (Zeigler Brothers Inc.

Gardner, PA) twice daily. All the fish used appeared clinically healthy, though the fish did have a subclinical trichodina infestation. It is normal for fish in ponds to have low numbers of external parasites; and the fish used in this study did not have skin lesions or other signs of irritation.

Water Quality:

In both the high density recirculating system and low density tanks the water quality was monitored as follows: pH, temperature, and ammonia were measured daily; alkalinity, hardness, nitrites, and nitrates were measured twice weekly. Dissolved oxygen (DO) was measured daily in the recirculating production system; and twice a week in the tank system. The pH was measured using a pocket pH meter (Hanna Instruments, Woonsocket, RI). Temperature and DO in the recirculating system were measured using an oxygen meter (YSI, Yellow Springs, OH). The remaining parameters, including oxygen in the tank system, were determined with a commercial kit (Hach Chemical, Loveland, CO). In the cage culture system, the water quality parameters listed above were measured each day that fish were sampled. All measurements were determined using the Hach kit. Ranges for water quality over the duration of the study are shown in Table 4-1.

Sampling Schedule:

In the high density production system, the fish were sampled four times at two to three week intervals. At each sampling time, 10 fish from each of the two tanks were quickly netted, anesthetized with tricaine methanesulfonate (MS-222 at 0.25 g/L, Sigma Chemical Co., Saint Louis, MO), and individually bled. In the tank system, all the fish from a tank were sampled at the same time. The water was lowered and the fish quickly netted and anesthetized in aerated buffered MS-222 (0.25 g/L). When the fish lost equilibrium, they were bled. Fish in the cage system were sampled by netting them quickly five or six at a time and transferring them to a tank with aerated buffered MS-222 (0.25 g/L) in pond water. Approximately 30 fish per day were sampled on three separate occasions during the summer. The fish from all culture systems were fasted 24 hours prior to sampling and sampled fish were not returned to the culture system.

Blood Handling:

Fish were bled from the caudal vessels with a 21 gauge needle and a plastic syringe. Blood from the fish in the recirculating system was placed into serum tubes without anticoagulant and was allowed to clot at room temperature for one hour. The serum was separated by centrifugation at

14,000g for three minutes and frozen at -5 °C. Blood from the fish in the tanks and cages was placed into 3 mL heparinized tubes and centrifuged immediately to separate the plasma. The plasma was promptly removed and frozen at -5 °C. Plasma was handled in this manner to allow measurement of blood ammonia. All samples were analyzed within one week of collection. The samples were thawed, centrifuged to remove fibrin, and analyzed using an automated system (Kodak Ektachem 700 serum analyzer, Rochester, NY) for the following: total protein, albumin, creatinine, total bilirubin, alkaline phosphatase (ALP), aspartate aminotransferase (AST), glucose, cholesterol, ammonia, sodium, chloride, potassium, calcium, magnesium, and phosphorus. Globulin was calculated from the total protein minus the albumin. Osmolality was determined with a Multiosmomette Micro-Osmometer 2430 (Precision Systems Inc. Natick MA). Cortisol was determined with a commercial enzyme-linked immunosorbent assay (Milenia Kinetic EIA, Diagnostic Products Corp., Los Angeles, CA).

Statistical Analysis:

Statistical analysis of the blood parameters was conducted using a commercial statistical program for the PC (Statistix version 4, Analytical Software, Tallahassee FL). The

reference interval for each parameter was determined following the guidelines proposed by the National Committee for Clinical Laboratory Standards for determining reference intervals in clinical laboratories (1991). Non-parametric methods were used to determine the reference intervals. The values were ranked, and the low and high 2.5 % discarded, with the range of the remaining values determining the reference interval. A Kruskal-Wallis test (for non-parametrically distributed analytes) and analysis of variance (ANOVA - for parametrically distributed analytes) was used to test for differences between reference intervals for the three groups of fish. If there was a significant difference ($P < 0.05$), a Tukey means comparison test was used to identify which group was different.

RESULTS

The reference intervals for the serum chemistry analytes of sunshine bass from the three culture systems are listed in Table 4-2. The reference intervals for the fish in the recirculating system in general had a wider reference interval than the fish from the tanks or cages. All the protein levels were higher for the fish in the recirculating system. There were considerable differences for chloride

and phosphorus of fish from recirculating systems; chloride concentrations were lower and phosphorus concentrations were higher than for fish in either of the other culture systems.

Differences in the reference intervals between the three groups of fish are shown in Table 4-3. The reference intervals from the three groups of fish were not statistically different for glucose. Fish in tanks and fish in cages had reference intervals which were not significantly different for creatinine and alkaline phosphatase and cortisol. Fish in recirculating systems and fish in tanks were not significantly different for cholesterol. Fish in recirculating systems and fish in cages were not statistically different for potassium. All the reference intervals for the remaining analytes were statistically different for each of the three groups.

Cortisol values were not correlated with other analytes; the value of r was $-0.33 < r < 0.39$ for fish in cages and $-0.32 < r < 0.31$ for fish in tanks. Glucose values were not correlated with other analytes in any of the production systems. Calcium and cholesterol were correlated with total protein in all three groups of fish. Correlations with total protein from recirculating systems, cages and tanks

respectively were $r = 0.76, 0.53, 0.77$ for calcium, and $r = 0.79, 0.59, 0.53$ for cholesterol. Sodium and chloride were also correlated in all three systems with respective r values of $0.68, 0.65, 0.75$.

DISCUSSION

The analytes measured in this study were chosen for a number of reasons. Detection of these analytes is readily available through commercial diagnostic laboratories throughout the country negating the need for possessing sophisticated laboratory equipment. Analytes are measured using standardized and automated equipment which reduces a source of variation in results. Automated analyzers are designed for human samples, but have been shown to be accurate for fish blood even when operated at mammalian temperatures of 37°C (Warner et al. 1978, 1979; Smith and Ramos 1980). Additionally, the analytes chosen are useful for diagnostic purposes in mammals, it is already known how these factors change in diseased states. It would save unnecessary background work if these analytes can be used diagnostically in fish as they are in mammals. To be useful in the diagnosis of disease, the analyte must be affected

early in the course of disease before the fish become moribund. The analyte must also deviate to the extent that it falls outside of the reference interval in order to be detected.

Reference intervals should be established for a select population and used to determine whether an individual's analyte is above or below the expected range for the population. For clinical diagnosis, sample means are not useful and should never be used. The reference interval must be broad enough to accommodate slight differences in values due to mild fluctuations of environmental conditions, yet narrow enough to be able to detect changes due to a diseased state.

Clinical reference intervals have been determined for rainbow trout bilirubin, calcium, magnesium, phosphorus and osmolality (Wedemeyer and Nelson 1975); striped bass osmolality, chloride, glucose, cortisol, total protein and cholesterol (Tisa and Strange 1983); milkfish (*Chanos chanos*) cholesterol, total protein, albumin sodium, and potassium (Bhaskar and Rao 1989) and complete biochemical profiles for channel catfish (Warner and Williams 1977; Bentinck-Smith et al. 1987). In these studies, the data collected at different sampling times were combined to

determine the reference interval. In determining the reference intervals for this study, we also combined data collected on different days for the same population of fish. Before combining the data from the fish in the high density recirculating system, we determined that there were no differences ($p < 0.05$) between the analytes of fish from the ozone treated water and from the untreated water. The data collected on different days were combined to provide a reference interval broad enough to accommodate variations in the analytes from slight changes in environmental conditions.

The reference intervals determined for the sunshine bass under the different culture conditions are similar to those determined for other species of fishes. They are narrower than the reference intervals determined for striped bass (Tisa and Strange 1983) and catfish in ponds (Warner and Williams 1977; Bentinck-Smith et al. 1987). This may be due to the relatively uniform environmental and water quality conditions of the indoor systems, and only sampling the caged fish during the summer.

Of all the serum components in fishes, the electrolytes are the most studied. Electrolyte changes and osmolality give an indication of the fishes ability to osmoregulate. This

ability is often compromised with stress, disease or gill pathology by increasing the permeability of the gills to ions. It has been demonstrated that sunshine bass when stressed by handling, first become hyperchloremic but after 24 hours the fish become hypochloremic and this hypochloremia can be profound (Tomasso et al. 1980). The mechanism for this increase may be that catecholamine and cortisol release cause a shift in the extracellular fluid volume to the intracellular space and a concurrent increase in the plasma sodium and chloride (McDonald and Milligan 1992). However, other studies with striped bass have not show an increase in chloride with stress (Davis et al. 1982, Davis and Parker 1990), although the fish became hypochloremic the day after the stressful event (Davis and Parker 1990). Stress did not affect the serum electrolytes of channel catfish (Ellsaesser and Clem 1987) indicating possible species differences in the response of electrolytes to stress.

In this study, fish from cages were sampled repeatedly from one cage each day. Repeated sampling has been shown to stress fish and affect the electrolyte levels (McDonald and Milligan 1992). Even though sampling was done as rapidly as possible in order to minimize stress and cortisol release, the blood values may reflect some changes due to stress as

demonstrated by the higher sodium and chloride levels. However, cortisol concentrations were not greater in caged fish and were not correlated with sodium and chloride concentrations. Cortisol would be expected to increase with stress prior to changes in the electrolytes. Other possible reasons for the difference in sodium and chloride could be differences in diet and water quality.

The lower serum chloride for the fish in the recirculating system is most likely due to factors associated directly or indirectly with the high density recirculating system as the fish were handled in a manner similar to the other groups. In other studies investigating serum chemistry changes with increased stocking density, chloride concentration did not change with stocking density in channel catfish (Warner and Williams 1977), or in Atlantic salmon (Kjartansson et al. 1988). An explanation for the reduced serum chloride may be the higher nitrite ($\text{NO}_2\text{-N}$) in the water of the recirculating system. Michael et al. (1987) induced hypertrophy and hyperplasia of gill epithelium in *Clarias lazera* chronically exposed to elevated nitrite concentrations of 3.2 mg/L $\text{NO}_2\text{-N}$. Byrne et al. (1989) demonstrated significantly lower serum chloride concentrations in rainbow trout with gill pathology due to increased loss of chloride across the

gills. Although, nitrite concentrations in the previous study were higher than the concentrations in our recirculating system, gill hyperplasia may have occurred at these lower doses, and caused increased loss of chloride to the water. Short term exposure in striped bass to 50 to 250 mg/L NO₂ for 24 hours did not cause a decrease in serum chloride (Mazik et al. 1991), however, the fish may not have been exposed to the nitrite long enough to develop gill hyperplasia and subsequent chloride loss. Further studies need to be conducted to correlate serum chloride levels with nitrite concentrations and gill pathology to draw definitive conclusions about the lower chloride concentrations seen in these fish.

Changes in the other electrolytes - calcium, phosphorus, potassium and magnesium, due to pathology or stress are not as well studied in fishes. Calcium levels are tightly controlled and are not affected by normal stressors (McDonald and Milligan 1992). A percentage of the calcium in the blood is bound to protein carriers; and as the protein concentration increases calcium also increases (McDonald and Milligan 1992). We found a positive correlation between total protein and calcium in all three production systems. The higher amounts of calcium in the

recirculating system fish are most likely due to the higher total protein concentration in these fish. The reason for the higher phosphorus levels in sunshine bass from the recirculating system is unknown. Although reference intervals for phosphorus in striped bass have not been determined, the means for the sunshine bass in the three production systems are comparable to means published for both striped bass (Table 4-4) and other fish species (Table 4-5).

Cortisol and glucose have been used to monitor primary and secondary stress responses, although, cortisol is a more sensitive indicator of stress (Silbergeld 1974; McDonald and Milligan 1992). Cortisol has both annual and daily variations (Kühn et al. 1986; Planas et al. 1990; McDonald and Milligan 1992) with the highest levels occurring just before dawn and during the warmer summer months. Cortisol has been measured in striped, sunshine and palmetto bass (Tomasso et al. 1980; Davis et al. 1982; and Noga et al. 1994) for both stressed and unstressed fish. The cortisol concentrations reported in this study were higher than reported in the above studies for unstressed fish. This may be due to the fish being sampled in the early morning and also at warmer water temperatures than in the previous studies. The cortisol values may also be higher due to the

test method used to determine cortisol concentrations. We used a commercial ELISA kit which has been shown to be comparable with the radioimmuno assay (RIA) for trout cortisol (Caldwell and Hinshaw 1990). The other studies on striped bass and the hybrids used a competitive protein binding assay (Tomasso et al. 1980; Davis et al. 1982) and a fluorescence polarization immunoassay (Noga et al. 1994). It is not known if these assays are comparable with the ELISA. Additionally, the fish in tanks were all sampled in less than 10 minutes; and changes in cortisol due to a stressful event are not detected for 10 minutes (McDonald and Milligan 1992). There was no statistical difference in the cortisol concentrations between fish in tanks and fish in cages, and there was no correlation of cortisol with the order in which the fish were sampled. For these reasons, we do not think that the cortisol values in our study are indicative of stress although this possibility can not be ruled out.

The blood enzymes ALP and AST are difficult to interpret in fishes because physiologic reasons for changes in the serum concentrations are not completely known. In mammals, an elevation of these enzymes usually indicates a specific organ dysfunction, however, under some circumstances can be caused by isozymes released from other tissues. Very few

studies have investigated the tissue origin of isozymes present in the blood of fish. Bucher (1990) described isozyme distribution in different tissues for a number of enzymes but did not indicate how these values related to amounts detected in the serum. Further studies are needed to determine the specific tissues of origin for serum enzymes. There are a number of studies which have correlated an increase in serum enzymes with tissue pathology (Asztalos 1986; Michael et al. 1987; Lemaire et al. 1991; Grizzle and Kiryu 1993). While others (Bucher and Hofer 1990) did not show increases in serum enzymes with tissue pathology. The outlook is promising for the clinical use of these analytes since most of the research has shown changes in serum enzymes to be associated with tissue pathology. The serum enzymes ALP and AST have not been measured in sunshine or striped bass previously. The reference intervals determined in this study are in the same range as the means determined for other species (Table 4-5).

We determined blood urea nitrogen (BUN) for the sunshine bass in recirculating systems, and in most cases the readings (0 - 3 mg/dL) were below the detectable limits of the analyzer. Blood urea nitrogen is routinely measured in mammals to monitor kidney function. The diagnostic value of BUN in fishes is questionable, as their main excretory

product is ammonia and not urea. Most teleosts, including bass, lack the enzymes needed to synthesize urea (Mommensen and Walsh 1989). The fishes that can synthesize urea by the ornithine-urea cycle still produce ammonia as their main excretory product (Saha and Ratha 1989). Fishes without ornithine-urea cycle enzymes can produce urea by uricolysis - hydrolysis of arginine by arginase, but the levels of urea produced are low (Mommensen and Walsh 1989). Even if sunshine bass could produce urea by uricolysis, the significance of an elevated concentration is not known. For these reasons, blood ammonia was measured in place of BUN for sunshine bass in tanks and cages. Blood ammonia may be a better diagnostic parameter than BUN for fishes. Since ammonia is excreted across the gills and not via the kidneys, and blood ammonia increases with increasing levels of environmental ammonia (McDonald and Milligan 1992), blood ammonia could possibly be used as an indicator of gill damage or environmental ammonia stress. In the cage culture systems, both the blood and water ammonia concentrations were higher than for fish from tanks. The blood ammonia may be higher in these fish due to the concentration of ammonia in the water, although other factors such as protein content in the diet, and gill function may contribute to serum levels.

The cholesterol concentrations determined in this study,

although higher than expected for catfish (Bentinck-Smith et al. 1987; Ellsaesser et al. 1987), are less than that reported for salmonids (McDonald and Milligan 1992) and less than those reported for striped bass (Table 4-4). This may be a species difference but could also be due to fact that the fish in this study were not sexually mature.

Cholesterol is highest in fishes prior to spawning (McDonald and Milligan 1992), and immature fish would not be expected to have cholesterol concentrations as high as those reported for mature and spawning fishes.

The reference intervals determined for sunshine bass raised in high density recirculating systems, cages and tanks were significantly different for the majority of the analytes. It is clear that separate reference intervals should be used for fish under different production systems. Further work needs to be conducted to determine if these reference intervals can be used clinically to diagnose disease and tissue pathology. For each culture system, it may be necessary to expand the intervals to include a wider variety of conditions, or narrow the intervals and create reference intervals for more specific conditions. Based on the width of these reference intervals, the individual variation seen in these fish is similar to the individual variation expected from other domestic animals. This indicates the

potential for generating clinically useful reference intervals in sunshine bass and other fish species.

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REFERENCES

- Asztalos, B. 1986. LDH enzyme as a tool on monitoring tissue necrosis in fishes caused by pesticides. *Acta Biologica Hungarica* 37:59-65.
- Bentinck-Smith, J., H. M. Bealeu, P. Waterstart, C. S. Tucker, F. Stiles, R. P. Bowser, and L. A. Brown. 1987. Biochemical reference ranges for commercially reared channel catfish. *The Progressive Fish-Culturist* 49:108-114.
- Bhaskar, B. R., and K. S. Rao. 1989. Influence of environmental variables on hematology, and compendium of normal hematological ranges of milkfish, Chanos chanos (Forsk.) in brackish culture. *Aquaculture* 83:123-136.
- Brown, C. L., G. Young, R. S. Nishioka, and H. A. Bern. 1987. Preliminary report on the physiological status of striped bass in the Carqinez Strait die-off. *Fisheries Research* 6:5-16.
- Bucher, F. 1990. Organ patterns and natural fluctuations of blood enzymes of rainbow trout (Salmo gairdneri Rich.). *Comparative Biochemistry and Physiology* 96B:795-799.
- Bucher, F., and R. Hofer. 1990. Effects of domestic waste water on the serum enzyme activities of brown trout (Salmo trutta). *Comparative Biochemistry and Physiology* 97C:381-385.
- Byrne, P., D. Speare, and H. W. Ferguson. 1989. Effects of a cationic detergent on the gills and blood chemistry of rainbow trout Salmo gairdneri. *Diseases of Aquatic Organisms* 6:185-196.
- Caldwell, C. A., and J. M. Hinshaw. 1990. Validation of a solid-phase enzyme immunoassay technique for the measurement of plasma cortisol in rainbow trout. *Journal of Aquatic Animal Health* 2:228-230.

- Courtois, L. A. 1975. Blood and serum analysis of adult striped bass, Morone saxatilis, captured in the Sacramento River. California Fish and Game 61:245-246.
- Courtois, L. A. 1976. Hematology of juvenile striped bass, Morone saxatilis (Walbaum), acclimated to different environmental conditions. Comparative Biochemistry and Physiology 54A:221-223.
- Davis, K. B., N. C. Parker, and M. A. Suttle. 1982. Plasma corticosteroids and chlorides in striped bass exposed to tricaine methanesulfonate, quinaldine, etomidate, and salt. Progressive Fish-Culturist 44:205-207.
- Davis, K.B., and Parker, N.C., 1990. Physiological stress in striped bass: effect of acclimation temperature. Aquaculture 91:349-358.
- Ellsaesser, C. F., and L. W. Clem. 1987. Blood serum chemistry measurements of normal and acutely stressed channel catfish. Comparative Biochemistry and Physiology 88A:589-594.
- Grizzle, J. M., and Y. Kiryu. 1993. Histopathology of gill, liver, and pancreas, and serum enzyme levels of channel catfish infected with Aeromonas hydrophila complex. Journal of Aquatic Animal Health 5:36-50.
- Haney, D. C., D. A. Hursh, M. C. Mix, and J. R. Winton. 1992. Physiological and hematological changes in chum salmon artificially with erythrocytic necrosis virus. Journal of Aquatic Animal Health 4:48-57.
- Hille, S. 1982. A literature review of the blood chemistry of rainbow trout, Salmo gairdneri Rich. Journal of Fish Biology 20:535-569.
- Hunn, J. B., and I. E. Greer. 1990. Colorimetric and refractometer estimates of total plasma protein in striped bass, Morone saxatilis (Walbaum). Journal of Fish Biology. 36:617-618.

- Hunn, J. B., R. H. Wiedmeyer, I. E. Greer, and A. W. Grady. 1992. Blood chemistry of laboratory-reared golden trout. *Journal of Aquatic Animal Health* 4:218-221.
- Kjartansson, H., S. Fivelstad, J. M. Thomassen, and M. J. Smith. 1988. Effects of different stocking densities on physiological parameters and growth of adult Atlantic salmon (Salmo salar L.) reared in circular tanks. *Aquaculture* 73:261-274.
- Kühn, E. R., S. Corneillie, and F. Ollevier. 1986. Circadian variations in plasma osmolality, electrolytes, glucose, and cortisol in carp (Cyprinus carpio). *General and Comparative Endocrinology* 61:459-468.
- Lane, H.C. 1979. Progressive changes in hematology and tissue water of sexually mature trout, Salmo gairdneri Richardson during the autumn and winter. *Journal of Fish Biology* 15:425-436.
- Lemaire, P., P. Draï, A. Mathieu, S. Lemaire, S. Carrière, J. Guidicelli, and M. Lafaurie. 1991. Changes with different diets in plasma enzymes (GOT, GPT, LDH, ALP) and plasma lipids (Cholesterol, triglycerides) of sea-bass (Dicentrarchus labrax). *Aquaculture* 93:63-75.
- Mazik, P. M., M. L. Hinman, D. A. Winkelmann, S. J. Klaine, and B. A. Simco. 1991. Influence of nitrite and chloride concentrations on survival and hematological profiles of striped bass. *Transactions of the American Fisheries Society* 120:247-254.
- Michael, M. I., A. M. Hilmy, N. A. El-Domiaty, and K. Wershana. 1987. Serum transaminase activity and histopathological changes in Clarias lazera chronically exposed to nitrite. *Comparative Biochemistry and Physiology* 86C:255-262.

- McDonald, D. G., and C. L. Milligan. 1992. Chemical Properties of Blood. Pages 56-135 in W. S. Hoar, D. J. Randall, and A. P. Farrell, editors. Fish Physiology Vol. 12 Part B, The Cardiovascular System. Academic Press, New York.
- Mommsen, T. P., and P. J. Walsh. 1989. Evolution of urea synthesis in vertebrates: the piscine connection. Science 243:72-75.
- NCCLS (National Committee for Clinical Laboratory Standards). 1992. How to define, determine, and utilize reference intervals in the clinical laboratory; proposed guidelines. NCCLS Document C28-P (ISBN 1-56238-143-1). NCCLS, Villanova, Pennsylvania.
- Noga, E. J., J. H. Kerby, W. King, D. P. Aucoin, and F. Giesbrecht. 1994. Quantitative comparison of the stress response of striped bass (Morone saxatilis) and hybrid striped bass Morone saxatilis X Morone chrysops and Morone saxatilis X Morone americana). American Journal of Veterinary Research 55:405-409.
- Nunley, C. E. 1992. Production of hybrid striped bass (Morone chrysops X Morone saxatilis) in a recirculating aquaculture system. M.S. Thesis, Virginia Polytechnic Institute and State University, Blacksburg, VA.
- Planas, J., J. Guiterrez, J. Fernandez, M. Carrillo, and P. Canals. 1990. Annual and daily variations of plasma cortisol in seabass, Dicentrarchus labrax L. Aquaculture 91:171-178.
- Saha, N., and B. K. Ratha. 1989. Comparative study of ureogenesis in freshwater, air-breathing teleosts. The Journal of Experimental Zoology 252:1-8.

- Sharma M. L., K. A. Goel, A. K. Awasthi, and S. K. Tyagi. 1982. Haematological and biochemical characteristics of Heteropneustes fossilis under the stress of congo red (diphenyl disazo binaphthionic acid). Toxicology Letters 14:237-241.
- Silbergeld, E. K. 1974. Blood glucose: a sensitive indicator of environmental stress in fish. Bulletin of environmental and Contamination Toxicology 11:20.
- Smith, A. C., and F. Ramos. 1980. Automated chemical analysis in fish health assessment. Journal of Fish Biology 17:445-450.
- Tisa, M. S., and R. J. Strange. 1983. Hematology of striped bass in fresh water. Progressive Fish-Culturist 45(1):41-44.
- Tomasso, J. R., K. B. Davis, and N. C. Parker. 1980. Plasma corticosteroid and electrolyte dynamics of hybrid striped bass (white bass X striped bass) during netting and hauling. Proceedings of the World Mariculture Society 11:303-310.
- Warner M. C., R. W. Williams. 1977. Comparison between serum values of pond and intensive raceway cultured channel catfish Ictalurus punctatus (Rafinesque). Journal of Fish Biology 11:385-391.
- Warner M. C., S. A. Diehl, and A. M. Tomb. 1978 Effects of dilution and temperature of analysis on blood serum values in rainbow trout Salmo gairdneri. Journal of Fish Biology 13:257-263.
- Warner, M. C., A. M. Tomb, and S. A. Diehl. 1979. Variability and stability of selected components in rainbow trout Salmo gairdneri serum and the precision of automated analysis in measuring these components. Journal of Fish Biology 15:141-151.

Wedemeyer G. A., and N. C. Nelson. 1975. Statistical methods for estimating normal blood chemistry ranges and variance in rainbow trout (Salmo gairdneri), shasta strain. Journal of the Fisheries Research Board of Canada 32:551-554.

Westin, D. T. 1978. Serum and blood from adult striped bass, Morone saxatilis. Estuaries 1:126-128.

Table 4-1. Ranges of water quality parameters at the sampling times for Sunshine bass under different culture systems.

Parameter	Recirculating	Cages	Tanks
Temperature (C)	24 - 26	24 - 28	25 - 28
pH	6.8 - 7.2	7.3 - 7.4	7.6 - 7.7
Ammonia ¹ (mg/L)	0.006 - 0.010	0.009 - 0.011	0.003 - 0.006
Nitrite (NO ₂ -N mg/L)	0.05 - 0.40	0.0 - 0.0	0.0 - 0.03
Nitrate (NO ₃ -N mg/L)	95 - 202	1 - 4	1 - 5
Alkalinity (mg/L)	57 - 150	51 - 51	86 - 103
Hardness (mg/L)	318 - 521	34 - 51	86 - 137
Dissolved Oxygen (mg/L)	7.4 - 12.5 ²	6.0 - 7.0	4.0 - 7.0

1. Un-ionized ammonia
2. Supplemental oxygen gas was used to maintain high oxygen levels in these systems.

Table 4-2. Reference intervals sunshine bass in high density recirculating systems, a cage culture system in a fresh water pond and in tanks.

Analyte	N ¹	Recirculating	Cages	Tanks
Total Protein (g/dL)	(78/79/51)	4.0 - 6.6	3.6 - 4.7	3.2 - 3.9
Albumin (g/dL)	(78/79/51)	1.7 - 2.4	1.4 - 1.8	1.2 - 1.5
Globulin (g/dL)	(78/79/51)	2.4 - 4.2	2.2 - 2.9	2.0 - 2.5
Creatinine (mg/dL)	(76/78/51)	0.2 - 1.3	0.1 - 0.2	0.1 - 0.3
Tot Bili ² (mg/dL)	(77/79/51)	0.1 - 0.7	0.0 - 0.3	0.0 - 0.4
ALP ³ (mU/mL)	(78/79/51)	36 - 105	49 - 73	46 - 66
AST ⁴ (mU/mL)	(77/79/51)	11 - 123	0.0 - 113	0.0 - 72
Glucose (mg/dL)	(78/79/51)	69 - 162	89 - 167	72 - 171
Cortisol (μg/dL)	(-/-51/48)	ND ⁵	5.5 - 53.8	4.3 - 43.7
Cholesterol (mg/dL)	(78/79/51)	164 - 285	152 - 222	172 - 247
Ammonia (μg/dL)	(-/-65/51)	ND	235 - 493	173 - 417
Osmolality (mOsm/kg)	(68/59/52)	327 - 421	329 - 379	286 - 363
Sodium (mEq/L)	(78/79/51)	150 - 188	159 - 173	153 - 165
Potassium (mEq/L)	(78/79/51)	1.4 - 8.3	2.4 - 4.5	2.4 - 3.7
Chloride (mEq/L)	(77/79/51)	91 - 123	136 - 157	137 - 148
Calcium (mg/dL)	(78/79/51)	11.78 - 15.52	11.43 - 13.10	10.35 - 12.07
Magnesium (mg/dL)	(-/-79/51)	ND	1.7 - 2.4	1.9 - 2.4
Phosphorus (mg/dL)	(78/79/51)	10.6 - 22.3	6.5 - 9.4	6.3 - 8.1

- 1) The number of fish used to generate the respective reference intervals.
2) Total bilirubin. 2) Alkaline phosphatase. 3) Aspartate aminotransferase.
4) Not determined

Table 4-3. Differences between the type of culture system on the serum chemistry reference interval of sunshine bass. P values were determined using a Kruskal-Wallis test. A Tukey Means Comparison Test was used to determine which groups were different (different letters indicates statistically different means, $\alpha = 0.05$).

Analyte	P	Recirculating	Cages	Tanks
Total Protein (g/dL)	<0.0001	5.1	A	4.1
Albumin (g/dL)	<0.0001	2.0	A	B
Globulin (g/dL)	<0.0001	3.1	A	B
Creatinine (mg/dL)	<0.0001	0.59	A	B
Tot Bili ¹ (mg/dL)	<0.0001	0.28	A	B
ALP (mU/mL)	<0.0001	64	A	B
AST (mU/mL)	<0.0001	46	A	B
Glucose (mg/dL)	0.0635	109	A	B
Cortisol (μ g/dL)	<0.0001	ND	A	102
Cholesterol (mg/dL)	<0.0001	220	A	23.7
Ammonia (μ g/dL)	<0.0001	ND	A	221
Osmolality (mOsm/kg)	<0.0001	363	A	265
Sodium (mEq/L)	<0.0001	164	A	328
Potassium (mEq/L)	<0.0001	3.78	A	159
Chloride (mEq/L)	<0.0001	104	A	2.97
Calcium (mg/dL)	<0.0001	13.49	A	142
Magnesium (mg/dL)	<0.0001	ND	A	11.20
Phosphorus (mg/dL)	<0.0001	15.6	A	2.15
			8.0	7.2

1) The same abbreviations are used as in Table 4-1

Table 4-4. Serum chemistry values taken from the literature for sunshine bass and striped bass, and reference intervals for striped bass for selected analytes.

Analyte	Value ¹	N	Analyte	Value	N	Reference
Sunshine bass Chloride	123	6	Cortisol ¹	0.8	6	Tomasso 1980
Striped bass Chloride	119	4(?)	Cortisol	29.9	10	Davis et al. 1982
AST (SGOT)	51	4	Calcium	10.2	4	Brown et al. 1987
ALP	51	4	Sodium	156	4	"
Total bilirubin	0.45	4	Potassium	6.1	4	"
Creatinine	0.55	4	Chloride	136	4	"
Cholesterol	315	4	Phosphorus	10.7	4	"
Glucose	109	4	Osmolality	327	4	"
Total protein	3.7	4	Albumin	1.3	4	"
Globulin	2.5	4	Cortisol	10.5	4	"
Potassium	0.4-2.1	4	Sodium	163-184	4	Courtois 1976
Potassium	3.7	6	Sodium	147	6	Courtois 1975
Phosphorus	8.2	6	Calcium	11.7	6	"
Cholesterol	367	6	Glucose	235	6	"
Total protein	4.9	6	AST (SGOT)	196	4	"
ALP	23	6				"
Total protein	5.18	22	Calcium	12.8	13	Westin 1978
Chloride	141.5	16				"

Reference intervals for striped bass in fresh water (Tisa and Strange 1983)

Analyte	Value	N	Analyte	Value	N
Osmolality	274-402	40	Chloride	112-172	40
Cholesterol	261-750	40	Cortisol	0-12.5	40
Total protein	3.1-6.0	40	Glucose	44-181	40

1) Values are means, if a range is given it indicates mean values differed for different environmental or other factors such as age or sex. Units are the same as used in this paper.

Table 4-4. cont.

Table 4-5. Serum chemistry values reported for other species of fishes. Values reported are means. If a range is given it indicates means for different water quality conditions or for different ages or sexes of fish.

Analyte	Value	N	Analyte	Value	N	Reference
<i>Oncorhynchus aguabonita</i>						
Osmolality	292-306	9-11	Magnesium	3.2-3.7	9-11	Hunn et al. 1992
Calcium	13.1-16.9	9-11				"
<i>Oncorhynchus mykiss</i>						
AST (SGOT)	236	15	ALP	390	14	Bucher 1990
Total protein	4.1	10	Albumin	1.6	10	Byrne et al. 1989
Osmolality	319	10	Sodium	161	9	"
Potassium	0.5	9	Chloride	127	9	"
Calcium ¹	3.36	10	Magnesium ¹	1.47	10	"
Phosphorus ¹	6.27	10				"
<i>Oncorhynchus keta</i>						
Cortisol	11.9-148	20	Glucose	72-84	20	Haney et al. 1992
Protein	3.3-5.1	20	Osmolality	299-380	20	"
<i>Salmo trutta</i>						
AST (SGOT)	214-219	6				Bucher and Hofer 1990
<i>Heteropneustes fossilis</i>						
Cholesterol	430	10	Calcium	1.7 ²	10	Sharma et al.1982
AST (SGOT)	31	10	Phosphorus	10.5	10	"
ALP	0.095 ³	10				"

Analyte	Value	N	Analyte	Value	N	Reference
<i>Ictalurus punctatus</i>						
Protein	2.9-4.8	9-70	Albumin	0.9-1.3	9-70	Ellsaesser et al.
ALP	34-88	9-70	AST (SGOT)	71-163	9-70	1987
Cholesterol	84-206	9-70	Glucose	21-46	9-70	"
Total bilirubin	0.1-0.2	9-70	Creatinine	0.3-0.9	9-70	"
Calcium	11.2-12.4	9-70	Chloride	102-120	9-70	"
Magnesium	2.7-4.0	9-70	Potassium	1.9-4.4	9-70	"
Phosphorus	10.3-14.5	9-70	Sodium	137-144	9-70	"
Total protein	3.7-4.5	~100	Glucose	78-129	~100	Warner and Williams
Albumin	0.32-0.78	~100	AST (SGOT)	122-277	~100	1977
Cholesterol	212-317	~100	ALP	45-93	~100	"
Total bilirubin	0-0.4	~100	Sodium	8-137	~100	"
Creatinine	0.01-1.78	~100	Potassium	0.7-2.1	~100	"
Chloride	95.5-98.9	~100	Calcium	9.2-13.1	~100	"
Phosphorus	16.2-19.2	~100				"

1)mmol/L. 2)mg/mL. 3)mgP/mL

Table 4-5. cont.

CHAPTER 5

The Effects of Temperature on the Hematology and Serum Chemistry of Sunshine Bass (*Morone chrysops* X *Morone saxatilis*)

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ABSTRACT

1. Standard hematology and serum chemistry profiles were determined for sunshine bass (reciprocal hybrid striped bass) acclimated to 10, 18, 24 and 29°C water. These values were compared with reference intervals previously developed for sunshine bass.
2. Significant differences in hematology and serum chemistry values were observed for fish at the different temperature conditions, however, most of the values were still within established reference intervals. The fish held at 10°C had the greatest number of divergent values.
3. The following values deviated sufficiently from the reference interval suggesting that separate reference intervals need to be developed for these temperatures: leukocytes, lymphocytes and monocytes at 10°C; total protein, albumin, globulin and chloride at 29°C; and glucose and calcium at 10 and 18°C.
4. Minor modifications to the previously established reference intervals for analytes with only slight variation due to temperature, will allow for the use of one reference intervals at different temperatures.

INTRODUCTION

Hematology and serum chemistry are fundamental tools used in human and veterinary medicine to diagnose diseases and other pathologic conditions. Many changes in physiologic states are reflected in blood, affecting both the hematology and serum chemistry profiles. Hematology and serum chemistry are not used extensively in fish medicine for diagnostic purposes due to the lack of reference intervals for the different fish species, and because external factors can affect some blood values. There have been numerous studies dealing with factors which can influence blood values in fishes. Environmental conditions (Lane, 1979; Bentinck-Smith et al., 1987; Bhaskar and Rao, 1989; Bucher, 1990; LeaMaster et al., 1990), culture conditions (Warner and Williams, 1977; Ellsaesser and Clem, 1987b; Kjartansson et al., 1988; Hrubec et al.), and capture and sampling techniques (Hunn et al., 1992; McDonald and Milligan, 1992) have been shown to affect hematologic values, yet there is little consensus on the influence of these factors. Often, too few animals were sampled to give a valid indication of the population. Many studies were field experiments, which introduce variations in values associated with catching fish and transporting the blood to the laboratory for analysis. Artifacts due to stress of capture with gill nets, hooks, or

electroshocking can mask true blood levels. With field studies, it is also difficult to correlate all the interacting environmental variables with the blood values. In laboratory studies, while similar environmental variables are present, they can be controlled to a greater extent than in the field. Additionally under laboratory conditions, the fish can usually be sampled with minimal stress.

Sunshine bass (reciprocal hybrid striped bass: female *Morone chrysops* X male *Morone saxatilis*) are raised throughout the United States for food and release as a sport fish. The culture of this species would be improved by the early detection and diagnosis of disease allowing for rapid therapeutic intervention. Hematology has a unique potential for use in the early detection of disease once a hematological data base is sufficiently developed to allow diagnosis of specific diseases or metabolic disorders. There are no published hematologic values for sunshine bass, and the only serum chemistry values published are chloride and cortisol (Tomasso et al., 1980). Hematological studies of striped bass are more numerous; but most are limited by the small number of analytes measured (Westin, 1978; Davis et al., 1982; Hunn and Greer, 1990), or the small number of fish sampled (Courtois, 1975, 1976; Brown et al., 1987). The most comprehensive study (Tisa and Strange, 1983)

determined reference intervals for PCV, hemoglobin, osmolality, chloride, glucose, cortisol, total protein, and cholesterol.

With the goal of developing hematology as an effective diagnostic tool for fish, hematologic and serum chemistry reference intervals were determined for sunshine bass in different production systems (Hrubec et al.). The reference intervals for fish under the different production systems were significantly different for a number of the blood analytes. Reasons for these differences may be caused by temperature and water quality, diet, genetic variation and stocking density. In this paper, we examine the effect of temperature on the hematology and serum chemistry of sunshine bass, and compare the blood values with the established reference interval. This will help establish a foundation for the development of hematology and serum chemistry as diagnostic tools in sunshine bass.

MATERIALS AND METHODS

Fish and Experimental Conditions

Sunshine bass were purchased from a commercial producer (Day Spring Farm, Etlan, VA) when the fish were 13 months old.

Fish were divided into four groups of seventy fish each and placed into separate 2000 L circular tanks with undergravel filters. Fish were fed a commercial pelleted fish feed (Zeigler Brothers Inc., Gardner, PA) daily at 1% body weight per day. Fish were allowed to acclimate to their laboratory setting for one week. Temperatures were then adjusted over the course of the second week and held constant at 10°C, 18°C, 24°C and 29°C for the duration of the experiment. All fish were maintained under a constant photoperiod of 13 hours light and 11 hours dark.

Water Quality

Water quality was monitored as follows: pH, temperature, and ammonia were measured daily; alkalinity, hardness, nitrites, nitrates and dissolved oxygen were measured twice weekly. The pH was measured using a pH meter (Hanna Instruments, Woonsocket, RI), other parameters were measured with a commercial kit (Hach Chemical, Loveland, CO). Ranges for water quality over the sampling time are shown in Table 5-1.

Blood Handling

Fish were anesthetized with tricaine methanesulfonate (MS-222 at 0.25 g/L, Sigma Chemical Co., Saint Louis, MO) and

bled using plastic syringes fitted with 21 gauge needles. The blood was divided between a plain serum tube and an ethylenediamine tetra-acetic acid (EDTA) blood tube. All hematological determinations were made with the EDTA treated blood. Packed cell volume (PCV) was determined in microhematocrit tubes by centrifugation (Clay Adams Autocrit-ultra 3, Parsippany, NJ) for 5 min. Plasma protein (total solids) was determined with a refractometer (Reichert-Jung, Leica, Buffalo, NY) from the plasma in the microhematocrit tube. Total cell count was determined manually with an improved Neubauer hemacytometer using Natt-Herrick's solution as a diluent (Natt and Herrick, 1952). Both erythrocytes and leukocytes were counted as a combined total cell count. Blood smears from the EDTA treated blood were stained with Wright's Geimsa stain (Hematek 1000 automatic stainer, Miles Inc., Elkhart, IN) and were used to determine the erythrocyte, leukocyte, thrombocyte and differential white cell counts. Erythrocytes were enumerated on the stained smear by counting the number of erythrocytes and "other cells" until 1500 cells were counted. The percentage of erythrocytes was multiplied by the total cell count to give the number of erythrocytes. The percentage of "other cells" was multiplied by the total cell count to determine the combined leukocyte and thrombocyte number. The differential white count was

determined by counting leukocyte types and thrombocytes until 200 leukocytes were enumerated. The number of thrombocytes was then subtracted from the combined leukocyte thrombocyte number to give the thrombocyte count and the total leukocyte count. This total leukocyte count was then used to determine the number of each different white cell type. Hemoglobin was determined using the cyanomethemoglobin method (Stanbio Laboratory Inc., San Antonio, TX). The erythrocyte indices of mean cell volume (MCV), mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) were calculated by the standard formulas (Blaxhall and Daisley, 1973).

Blood in the serum tubes clotted at room temperature for one hour. Serum was separated by centrifugation at 14,000g for five minutes and frozen at -5°C. After all fish were sampled, the serum samples were thawed, centrifuged to remove fibrin, and analyzed using an automated system (Kodak Ektachem 700 serum analyzer, Rochester, NY). The following analytes were determined: total protein, albumin, creatinine, total bilirubin, alkaline phosphatase (ALP), aspartate aminotransferase (AST), glucose, cholesterol, sodium, chloride, potassium, calcium, magnesium, and phosphorus. Globulin was calculated from the total protein

minus the albumin. Cortisol was determined with a commercial enzyme-linked immunosorbent assay (Milenia Kinetic EIA, Diagnostic Products Corp., Los Angeles, CA).

Sampling Schedule

The fish were maintained at their experimental conditions for four weeks prior to sampling. Four fish from each treatment were sampled every 8 days for three weeks and then every 14 days for an additional 8 weeks, a total of 8 sampling times. Sampled fish were not returned to the tank. Serum from the four fish at each sampling time was pooled to give a composite serum sample. The PCV, plasma protein and hemoglobin were determined on 24 fish in each temperature group; cell counts and differential were determined on 12 fish in each group.

Development of Reference Intervals

Reference intervals were determined previously for sunshine bass under different culture conditions (Hrubec et al.) following the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS, 1992). The guidelines state that reference intervals should be as broad as possible, but still maintain the ability to detect pathologic changes. Therefore, when an analyte was not statistically different between the different culture

systems, a composite interval giving the broadest range was used as the reference interval for this study. The water temperature range of the fish used to determine the reference interval in the previous study was 24 - 28°C

Statistical Analysis

One-way analysis of variance (ANOVA) was used to determine differences between the temperature groups for each blood value using a commercial statistical program (Statistix Version 4, Analytical Software, Tallahassee, FL). When a significant difference was detected ($P < 0.05$), the means were compared with a Tukey's means comparison test.

RESULTS

The hematology results for the fish maintained at different temperatures are shown in Table 5-2. Differences in the values due to temperature were seen predominately in the fish at 10°C which had fewer total leukocytes, small and large lymphocytes and monocytes. At 18°C, 24°C and 29°C, the counts for the above cell types were not significantly different. The MCHC demonstrated a significant trend with higher values at the lower temperatures. The eosinophil

count also demonstrated a trend with lower counts in both the 10 and 18°C tanks. Other hematological analytes and cell counts were not affected by the temperature.

Serum chemistry results for the fish in the different temperature groups are shown in Table 5-3. Most of the analytes were significantly different between the temperature groups; with only creatinine, total bilirubin, and ALP not affected by temperature. The total protein, and albumin were higher in the 29°C group but not significantly different at the other temperatures. Globulin was higher in fish maintained at 10 and 29°C than at 18 and 24°C. Glucose was higher in fish maintained at 10 and 18°C than at the warmer temperatures, but another indicator of stress, cortisol, was higher at warmer temperatures. Chloride was highest at 29°C, while calcium increased with increasing temperature. Six of the analytes - AST, cholesterol, sodium, potassium, magnesium and phosphorus, although different at the different temperatures, did not demonstrate a pattern or trend in the values.

When the hematology values for fish at different temperatures were compared to reference intervals developed for sunshine bass (Table 5-4), most of the values for fish

in this study were within the established reference intervals. The erythrocyte counts were slightly higher; while total leukocyte, small lymphocyte, monocyte and thrombocyte counts were lower than the established reference interval in the 10°C tank. The monocyte count of fish at 29°C was also lower than the reference interval.

Serum chemistry values for fish at the different temperatures, were compared with established reference intervals (Table 5-5). The total protein, albumin and globulin were slightly higher than the reference interval at 29°C. Glucose values were higher in the two colder tanks, while ALP values were slightly higher at all temperatures than the established reference intervals. Electrolytes demonstrated the greatest changes with temperature. Sodium and chloride were slightly higher at 29°C. Calcium was lower at 10 and 18°C, and magnesium was higher at 10 and 29°C. All potassium levels were lower and all phosphorus levels were higher than the reference intervals.

DISCUSSION

Our goal is to develop hematology and serum chemistry as a

diagnostic tool for use with fishes. The sequential steps in this process are to 1. determine reference intervals (the range of individual variation expected in a normal healthy population) for the blood analytes, and 2. determine how the analytes change with pathologic conditions. Since fishes are ectothermic, a third step is required. This is to investigate the effect of temperature and environmental variables in order to determine if one reference interval can be used at all temperatures and all water qualities.

Previous studies have shown the effect of temperature on blood values in fishes, but the changes were not compared with established reference intervals. In most cases, reference intervals were not even determined. This has led to the misconception that too many factors influence the blood values of fishes for hematology to be useful as a diagnostic tool. However, if a change caused by temperature does not deviate the blood analyte outside the reference interval, then temperature variation should not interfere with the ability to use blood values to diagnose disease. Conversely, if the change caused by the temperature deviates the blood analyte outside the reference interval, potentially masking changes due to disease, a new reference interval must be developed for that temperature. It is useful to know the effect of temperature on the blood

analytes even if the value is not deviated outside the reference interval. This allows the diagnostician to make adjustments in the interpretation of the hemogram in light of environmental factors.

Standard hematology (Blaxhall and Daisley 1973) and serum chemistry profiles were used in this study to investigate the influence of temperature on blood values. Serum chemistry profiles were determined using automated blood chemistry analyzers which are accurate for fish blood even when operated at mammalian temperatures of 37°C (Warner et al., 1978, 1979; Smith and Ramos, 1980) and are readily available in commercial diagnostic laboratories. The analytes measured are used routinely in human and veterinary medicine to diagnose pathologic disorders.

At temperatures above and below the optimal temperature, the fish may be stressed and demonstrate a stress response in the blood. Optimal temperature for sunshine bass is difficult to define as numerous factors can be optimized such as growth, feed conversion, disease resistance and physiologic homeostasis. However, much evidence points to an optimum temperature for hybrid striped bass in the low to mid 20s. Maximal growth rate of hybrid striped bass was at

21°C while growth declined above and below this temperature (Woiwode and Adelman, 1991). Hybrid striped bass congregated at the cooler temperatures of 20-25°C during the warmer months, tolerating reduced oxygen concentrations of 2 mg/L to avoid water temperatures above 27°C (Douglas and Jahn, 1987). It has also been shown that hybrid striped bass and other fish will congregate in the heated water outlets of industrial plants in the colder months (Glass and Maughan, 1985). In these instances, the fish are presumably moving to temperatures which are less stressful and allow for more optimal physiologic function.

Stress can be defined as an event which induces the release of catecholamines and cortisol. In fishes, stressful events include handling, confinement, transport, angling, disease, and unsuitable water conditions. The stress response is well characterized in fishes, changing both hematological and serum chemistry components. The physiologic response to catecholamines and cortisol can cause splenic contraction, increased blood flow and demargination of vascular leukocytes. The results are an erythrocytosis, thrombocytosis, lymphopenia, neutrophilia, decrease in clotting time, and increase in PCV due to erythrocyte swelling (Casillas and Smith, 1977; Ellsaesser and Clem,

1986, 1987a; McDonald and Milligan, 1992; Randall and Perry, 1992). Serum chemistry changes with acute stress include a hypercortisolemia, hyperglycemia, hyperproteinemia, hyponatremia, hypochloremia, and a hypercalcemia (McDonald and Milligan, 1992). Chronic stress can induce a hypoproteinemia, hypoglycemia, and a hypocortisolemia (McDonald and Milligan, 1992). Although the stress response has not been fully characterized in sunshine bass, both sunshine and striped bass become hypochloremic with stress (Tomasso *et al.*, 1980; Davis *et al.*, 1982; Davis and Parker, 1990).

In this study, only sunshine bass at 10°C demonstrated a marked response to temperature differences characterized by a leukopenia, lymphopenia and a monocytopenia. Eosinophil count, MCV and MCHC, although significantly lower in fish at 10°C, were not outside the established reference interval. Studies with other fish species have also demonstrated hematologic changes only at temperature extremes. Goldfish, whose optimal temperature is 25°C, were acclimated to 5, 15, 25 and 35°C (Dunn *et al.*, 1989). The goldfish exhibited a lymphopenia, a neutropenia and an eosinopenia at 5°C. The response to colder temperatures occurs rapidly; short term exposure of tilapia to temperatures shifts from 30°C to 14°C

for 24 or 86 hours decreased the leukocrit within 24 hours (Sun et al., 1992). Moderate temperatures that are not at extreme limits for a fish do not seem to affect hematologic values. Milkfish cultured within a small temperature range of 22 - 30°C demonstrated no differences in PCV, hemoglobin, MCV, MCH, MCHC, erythrocyte, leukocyte and differential counts (Bhaskar and Rao, 1989).

Studies monitoring seasonal changes in hematologic values give conflicting results. In a reservoir population of striped bass, the PCV, hemoglobin, erythrocyte counts, MCHC and leukocrit were highest during fall and winter while the MCV and MCH were lowest in the winter (Lochmiller et al., 1989). Similarly in carp, greater erythrocyte counts, hemoglobin, and plasma protein were seen in the winter and spring (Fourie and Hattingh, 1976). However, no changes were seen in the PCV, MCV, MCH, MCHC and the leukocyte counts. Rainbow trout on the other hand, had lower erythrocyte counts, PCV, hemoglobin, and higher MCV, MCH, MCHC in the colder months (Lane, 1979). Unfortunately, some variation seen in the seasonal data probably arise from capture and sampling differences.

A greater number of serum chemistry analytes were

significantly different at the four temperatures. This is not surprising as chemistries monitor metabolic function which is directly controlled by temperature in an ectothermic animal. In this study, protein levels (total protein, albumin and globulin) and serum chloride were highest and outside the reference interval at the warmest temperature. Glucose was highest and outside the reference interval at the colder temperatures. Similar results have been demonstrated in other species. Striped bass acclimated to 5, 10, 16, 21, 25, 30°C (Davis and Parker, 1990) had lower chlorides and higher glucose at the colder temperatures, while cortisol was highest at 30°C. Red sea bream acclimated to 10, 20 25, 30°C (Woo, 1990) had higher plasma protein at warmer temperature, and higher glucose levels at colder temperatures. Cortisol levels in sea bass ranged from 10-60 µg/dL and was lower in colder months and higher in summer (Planas et al., 1990). In carp, minor temperature differences of 11 and 18°C (Kühn et al., 1986), did not affect sodium, calcium and glucose levels, but cortisol was higher at the warmer temperature. As with hematological changes, the serum chemistry changes are relatively rapid. Short term exposure of tilapia to temperatures shifts from 30°C to 14°C demonstrated decreases in the chloride and cortisol, and increases in the glucose

by 86 hours (Sun et al., 1992).

Sunshine bass demonstrated a significant decrease in calcium at 10 and 18°C. It is unknown if this trend in calcium levels with temperature will persist across species as do the trends in glucose, cortisol and chloride. Calcium was determined only in carp (Kühn et al., 1986) and demonstrated no difference at 11 and 18°C, however, warmer temperatures were not monitored. McDonald and Milligan (1992) state that the greatest changes in blood calcium occur with vitellogenesis. It is possible that sunshine bass in the warmer temperatures were developing faster and beginning vitellogenesis, however, we saw no differences in the calcium levels between males and females.

Studies monitoring seasonal changes in the blood enzymes of rainbow trout (Bucher, 1990) have shown ALP and AST to be significantly lower in the summer; however, ALP in channel catfish increased in the summer (Ellsaesser and Clem, 1987b). We saw no differences in ALP with temperature and only minor differences in the AST which were not outside the reference interval.

Although the changes in sunshine bass with colder

temperatures are reminiscent of a chronic stress response, they do not match completely. Whereas a classic stress response results in a lymphopenia and a neutrophilia, the response to colder temperatures results in a lymphopenia, monocytopenia and eosinopenia. A neutropenia as described in goldfish (Dunn et al., 1989) was not observed in sunshine bass, but this may represent a species difference in the response to low temperatures. Serum chemistry changes are not indicative of a stress response either. Changes due to stress include a hypercortisolemia, hyperglycemia, hyperproteinemia, hyponatremia, hypochloremia, and a hypercalcemia. In sunshine bass at the colder temperatures, only the hyperglycemia was demonstrated. In the fish at 29°C only the hyperproteinemia was clearly demonstrated. Cortisol and calcium, although higher at the warmer temperatures, were not outside the reference interval.

A number of blood analytes (monocytes, thrombocytes, AST, cholesterol, sodium, and magnesium) were significantly different, but did not show a trend with temperature and were within or just outside the range of the reference intervals. These differences were probably due to individual variation. The established reference intervals were determined for sunshine bass in a relatively uniform

environment. The minor differences seen here indicate that the reference intervals may need to be broadened to accommodate the individual variation seen in fish from slightly different environments. Until it is known how the hematologic and serum chemistry values change with pathologic conditions it cannot be determined if these minor differences will mask pathologic changes. Potassium and phosphorus did not show consistent changes with temperature, but were outside the reference intervals at all temperatures. The reason for this is not known, but may be from the influence of other water quality parameters, such as hardness or pH.

The hematology and serum chemistry data taken together do not indicate a stress response in the sunshine bass at elevated temperatures, and exhibit only some of the features of a stress response at 10°C. The consistencies between our data and that of other studies at the temperatures extremes indicate that separate reference intervals should be generated for leukocytes, lymphocytes, monocytes, glucose and calcium at colder temperatures. At warmer temperatures, separate reference intervals should be determined for total protein, albumin, globulin and chloride. Additionally, changes in the blood analytes with disease need to be

established before hematology and serum chemistry can function fully as a diagnostic tool.

REFERENCES

- Bentinck-Smith J., Bealeau H. M., Waterstart P., Tucker C. S., Stiles F., Bowser R. P. and Brown L. A. (1987) Biochemical reference ranges for commercially reared channel catfish. *Prog. Fish-Cult.* **49**:108-114.
- Bhaskar B. R. and Rao K. S. (1989) Influence of environmental variables on hematology, and compendium of normal hematological ranges of milkfish, Chanos chanos (Forsk.) in brackish culture. *Aquaculture* **83**:123-136.
- Blaxhall P. C. and Daisley K. W. (1973) Routine haematological methods for use with fish blood. *J. Fish Biol.* **5**:771-781.
- Brown C. L., Young G., Nishioka R. S. and Bern H. A. (1987) Preliminary report on the physiological status of striped bass in the Carginez Strait die-off. *Fish. Res.* **6**:5-16.
- Bucher F. (1990) Organ patterns and natural fluctuations of blood enzymes of rainbow trout (Salmo gairdneri Rich.) *Comp. Biochem. Physiol.* **96B**:795-799.
- Casillas E. and Smith L. S. (1977) Effect of stress on blood coagulation and hematology in rainbow trout (Salmo gairdneri). *J. Fish Biol.* **10**:481-491.
- Courtois L. A. (1975) Blood and serum analysis of adult striped bass, Morone saxatilis, captured in the Sacramento River. *Calif. Fish and Game* **61**:245-246.
- Courtois L. A. (1976) Hematology of juvenile striped bass, Morone saxatilis (Walbaum), acclimated to different environmental conditions. *Comp. Biochem. Physiol.* **54A**:221-223.

- Davis K. B., Parker N. C. and Suttle M. A. (1982) Plasma corticosteroids and chlorides in striped bass exposed to tricaine methanesulfonate, quinaldine, etomidate, and salt. *Prog. Fish-Cult.* **44**:205-207.
- Davis K. B. and Parker N. C. (1990) Physiological stress in striped bass: effect of acclimation temperature. *Aquaculture* **91**:349-358.
- Douglas D. R. and Jahn L. A. (1987) Radiotracking hybrid . striped bass in Spring Lake, Illinois, to determine temperature and oxygen preferences. *N. Amer. J. Fish. Manag.* **7**:531-534.
- Dunn S. E., Murad A. and Houston A. H. (1989) Leucocytes and leucopoietic capacity in thermally acclimated goldfish, Carassius auratus L. *J. Fish Biol.* **34**:901-911.
- Ellsaesser C. F. and Clem L. W. (1986) Hematological and immunological changes in channel catfish stressed by handling and transport. *J. Fish Biol.* **28**:511-521.
- Ellsaesser C. F. and Clem L. W. (1987a) Cortisol-induced hematologic and immunologic changes in channel catfish (Ictalurus punctatus). *Comp. Biochem. Physiol.* **87A**:405-408.
- Ellsaesser C. F. and Clem L. W. (1987b) Blood serum chemistry measurements of normal and acutely stressed channel catfish. *Comp. Biochem. Physiol.* **88A**:589-594.
- Fourie F. L. R. and Hattingh J. (1976) A seasonal study of the hematology of carp (Cyprinus carpio) from a locality in the transvaal, South Africa. *Zool. Afric.* **11**:75-80.
- Glass R. D. and Maughan O. E. (1985) Concentrated harvest of Striped Bass X White bass near a heated water outlet. *N. Amer. J. Fish. Manag.* **5**:105-107.

- Hrubec T. C., Smith S. A., Feldman B., Veit H. P., Libey G., Tinker M. K., Robertson J. L. Hematologic reference intervals for hybrid striped bass: Comparisons between culture system and type of hybrid. Submitted to *J. Aquat. Animal Health* 1994.
- Hrubec T. C., Smith S. A., Feldman B., Veit H. P., Libey G., Tinker M. K., Robertson J. L. Serum chemistry reference intervals for sunshine bass (Morone chrysops X Morone saxatilis): A comparison of different culture systems. Submitted to *J. Aquat. Animal Health* 1994.
- Hunn J. B. and Greer I. E. (1990) Colorimetric and refractometer estimates of total plasma protein in striped bass, Morone saxatilis (Walbaum). *J. Fish Biol.* **36**:617-618.
- Hunn J. B., Wiedmeyer R. H., Greer I. E. and Grady A. W. (1992) Blood chemistry of laboratory-reared golden trout. *J. Aquat. Animal Health* **4**:218-221.
- Kjartansson H., Fivelstad S., Thomassen J. M. and Smith M. J. (1988) Effects of different stocking densities on physiological parameters and growth of adult Atlantic salmon (Salmo salar L.) reared in circular tanks. *Aquaculture* **73**:261-274.
- Kühn E. R., Corneillie S. and Ollevier F. (1986) Circadian variation in plasma osmolality, electrolytes, glucose, and cortisol in carp (Cyprinus carpio). *Gen. Comp. Endocrinol.* **61**:459-468.
- Lane H. C. (1979) Progressive changes in hematology and tissue water of sexually mature trout, Salmo gairdneri Richardson during the autumn and winter. *J. Fish Biol.* **15**:425-436.

- LeaMaster B. R., Brock J. A, Fugioka R. S. and Nakanura R. M. (1990) Hematologic and blood chemistry values for Sarotherodon melanotheron and a red hybrid tilapia in fresh water and seawater. *Comp. Biochem. Physiol.* **97A**:523-529.
- Lochmiller R. L., Weichman J. D. and Zale A. V. (1989) Hematological assessment of temperature and oxygen stress in a reservoir population of striped bass (Morone saxatilis). *Comp. Biochem. Physiol.* **93A**:535-541.
- McDonald D. G. and Milligan C. L. (1992) Chemical Properties of Blood. In: *Fish Physiology* (Edited by Hoar W. S., Randall D. J. and Farrell A. P.), Vol. 12 Part B, pp. 56-135. Academic Press, New York.
- Natt M. P. and Herrick C. A. (1952) A new blood diluent for counting erythrocytes and leukocytes of the chicken. *Poul. Sci.* **31**:735-738.
- NCCLS (National Committee for Clinical Laboratory Standards) (1992) How to define, determine, and utilize reference intervals in the clinical laboratory; proposed guidelines. *NCCLS Document C28-P* (ISBN 1-56238-143-1). NCCLS, Villanova, Pennsylvania.
- Planes J., Gutierrez J., Fernandez J., Carrillo M. and Canals P. (1990) Annual and daily variation of plasma cortisol in seabass, Dicentrarchus labrax L. *Aquaculture* **91**:171-178.
- Randall D. J. and Perry S. F. (1992) Catecholamines. In: *Fish Physiology* (Edited by Hoar W. S., Randall D. J. and Farrell A. P.), Vol. 12 Part B, pp. 255-300. Academic Press, New York.
- Smith A. C. and Ramos F. (1980) Automated chemical analysis in fish health assessment. *J. Fish Biol.* **17**:445-450.

- Sun L. T., Chen G. R. and Chang C. F. (1992) The physiological response of tilapia exposed to low temperatures. *J. Therm. Biol.* **17**:149-153.
- Tisa M. S. and Strange R. J. (1983) Hematology of striped bass in fresh water. *Prog. Fish-Cult.* **45**:41-44.
- Tomasso J. R., Davis K. B. and Parker N. C. (1980) Plasma corticosteroid and electrolyte dynamics of hybrid striped bass (white bass X striped bass) during netting and hauling. *Proc. World Maricult. Soc.* **11**:303-310.
- Warner M. C., Diehl S. A. and Tomb A. M. (1978) Effects of dilution and temperature of analysis on blood serum values in rainbow trout Salmo gairdneri. *J. Fish Biol.* **13**:257-263.
- Warner M. C., Tomb A. M. and Diehl S. A. (1979) Variability and stability of selected components in rainbow trout Salmo gairdneri serum and the precision of automated analysis in measuring these components. *J. Fish Biol.* **15**:141-151.
- Warner M. C. and Williams R. W. (1977) Comparison between serum values of pond and intensive raceway cultured channel catfish Ictalurus punctatus (Rafinesque). *J. Fish Biol.* **11**:385-391.
- Westin D. T. (1978) Serum and blood from adult striped bass, Morone saxatilis. *Estuaries* **1**:126-128.
- Woo N. Y. S. (1990) Metabolic and osmoregulatory changes during temperature acclimation in the red sea bream, Chrysophrys major: Implications for its culture in the subtropics. *Aquaculture* **87**:197-208.
- Woiwode J. G. and Adelman I. R. (1991) Effects of temperature, photoperiod, and ration size on growth of hybrid striped bass X white bass. *Trans. Amer. Fish. Soc.* **120**:217-229.

Table 5-1. Range of water quality parameters

PARAMETER	10°C ¹	18°C	24°C	29°C
Temperature (C)	9-11	17-18	23-25	27-30
pH	7.4-8.3	7.4-8.1	7.1-8.1	7.3-8.1
Ammonia (NH ₃ mg/L)	0-0.005	0-0.006	0-0.009	0-0.009
Nitrite (NO ₂ -N mg/L)	0-0.06	0-0.05	0-0.15	0-0.05
Nitrate (NO ₃ -N mg/L)	2-6	3-7	3-6	3-6
Alkalinity (mg/L)	86-103	86-120	86-137	86-137
Hardness (mg/L)	86-154	103-154	103-154	86-137
Dissolved Oxygen (mg/L)	8-9	7-9	5-6	5-6

1) The temperature designation used for each group was determined by the mean temperature over the course of the experiment.

Table 5-2. Hematological values for sunshine bass at different temperatures.

ANALYTE	N	10°C	18°C	24°C	29°C
PCV ² %	24	40	a ¹	41	a
Plasma protein (mg/dL)	24	6.2	a	5.9	a
Hemoglobin (g/dL)	12	10	a	10	a
MCV ³ (fL)	12	81	a	95	b
MCH ⁴ (pg)	12	22.4	a	22.1	a
MCHC ⁵ (g/dL)	12	27	a	25	a,b
Erythrocytes (x10 ⁶ /μL)	12	4.97	a	4.46	a
Leukocytes (x10 ³ /μL)	12	20.64	a	60.28	b
Lymphocytes (x10 ³ /μL)	12	5.49	a	26.08	b
Small	12	0.89	a	2.82	b
Large	12	0.58	a	0.59	a
Neutrophils (x10 ³ /μL)	12	0.20	a	0.81	b
Monocytes (x10 ³ /μL)	12	0.09	a	0.86	b
Eosinophils (x10 ³ /μL)	12	0.37	a	0.65	a
TLC ⁶ (x10 ³ /μL)	12	37.92	a	54.92	a,b
Thrombocytes (x10 ³ /μL)	12	59.82	b	65.35	b

1) Similar letters indicate statistically (P < 0.05) similar groups. 2) Packed cell volume. 3) Mean cell volume. 4) Mean cell hemoglobin. 5) Mean cell hemoglobin concentration. 6) Thrombocyte like cell.

Table 5-3. Serum chemistry values for sunshine bass at different temperatures.

ANALYTE	N ¹	10°C	18°C	24°C	29°C
Total Protein (g/dL)	8	3.9	3.9	3.9	4.3
Albumin (g/dL)	8	1.4	1.5	1.5	1.6
Globulin (g/dL)	8	2.5	2.4	2.4	2.7
Creatinine (mg/dL)	8	0.2	0.2	0.2	0.2
Total Bilirubin (mg/dL)	8	0.2	0.2	0.2	0.2
ALP ³ (mU/mL)	8	74	80	78	81
AST ⁴ (mU/mL)	8	60	28	16	34
Glucose (mg/dL)	8	211	200	110	102
Cortisol (µg/dL)	8	16	30	35	35
Cholesterol (mg/dL)	8	205	174	204	231
Sodium (mEq/L)	8	165	161	165	168
Potassium (mEq/L)	8	1.3	1.5	1.0	0.9
Chloride (mEq/L)	8	147	142	147	153
Calcium (mg/dL)	8	9.33	9.09	11.64	11.84
Magnesium (mg/dL)	8	2.6	2.0	2.2	2.8
Phosphorus (mg/dL)	8	11.6	13.2	10.4	11.5

1) Each sample pooled from 4 fish. 2) Similar letters indicate statistically (P < 0.05) similar groups. 3) Alkaline phosphatase. 4) Aspartate aminotransferase (SGOT).

Table 5-4. Hematology values at different temperatures compared with reference intervals developed for sunshine bass in tanks.

ANALYTE	REFERENCE ¹ INTERVAL	10°C	18°C	24°C	29°C
PCV ² %	34-47	*	*	*	*
Plasma protein (mg/dL)	5.1-6.5	*	*	*	*
Hemoglobin (g/dL)	8.0-12.0	*	*	*	*
MCV ³ (fL)	81-106	*	*	*	*
MCH ⁴ (pg)	19.6-26.4	*	*	*	*
MCHC ⁵ (g/dL)	22-30	*	*	*	*
Erythrocytes (x10 ⁶ /μL)	3.66-4.96	4.97	*	*	*
Leukocytes (x10 ³ /μL)	32.6-118.2	20.64	*	*	*
Lymphocytes (x10 ³ /μL)					
Small	9.8-54.1	5.49	*	*	*
Large	0.5-6.9	*	*	*	*
Neutrophils (x10 ³ /μL)	0-4.0	*	*	*	*
Monocytes (x10 ³ /μL)	0.8-5.2	0.20	*	*	0.56
Eosinophils (x10 ³ /μL)	0-1.3	*	*	*	*
TLC ⁶ (x10 ³ /μL)	0.6-3.7	0.37	*	*	*
Thrombocytes (x10 ³ /μL)	21.3-104.3	*	*	*	*

An * indicates that the value falls within the established reference interval. 1) Hrubec et al. 2) Packed cell volume. 3) Mean cell volume. 4) Mean cell hemoglobin. 5) Mean cell hemoglobin concentration. 6) Thrombocyte-like-cell.

Table 5-5. Serum chemistry values at different temperatures compared with reference intervals developed for sunshine bass in tanks.

ANALYTE	REFERENCE ¹ INTERVAL	10°C	18°C	24°C	29°C
Total Protein (g/dL)	3.2-3.9	*	*	*	4.3
Albumin (g/dL)	1.2-1.5	*	*	*	1.6
Globulin (g/dL)	2.0-2.5	*	*	*	2.7
Creatinine (mg/dL)	0.1-0.3	*	*	*	*
Total Bilirubin (mg/dL)	0-0.4	*	*	*	*
ALP ² (mU/mL)	46-73	74	80	78	81
AST ³ (mU/mL)	0-72	*	*	*	*
Glucose (mg/dL)	69-171	211	200	*	*
Cortisol (µg/dL)	4.3-53.8	*	*	*	*
Cholesterol (mg/dL)	164-285	*	*	*	*
Sodium (mEq/L)	153-165	*	*	*	168
Potassium (mEq/L)	2.4-3.7	1.3	1.5	1.0	0.9
Chloride (mEq/L)	137-148	*	*	*	153
Calcium (mg/dL)	10.35-12.07	9.3	9.1	*	*
Magnesium (mg/dL)	1.9-2.4	2.6	*	*	2.8
Phosphorus (mg/dL)	6.3-8.1	11.6	13.2	10.4	11.5

An * indicates that the value falls within the established reference interval. 1) Hrubec et al. 2) Alkaline phosphatase. 3) Aspartate aminotransferase (SGOT).

CHAPTER 6

The Effects of Water Quality on the Hematology
and Serum Chemistry of Sunshine Bass
(*Morone chrysops* X *Morone saxatilis*)

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ABSTRACT

1. Standard hematology and serum chemistry values were determined for sunshine bass (reciprocal hybrid striped bass) acclimated to elevated ammonia (0.15 mg/L) and nitrate (200 mg/L) levels. The blood values were compared with controls and with reference intervals established for sunshine bass.

2. Differences in hematology and serum chemistry analytes were observed in the elevated ammonia and nitrate groups, however, most of the values were within the established reference intervals.

3. In fish from the elevated nitrate tank, serum creatinine levels were elevated above controls, and chloride levels were lower than controls, with both values outside the reference intervals. These are considered to be pathologic changes associated with the elevated nitrate levels.

INTRODUCTION

Sunshine bass (reciprocal hybrid striped bass: female *Morone chrysops* X male *Morone saxatilis*) are an important aquaculture species raised throughout the United States. Very little is known about their basic physiology and response to disease. Additionally, there are few diagnostic tools for the early detection and diagnosis of disease in sunshine bass and other fishes. Developing hematology and serum chemistry for use in fishes would enhance the culture and husbandry practices of sunshine bass and other striped bass hybrids.

Little is known about the blood chemistry profile of sunshine bass, with chloride and cortisol the only published analytes (Tomasso et al., 1980). Striped bass have been studied more extensively. Most studies, however, are limited in value due to the number of analytes measured (Westin, 1978; Davis et al., 1982; Hunn and Greer, 1990), or by the small number of fish sampled (Courtois, 1975, 1976; Brown et al., 1987). The two most comprehensive studies on striped bass hematology and clinical chemistry are by Tisa and Strange (1983), and Lochmiller et al. (1989).

Poor water quality is thought to stress fish and increase

their susceptibility to disease. Additionally, environmental conditions (Lane, 1979; Bentinck-Smith *et al.*, 1987; Bhaskar and Rao, 1989; Bucher, 1990; McDonald and Milligan, 1992), and culture conditions (Warner and Williams, 1977; Kjartansson *et al.*, 1988, Hrubec *et al.*) have been shown to affect blood analytes. Although these studies have investigated the effect of environmental influences, the changes seen have not been compared with established reference intervals, and cannot aid in the diagnosis of pathological conditions. Fishes are exposed to environmental ammonia and nitrate from excretory waste, runoff from agricultural lands and waste water effluent from sewage treatment plants. Elevated levels of ammonia and nitrate can present health problems for fish unless measures are taken to control levels of these compounds.

In order to develop hematology and serum chemistry as diagnostic tools for use with fishes, the effect of water quality on the blood analytes must be determined. If environmental conditions do not deviate blood values outside the reference interval, then a single reference interval can be used regardless of the water quality. Deviation of a blood value outside the reference interval represents a normal physiologic response or a pathological change. If a deviation is due to a normal physiologic response, then a

separate reference interval should be determined for that specific condition. Reference intervals have been determined for sunshine bass under different culture conditions (Hrubec et al.) and are used in this study to evaluate the effect of elevated ammonia and nitrate levels on the hematology and serum chemistry of sunshine bass.

MATERIALS AND METHODS

Fish and Experimental Conditions

Sunshine bass were obtained from the Department of Fisheries and Wildlife at Virginia Polytechnic Institute and State University when the fish were 16 months old. Fish were divided into three groups, each consisting of fifty fish in separate 2000 L tanks at 24°C with an undergravel filter. Fish were fed a commercial pelleted fish feed (Zeigler Brothers Inc., Gardner, PA) daily at 1% body weight per day. The fish were allowed to acclimate to the laboratory setting for one week before the water quality was adjusted. Tanks were randomly designated as a control tank (normal water), an elevated ammonia tank and an elevated nitrate tank. Ammonia and nitrate levels were gradually increased over a two week acclimation period and then maintained at elevated

levels for the duration of the experiment. In the elevated ammonia tank, ammonium chloride (Sigma Chemical, Saint Louis, MO) was added daily to bring the total ammonia concentration to 2.6 mg/L, giving an un-ionized ammonia range of 0.05 to 0.15 mg/L. Ammonia levels dropped from nitrification to a mean of 0.005 mg/L after 10 hours. In the elevated nitrate tank, sodium nitrate (Sigma Chemical) was added to bring the levels to 200 mg/L $\text{NO}_3\text{-N}$. Additional sodium nitrate was added after routine water changes to keep nitrate levels at 200 mg/L. All fish were maintained under a constant photoperiod of 13 hours light and 11 hours dark.

Water Quality

Water quality was monitored as follows: pH, temperature, and ammonia were measured daily; alkalinity, hardness, nitrites, nitrates and dissolved oxygen were measured twice weekly. Nitrate levels were measured daily in the elevated nitrate tank. The pH was measured using a pH meter (Hanna Instruments, Woonsocket, RI). The other parameters were measured with a commercial kit (Hach Chemical, Loveland, CO). Ranges for water quality over the experiment are shown in Table 6-1.

Blood Handling

Fish were anesthetized with tricaine methanesulfonate (MS-222 at 0.25 g/L, Sigma Chemical Co., Saint Louis, MO) and bled, blood was handled as described previously (Hrubec et al., companion paper). A brief summary of the blood handling follows. Whole blood with ethylenediamine tetraacetic acid (EDTA) as an anticoagulant and serum were collected. The EDTA treated blood was used for the following hematological determinations: packed cell volume (PCV), plasma protein, hemoglobin, erythrocyte, leukocyte, thrombocyte and differential white cell counts. The erythrocyte indices of mean cell volume (MCV), mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) were calculated by standard formulas (Blaxhall and Daisley, 1973). The serum was used to determine the following: total protein, albumin, creatinine, total bilirubin, alkaline phosphatase (ALP), aspartate aminotransferase (AST), glucose, cholesterol, sodium, chloride, potassium, calcium, magnesium, and phosphorus. Globulin was calculated from the total protein minus the albumin. Cortisol was determined with a commercial enzyme-linked immunosorbent assay (Milenia Kinetic EIA, Diagnostic Products Corp., Los Angeles, CA).

Sampling Schedule

The fish were maintained at their experimental conditions for four weeks prior to sampling. Four fish from each water quality were sampled every 4 days for two weeks, and then every 14 days for 8 weeks, for a total of 8 sampling times. Fish were only sampled 6 times in the nitrate tank due to mortalities. Serum chemistries were determined on serum pooled from the four fish in each group at the sampling times. The PCV, plasma protein and hemoglobin were determined for 24 fish in the control and ammonia groups and 16 fish in the nitrate group; cell counts and differential were determined for 12 fish in each group.

Statistical Analysis

One-way analysis of variance (ANOVA) was used to determine differences in the blood analytes between the water quality groups using commercial statistical software (Statistix Version 4, Analytical Software, Tallahassee, FL). When a significant difference was detected ($P < 0.05$), the means were compared with a Tukey's means comparison test.

Development of Reference Intervals

Reference intervals were determined previously for sunshine bass under different culture conditions (Hrubec et al.)

following the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS, 1992). The guidelines state that reference intervals should be as broad as possible, but still maintain the ability to detect pathologic changes. Therefore, when an analyte was not statistically different between the different culture systems, a composite interval giving the broadest range was used as the reference interval for this study. The water temperature range of the fish used to determine the reference interval in the previous study was 24 - 28°C.

RESULTS

The hematology results for the fish in the different water qualities are shown in Table 6-2. Only two of the hematologic values were affected by ammonia or nitrate. Neutrophil and monocyte counts were slightly elevated in the ammonia group and elevated to a greater extent in the nitrate group. The PCV was different between the ammonia and nitrate groups, but neither value was significantly different ($P < 0.05$) from the control. There were increased numbers of reticulocytes in the nitrate exposed fish. In control fish, there were 1 to 2 reticulocytes per microscope

field under oil immersion, while in the elevated nitrate group there were 5 to 6 reticulocytes per field.

The serum chemistry results are shown in Table 6-3. Total protein, albumin, globulin, ALP, glucose, calcium and phosphorus were not affected by elevated ammonia or nitrate conditions. The nitrate treated fish had higher creatinine and potassium levels and lower total bilirubin, AST and cholesterol. Cortisol, sodium and chloride were slightly decreased in fish from the ammonia group, and decreased to a greater extent in the nitrate group. Serum magnesium was higher in the ammonia group and lower in the nitrate group, but neither were significantly different from the control.

When the hematology values for sunshine bass from the different water qualities were compared to the reference intervals developed for sunshine bass (Table 6-4), most of the values were within the reference intervals. The MCV for control and nitrate groups was slightly lower than the reference interval, while the erythrocyte count was slightly higher in the control and ammonia groups. The thrombocyte-like-cell counts were lower than the reference interval for the ammonia and nitrate groups.

A comparison of serum chemistry values with the established

reference intervals is shown in Table 6-5. Total protein, albumin, globulin, ALP and phosphorus were higher, and potassium lower than the reference interval for all groups including the control. Fish in the control group also had sodium, chloride and magnesium levels higher than the reference interval. In the ammonia group, sodium and magnesium were higher than the reference interval. In the nitrate group, creatinine and calcium were higher and chloride lower than the reference intervals.

To check variation between the fish used for this water quality experiment and fish used in the previous temperature study (see companion paper), data from the control fish were compared with data from fish maintained at 24°C in the temperature study. Analysis of variance demonstrated no difference between the two groups of fish for all analytes except monocyte and eosinophil counts, cholesterol and cortisol. The monocyte count was higher (1739 vs. 810 / μ L) and the eosinophil count was lower (77 vs. 863 / μ L) in the water quality group as compared to the 24°C temperature group. The cortisol was lower (22 vs. 35 μ g/dL) and the cholesterol was higher (228 vs. 203 mg/dL) in the control water quality group compared to the 24°C temperature fish. The differences in the hematology and serum chemistry values

between the two tanks, however, were not outside the reference interval for these analytes (Tables 6-4 and 6-5) and probably indicate normal individual variation.

The fish in the elevated nitrate group appeared to become blind three weeks into the experiment, one week after the nitrate concentration reached 200 mg/L. The fish would often swim into objects and the walls of the tank. The skin color became darker, but there were no obvious gross lesions or changes to the eyes. After seven weeks at the elevated nitrate level, the fish began to die. Each day approximately 3% of the fish were found moribund, swimming weekly upside down or lying on the bottom. About 50% of the moribund fish were icteric, with a yellow color around the operculum, gills, ventral abdomen and in the viscera and fat. The other moribund fish showed no clinical signs. The icteric fish were anemic with a packed cell volume (PCV) of 20 - 25%, while the other moribund fish had a PCV of 30 - 45%. The serum chemistry profile of icteric fish demonstrated an elevated creatinine (1.8 mg/dL), total bilirubin (2.5 mg/dL) and AST (585 mU/mL) levels, and a decreased sodium (114 mEq/L). Histopathological changes included inflammation of the gills, and lamellar hyperplasia and fusion. The spleen was hypocellular with erythrocytic and lymphoid depletion, and multifocal necrosis. The liver

was vacuolated with necrotic foci and the head kidney was depleted of lymphoid tissue. The posterior kidney was edematous with dilated tubules; and there was mineralization and necrosis of the tubules. Bacterial isolation from the posterior kidney and parasite examination were negative. All fish in the elevated nitrate group were dead by 14 weeks. Blood from moribund fish was not included in the hematological or serum chemistry determinations for this study. Examination of the sodium nitrate used to elevate nitrate levels in the water revealed no analytical evidence of contamination.

DISCUSSION

Reference intervals are the range of individual variation expected in a normal healthy population. In this study, there were analytes which were significantly different from controls, but did not deviate outside the range of the reference intervals. These include chloride levels in the elevated ammonia group, and neutrophil and monocyte counts, total bilirubin, AST, cortisol, cholesterol and magnesium in the elevated nitrate group. These differences, although statistically significant, are probably not clinically significant as the values were within the range expected in

a normal population. The MCV, erythrocyte and thrombocyte-like-cell counts, total protein, albumin, globulin, ALP, sodium, potassium, phosphorus and magnesium levels for all groups of fish, and the chloride level for control fish were slightly outside the reference interval. These differences were most likely due to the reference interval being too narrow. The reference interval was established for sunshine bass in a relatively uniform environment and may exclude normal individual variation seen in fish from slightly different environments. Reference intervals should be as broad as possible, but still maintain the ability to detect pathologic changes. Until it is known how the hematologic and serum chemistry values change with pathologic conditions it cannot be determined if these minor differences seen will mask pathologic changes.

Ammonia

Monitoring water quality is an important part of fish husbandry. The primary nitrogenous excretory product in fishes is ammonia. Most teleosts, including bass, lack the enzymes needed to synthesize urea or uric acid (Mommensen and Walsh, 1989). Ammonia is highly toxic to fishes and can reach lethal concentrations in all culture systems. Ammonia toxicity is thought to occur from osmoregulatory imbalance causing renal failure, gill epithelial damage leading to

suffocation, and suppressed excretion of endogenous ammonia resulting in neurological and cytological failure (Ruffier et al., 1981). Ammonia toxicity has been reviewed by Meade (1985). Unfortunately, there is disagreement about safe levels of ammonia for fishes. In one study, gill hyperplasia occurred at 0.005 mg/L unionized ammonia, while in another study levels up to 0.3 mg/L did not cause gill hyperplasia (Meade, 1985). Levels of 0.05 - 0.2 mg/L unionized ammonia has been shown to reduce growth (Meade 1985). In striped bass and hybrid striped bass, the total ammonia (un-ionized plus ionized forms) 96 hour LC_{50} is 1.5-2.8 mg/L (Nicholson et al., 1990). Total ammonia concentrations of > 0.6 mg/L reduced feeding, slowed growth, caused histologic changes to the gill filaments and reduced resistance to disease (Nicholson et al., 1990). In our study, ammonia concentrations were brought to the 96 hour LC_{50} each day, however, the ammonia concentrations declined over the 24 hour period due to bacterial degradation. The fish maintained normal behavior and appetite over the course of the experiment at this dose of ammonia.

The hematology and serum chemistry profiles for fish in the elevated ammonia group were similar to controls; chloride was decreased but not outside the reference interval.

Hematological changes have been reported from increased environmental ammonia, however, the changes were slight and only evident after prolonged exposure (Meade, 1985). The PCV and hemoglobin decreased after 11 months exposure to elevated ammonia at levels of 0.067 and 0.076 mg/L. In another study, ammonium chloride injected IP at 5-9 mM/kg body weight caused significant decreases in the serum protein levels (Mugiyana and Sugano, 1991).

The effects of ammonia and nitrate on serum enzymes have been investigated using domestic waste water which has high levels of these two compounds. Domestic waste water caused increases in the SGOT (AST) and SGPT (ALT) of rainbow trout caught near the sewage outlet (Weiser and Hinterleitner, 1980). Fish with the highest serum enzymes were found where ammonia (NH₃) and the sum of nitrate and nitrite were highest. In another study, domestic waste water did not increase serum enzymes, but did cause histopathological changes in the kidney (heavy hyaline droplet degeneration of the tubular cells of the proximal segment) (Bucher and Hofer, 1990). These lesions progressed to necrosis of the kidney tubules and hepatocytes.

Nitrate

Nitrate is generally considered non-toxic to fishes (Boyd,

1990). In most aquaculture systems, nitrate levels are generally below 50 mg/L, but in intensive recirculation systems nitrate levels often exceed 100 mg/L. Bonn (1976) reported that adult striped bass tolerated nitrate levels up to 800 mg/L, while fry showed signs of stress at 200 mg/L, however, both adult and fry fed and grew better at levels below 38 mg/L. Unfortunately, the original study was not referenced; and the duration of exposure and clinical signs are not known. There is no information available on the nitrate tolerance levels for striped bass hybrids such as sunshine or palmetto bass.

The only serum components in the fish from the nitrate group which deviated from the reference intervals were creatinine (higher than controls) and chloride (lower than controls). In mammals, elevated creatinine is indicative of impaired renal function. In fishes, creatinine is excreted by the kidneys, however, it is not known if blood levels become elevated with impaired renal function (McDonald and Milligan, 1992). The elevated creatinine levels observed in fish with renal pathology demonstrate that creatinine may be an indicator of renal dysfunction in fishes as well.

A severe hypochloremia has been demonstrated in response to handling stress (Tomasso et al., 1980) and a mild

hypochloremia occurs with gill injury (Byrne et al., 1989). The hypochloremia is due to increased loss of chloride across the gills. Whether the hypochloremia demonstrated in the nitrate exposed fish is in response to stress, gill damage or both is unclear. A possible explanation for the hypochloremia is that chloride moves out of the fish to balance the sodium added to the water as sodium nitrate. If the hypochloremia was due to ionic imbalance between the fish and the water, one might expect a hypernatremia from the elevated sodium in the water. When acclimated to salt water, striped bass (Courtois, 1976), milkfish (Bhaskar and Rao, 1989) and red tilapia (LeaMaster et al., 1990) had higher blood sodium levels than in the fresh water. Higher sodium levels were not observed in the nitrate treated fish, however, it is possible that sunshine bass can regulate sodium influx more efficiently than chloride efflux.

The changes in the creatinine and chloride may be a pathophysiologic response of sunshine bass to elevated nitrates. Similar hematological and serum chemistry changes (increased creatinine, hypochloremia, and reticulocytosis) were also seen in sunshine bass raised in a high density recirculating system where the nitrate level was 95 - 202 mg/L (Hrubec et al.).

The cause of the clinical signs and mortality in the nitrate treated fish is unclear. Some fish suffered from a hemolytic crisis, becoming anemic and icteric prior to death, but whether this was a direct result of exposure to nitrate is not known. There is a report of jaundice in cultured eels associated with a hemolytic crisis, although, excessive destruction of erythrocytes was not the primary cause of jaundice as the bilirubin was mainly conjugated (Endo et al., 1992). The cause of the cholestasis in eels was not determined, but based on histologic changes, the authors felt there was an intrahepatic disorder. The water quality parameters in this report were not mentioned, so it is not known if the nitrate levels were elevated.

In mammals the stress response is well characterized. There are three stages; an initial alarm phase, a stage of resistance and a final stage of exhaustion. Exhaustion is reached if the stress is sufficiently prolonged or severe, and is characterized by decreased levels of cortisol, depletion of liver glycogen, immunosuppression and other changes reducing the survivorship of the organism. This progression has been demonstrated in fish when exposed long term to environmental pollutants (Hontela et al., 1992). Fish from polluted sites had decreased cortisol and atrophied pituitaries, indicating an exhaustion of the

cortisol producing system. Hematologic changes associated with stress include a leukopenia characterized by a lymphopenia, and a neutrophilia (Ellsaesser and Clem, 1986, 1987a). Serum chemistry changes include elevated protein, cortisol and glucose levels, and decreases in sodium, chloride and calcium from increased permeability of the gills (McDonald and Milligan, 1992). We did not find any significant changes in the hematological profile of ammonia or nitrate treated fish. The serum chemistry profiles were also not indicative of a stress response as glucose and cortisol levels were not elevated. It is possible that the fish had reached an exhausted state and were unable to elevate glucose and cortisol levels, however, both values were well within the reference interval. Thus it is unlikely that the changes seen in the nitrate treated fish were stress induced.

To summarize, the hematology and serum chemistry of sunshine bass was not affected by elevated ammonia levels up to 0.15 mg/L un-ionized ammonia. The values from the ammonia exposed fish which were outside the reference interval, were not different from controls. Fish from the elevated nitrate group had hematology and serum chemistry profiles characterized by an increased creatinine level, a hypochloremia and a reticulocytosis. The observed changes

in the hematology and serum chemistry of nitrate exposed fish have also been observed in cultured fish with elevated nitrate levels (Hrubec et al.). These changes are most likely a pathological response to the elevated nitrate and are not a generalized stress response. As in the ammonia group, some values for the nitrate treated fish were only deviated slightly from the reference interval and were not significantly different from controls. These values with only slight deviation were most likely an indication of normal variation within fish. The reference intervals were determined on fish from a uniform environment and may not be sufficiently broad to apply to all fish. The deviations were slight enough, however, that minor adjustments to accommodate fish from slightly different environments should make the reference intervals applicable to most sunshine bass.

LITERATURE CITED

- Bentinck-Smith J., Bealeau H. M., Waterstart P., Tucker C. S., Stiles F., Bowser R. P. and Brown L. A. (1987) Biochemical reference ranges for commercially reared channel catfish. *Prog. Fish-Cult.* **49**:108-114.
- Bhaskar B. R. and Rao K. S. (1989) Influence of environmental variables on hematology, and compendium of normal hematological ranges of milkfish, Chanos chanos (Forsk.) in brackish culture. *Aquaculture* **83**:123-136.
- Blaxhall P. C. and Daisley K. W. (1973) Routine haematological methods for use with fish blood. *J. Fish Biol.* **5**:771-781.
- Bonn E. W., Bailey W. M., Bayless J. D., Erickson K. E. and Stevens R. E. (1976) *Guidelines for striped bass culture*. Striped Bass Committee, Southern Division, American Fisheries Society, Bethesda, Maryland.
- Boyd, C. E. (1990) *Water Quality for Ponds in Aquaculture*. Birmingham Publishing Co. Birmingham, Alabama.
- Brown C. L., Young G., Nishioka R. S. and Bern H. A. (1987) Preliminary report on the physiological status of striped bass in the Carginez Strait die-off. *Fish. Res.* **6**:5-16.
- Bucher F. (1990) Organ patterns and natural fluctuations of blood enzymes of rainbow trout (Salmo gairdneri Rich.) *Comp. Biochem. Physiol.* **96B**:795-799.
- Bucher F. and Hofer R. (1990) Effects of domestic waste water on the serum enzyme activities of brown trout (Salmo trutta). *Comp. Biochem. Physiol.* **97C**:381-385.

- Byrne P., Speare D. and Ferguson H. W. (1989) Effects of a cationic detergent on the gills and blood chemistry of rainbow trout Salmo gairdneri. *Dis. Aquat. Org.* **6**:185-196.
- Courtois L. A. (1975) Blood and serum analysis of adult striped bass, Morone saxatilis, captured in the Sacramento River. *Calif. Fish and Game* **61**:245-246.
- Courtois L. A. (1976) Hematology of juvenile striped bass, Morone saxatilis (Walbaum), acclimated to different environmental conditions. *Comp. Biochem. Physiol.* **54A**:221-223.
- Davis K. B., Parker N. C. and Suttle M. A. (1982) Plasma corticosteroids and chlorides in striped bass exposed to tricaine methanesulfonate, quinaldine, etomidate, and salt. *Prog. Fish-Cult.* **44**:205-207.
- Ellsaesser C. F. and Clem L. W. (1986) Hematological and immunological changes in channel catfish stressed by handling and transport. *J. Fish Biol.* **28**:511-521.
- Ellsaesser C. F. and Clem L. W. (1987a) Cortisol-induced hematologic and immunologic changes in channel catfish (Ictalurus punctatus). *Comp. Biochem. Physiol.* **87A**:405-408.
- Endo M., Sakai T., Yamaguchi T. and Nakajima H. (1992) Pathology of jaundice in the cultured eel Anguilla japonica. *Aquaculture* **103**:1-7.
- Hontela A., Rasmussen J. B., Audet C. and Chevalier G. (1992) Impaired cortisol stress response in fish from environments polluted by PAHs, PCBs, and mercury. *Arch. Environ. Contam. Toxicol.* **22**:278-283.

- Hrubec T. C., Smith S. A., Feldman B., Veit H. P., Libey G., Tinker M. K., Robertson J. L. Hematologic reference intervals for hybrid striped bass: Comparisons between culture system and type of hybrid. Submitted to *J. Aquat. Animal Health* 1994.
- Hrubec T. C., Smith S. A., Feldman B., Veit H. P., Libey G., Tinker M. K., Robertson J. L. Serum chemistry reference intervals for sunshine bass (Morone chrysops X Morone saxatilis): A comparison of different culture systems. Submitted to *J. Aquat. Animal Health* 1994.
- Hunn J. B. and Greer I. E. (1990) Colorimetric and refractometer estimates of total plasma protein in striped bass, Morone saxatilis (Walbaum). *J. Fish Biol.* **36**:617-618.
- Kjartansson H., Fivelstad S., Thomassen J. M. and Smith M. J. (1988) Effects of different stocking densities on physiological parameters and growth of adult Atlantic salmon (Salmo salar L.) reared in circular tanks. *Aquaculture* **73**:261-274.
- Lane H. C. (1979) Progressive changes in hematology and tissue water of sexually mature trout, Salmo gairdneri Richardson during the autumn and winter. *J. Fish Biology* **15**:425-436.
- LeaMaster B. R., Brock J. A, Fugioka R.S. and Nakanura R. M. (1990) Hematologic and blood chemistry values for Sarotherodon melanocheilus and a red hybrid tilapia in fresh water and seawater. *Comp. Biochem. Physiol* **97A**:523-529.
- Lochmiller R. L. Weichman J. D. and Zale A. V. (1989) Hematological assessment of temperature and oxygen stress in a reservoir population of striped bass (Morone saxatilis). *Comp. Biochem. Physiol.* **93A**:535-541.

- McDonald D. G. and Milligan C. L. (1992) Chemical Properties of Blood. In: *Fish Physiology* (Edited by Hoar W. S., Randall D. J. and Farrell A. P.), Vol. 12 Part B, pp. 56-135. Academic Press, New York.
- Meade J. W. (1985) Allowable ammonia for fish culture. *Prog. Fish. Cult.* **47**:135-145.
- Mommsen T. P. and Walsh P. J. (1989) Evolution of urea synthesis in vertebrates: the piscine connection. *Science* **243**:72-75.
- Mugiya Y. and Sugano K. (1991) Short- and long-term effects of ammonium chloride on RNA, DNA and protein concentrations in muscle and serum in the rainbow trout, *Oncorhynchus mykiss*. *Comp Biochem. Physiol.* **99B**:899-902.
- NCCLS (National Committee for Clinical Laboratory Standards) (1992) How to define, determine, and utilize reference intervals in the clinical laboratory; proposed guidelines. *NCCLS Document C28-P* (ISBN 1-56238-143-1). NCCLS, Villanova, Pennsylvania.
- Nicholson L. C., Woods L. C. and Woiwode J. G. (1990) Intensive culture techniques for the striped bass and its hybrids. In: *Culture and Propagation of Striped Bass and its Hybrids* (Edited by Harrell R. M., Kerby J. H. and Minton R. V.), pp. 141-158. American Fisheries Society, Bethesda, MD.
- Ruffier P. J., Boyle W. C. and Kleinschmidt J. (1981) Short-term acute bioassays to evaluate ammonia toxicity and effluent standards. *J. Water Pollut. Control Fed.* **53**:367-377.
- Tisa M. S. and Strange R. J. (1983) Hematology of striped bass in fresh water. *Prog. Fish-Cult.* **45**:41-44.

- Tomasso J. R., Davis K. B. and Parker N. C. (1980) Plasma corticosteroid and electrolyte dynamics of hybrid striped bass (white bass X striped bass) during netting and hauling. *Proc. World Maricult. Soc.* **11**:303-310.
- Warner M. C. and Williams R. W. (1977) Comparison between serum values of pond and intensive raceway cultured channel catfish Ictalurus punctatus (Rafinesque). *J. Fish Biol.* **11**:385-391.
- Westin D. T. (1978) Serum and blood from adult striped bass, Morone saxatilis. *Estuaries* **1**:126-128.
- Weiser W. and Hinterleitner S. (1980) Serum enzymes in rainbow trout as tools in the diagnosis of water quality. *Bull. Environ. Contam. Toxicol.* **25**:188-193.

Table 6-1. Range of water quality parameters.

PARAMETER	CONTROL	AMMONIA ¹	NITRATE ¹
Temperature (°C)	24-25	24-25	24-25
pH	7.4-7.9	7.5-7.9	7.5-8.0
Ammonia (NH ₃ mg/L)	0-0.011	0-0.150	0-0.011
Nitrite (NO ₂ -N mg/L)	0-0.03	0-0.06	0-0.20
Nitrate (NO ₃ -N mg/L)	2-8	4-16	180-220
Alkalinity (mg/L)	68-86	68-86	68-86
Hardness (mg/L)	86-171	103-171	103-188
Dissolved Oxygen (mg/L)	6-7	6-7	7-8

1) Ammonium chloride and sodium nitrate were added to the respective tanks to elevate these water quality parameters.

Table 6-2. Hematology values for fish in different water qualities.

ANALYTE	N ¹	CONTROL		AMMONIA		NITRATE	
PCV ³ %	24/16	38	a, b ²	40	a	36	b
Plasma protein (mg/dL)	24/16	6.1	a	6.1	a	5.8	a
Hemoglobin (g/dL)	24/14	10.7	a	10.8	a	9.5	a
MCV ⁴ (fL)	12	78.9	a	84.0	a	80.0	a
MCH ⁵ (pg)	12	25.3	a	25.1	a	21.9	a
MCHC ⁶ (g/dL)	24/14	27.9	a	27.0	a	26.7	a
Erythrocytes (x10 ⁶ /μL)	12	5.00	a	5.03	a	4.56	a
Leukocytes (x10 ³ /μL)	12	60.38	a	65.06	a	80.00	a
Lymphocytes (x10 ³ /μL)							
Small	12	23.31	a	24.48	a	32.96	a
Large	12	2.19	a	2.69	a	3.38	a
Neutrophils (x10 ³ /μL)	12	0.49	a	1.37	a, b	2.47	b
Monocytes (x10 ³ /μL)	12	1.74	a	2.74	a, b	4.96	b
Eosinophils (x10 ³ /μL)	12	0.08	a	0.06	a	0.10	a
TLC ⁷ (x10 ³ /μL)	12	0.71	a	0.40	a	0.44	a
Thrombocytes (x10 ³ /μL)	12	72.20	a	73.26	a	73.96	a

1) Number of fish/number of nitrate treated fish, when different. 2) Similar letters indicate statistically (P < 0.05) similar groups. 3) Packed cell volume. 4) Mean cell volume. 5) Mean cell hemoglobin. 6) Mean cell hemoglobin concentration. 7) Thrombocyte-like-cell.

Table 6-3. Serum chemistry values for fish in different water qualities.

ANALYTE	N ¹	CONTROL	AMMONIA	NITRATE
Total Protein (g/dL)	8/6	4.1	4.2	4.1
Albumin (g/dL)	8/6	1.6	1.6	1.6
Globulin (g/dL)	8/6	2.6	2.6	2.6
Creatinine (mg/dL)	8/6	0.2	0.2	0.9
Total Bilirubin (mg/dL)	8/6	0.17	0.19	0.02
ALP ³ (mU/mL)	8/6	82	78	82
AST ⁴ (mU/mL)	8/6	35	42	6
Glucose (mg/dL)	8/6	132	111	109
Cortisol (μg/dL)	8/6	22.1	16.7	10.6
Cholesterol (mg/dL)	8/6	228	217	181
Sodium (mEq/L)	8/6	170	166	158
Potassium (mEq/L)	8/6	1.2	1.3	2.4
Chloride (mEq/L)	8/6	153	143	53
Calcium (mg/dL)	8/6	11.84	11.95	12.20
Magnesium (mg/dL)	8/6	2.6	2.7	2.2
Phosphorus (mg/dL)	8/6	11.9	12.1	11.0

1) Number of samples/number of nitrate treated samples. Each sample pooled from 4 fish. 2) Similar letters indicate statistically (P < 0.05) similar groups. 3) Alkaline phosphatase. 4) Aspartate aminotransferase (SGOT).

Table 6-4. Hematology values at different water qualities compared with reference intervals developed for sunshine bass in tanks.

ANALYTE	REFERENCE ¹ INTERVAL	CONTROL	AMMONIA	NITRATE
PCV ² %	34-47	*	*	*
Plasma protein (mg/dL)	5.1-6.5	*	*	*
Hemoglobin (g/dL)	8.0-12.0	*	*	*
MCV ³ (fL)	81-106	79	*	80
MCH ⁴ (pg)	19.6-26.4	*	*	*
MCHC ⁵ (g/dL)	22-30	*	*	*
Erythrocytes (x10 ⁶ /μL)	3.66-4.96	5.00	5.03	*
Leukocytes (x10 ³ /μL)	32.6-118.2	*	*	*
Lymphocytes (x10 ³ /μL)				
Small	9.8-54.1	*	*	*
Large	0.5-6.9	*	*	*
Neutrophils (x10 ³ /μL)	0-4.0	*	*	*
Monocytes (x10 ³ /μL)	0.8-5.2	*	*	*
Eosinophils (x10 ³ /μL)	0-1.3	*	*	*
TLC ⁶ (x10 ³ /μL)	0.6-3.7	*	0.4	0.44
Thrombocytes (x10 ³ /μL)	21.3-104.3	*	*	*

An * indicates that the value falls within the established reference interval. 1) Hrubec et al. 2) control water quality tank. 3) Packed cell volume. 4) Mean cell volume. 5) Mean cell hemoglobin. 6) Mean cell hemoglobin concentration. 7) Thrombocyte-like-cell.

Table 6-5. Serum chemistry values at different water qualities compared with reference intervals developed for sunshine bass in tanks.

ANALYTE	REFERENCE ¹ INTERVAL	CONTROL	AMMONIA	NITRATE
Total Protein (g/dL)	3.2-3.9	4.1	4.2	4.1
Albumin (g/dL)	1.2-1.5	1.6	1.6	1.6
Globulin (g/dL)	2.0-2.5	2.6	2.6	2.6
Creatinine (mg/dL)	0.1-0.3	*	*	0.9
Total Bilirubin (mg/dL)	0-0.4	*	*	*
ALP ² (mU/mL)	46-73	82	78	82
AST ³ (mU/mL)	0-72	*	*	*
Glucose (mg/dL)	69-171	*	*	*
Cortisol (µg/dL)	4.3-53.8	*	*	*
Cholesterol (mg/dL)	164-285	*	*	*
Sodium (mEq/L)	153-165	170	166	*
Potassium (mEq/L)	2.4-3.7	1.2	1.3	2.4
Chloride (mEq/L)	137-148	153	*	53
Calcium (mg/dL)	10.35-12.07	*	*	12.2
Magnesium (mg/dL)	1.9-2.4	2.6	2.7	*
Phosphorus (mg/dL)	6.3-8.1	11.9	12.1	11.0

An * indicates that the value falls within the established reference interval. 1) Hrubec et al. 2) Alkaline phosphatase. 3) Aspartate aminotransferase (SGOT).

CHAPTER 7

SUMMARY

In summary, the kinetics of the humoral immune response in sunshine bass were affected by temperature, being slower and of less magnitude at 10 and 18°C than at warmer temperatures. Temperatures elevated above optimal and elevated ammonia levels did not inhibit antibody production, while elevated nitrate levels inhibited antibody production to the same extent as 18°C. It is not known if antibody production was inhibited directly by nitrate, or whether the fish were compromised physiologically and unable to mount a full response.

Hematologic reference intervals were different for sunshine in different production systems and between sunshine and palmetto bass. A greater number of analytes were different between sunshine bass in the two production systems than between the two types of hybrid. Specifically, leukocyte, lymphocyte, monocyte and neutrophil counts were higher in sunshine bass from recirculating systems than either hybrid

in tanks. Palmetto bass were different from sunshine bass with a lower PCV and hemoglobin, and more neutrophils than monocytes, while sunshine bass had more monocytes than neutrophils.

Blood chemistry reference intervals were different for sunshine bass in different culture systems. Fish in recirculating systems had higher levels of total protein, albumin, globulin, creatinine and phosphorus and lower levels of chloride than fish from tanks or cages. Other analytes were statistically different between fish in the three production systems but the differences were slight and may be due to influences such as water quality and diet. The fish sampled were from relatively uniform populations in each production system and the reference intervals may need to be broadened to encompass the individual variation seen in more diverse populations.

Temperature and water quality may be responsible for some of the differences seen in reference intervals for fish from various production systems. Total leukocyte, small lymphocyte, and monocyte counts were lower and outside the reference intervals at 10°C; and calcium levels were lower and glucose levels higher at 10 and 18°C. Separate

reference intervals should be developed for fish at these temperatures. Elevated ammonia did not significantly affect the reference interval. Analytes from the ammonia treated fish were either within the reference intervals or similar to analytes from control fish. Elevated nitrate on the other hand, induced a reticulocytosis, elevated creatinine level and a hypochloremia. These changes were also observed in fish from the recirculating system where the nitrate levels were elevated. This reaction to elevated nitrate may be responsible for some of the differences seen in reference intervals between fish from different culture systems.

CHAPTER 8

FUTURE CONSIDERATIONS

One of the advantages of working with an animal that has not been studied previously, is the many opportunities for future research. The work in this dissertation collectively serves as a foundation to generate a body of work on the immunology and clinical hematology of sunshine bass. Developing this knowledge base is critical for future vaccine development, modification of the immune response, development of therapeutic drugs, and building clinical pathology into a tool usable with fishes.

The first area which needs to be addressed is further general information on the immune response, hematology and serum chemistry. My dissertation only addresses the humoral immune response. A logical next step is to examine different aspects of cell mediated immunity. Tests have been developed for fish such as scale graft rejection, the mixed leukocyte response, and delayed-type hypersensitivity that measure cell mediated response, but have not been used with sunshine or striped bass. Temperature and water

quality studies, similar to ones for characterizing humoral response should be conducted with cell mediated response. Additionally, characterizing changes in both humoral and cell mediated immunity with developmental stage and age will provide additional baseline data. I am currently developing a test for cell mediated immunity and investigating developmental changes in immune function through a grant from the Virginia Seagrant Program.

Developing hematology and serum chemistry into a diagnostic tool requires further basic studies. The effects of water hardness, alkalinity and nitrite on the hematology and serum chemistry still need to be conducted to complete investigation of routine water parameters. Then, work on determining specific hematological and serum chemistry changes associated with specific diseases should be conducted.

Further studies are needed to examine nitrate toxicity in more depth. The severe pathologic changes associated with elevated nitrate exposure were completely unexpected as nitrates are generally considered to be nontoxic. Future studies should examine the effects of different sources and concentrations of nitrate.

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Hrubec, T.C., J.L. Robertson, and S.A. Smith. The effects of temperature on the hematology and serum chemistry of sunshine bass (*Morone chrysops* X *Morone saxatilis*). Submitted to *Comparative Biochemistry and Physiology* 1994.

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Hrubec, T.C., J.L. Robertson, S.A. Smith, and M.K. Tinker 1995. The effect of temperature and water quality on antibody response to *Aeromonas salmonicida* in sunshine bass (*Morone chrysops* X *Morone saxatilis*). *Veterinary Immunology and Immunopathology*, in press.

T. Caceci, T.C. Hrubec 1990. Histology and Ultrastructure of the gut of the Black Mollie (*Poecilia* spp.), a Hybrid Teleost. *The Journal of Morphology* 204, 265-280.

T. Caceci, T.C. Hrubec 1989. Ultrastructure of the Digestive Tube of the Black Mollie. In: *Proceedings of the 47th Annual Meeting of the Electron Microscopy Society of America*, Ed. G.W. Bailey pp. 914-915.

T.J. Buckhout, T.C. Hrubec 1986. Pyridine Nucleotide-Dependent Ferricyanide Reduction Associated with Isolated Plasma Membranes of Maize (*Zea mays* L.) Roots. *Protoplasma* 135, 144-154.

T.C. Hrubec, J.M. Robinson, R.P. Donaldson 1985. Effects of CO₂ Enrichment and Carbohydrate Content on the Dark Respiration of Soybeans. *Plant Physiology* 79, 684-689.

T.C. Hrubec, J.M. Robinson, R.P. Donaldson 1985. Isolation of Mitochondria from Soybean Leaves on Discontinuous Percol Gradients. *Plant Physiology* 77, 1010-1012.

Presentations and Posters:

Hrubec, T.C., S.A. Smith, J.L. Robertson, H.P. Veit, and G. Libey. (1994) Comparison of the hematologic reference intervals for cultured hybrid striped bass. International Symposium on Aquatic Animal Health. Seattle, WA, Sept. 4-8, 1994.

T.C. Hrubec, S.A. Smith, J.L. Robertson, B. Feldman, M. Tinker, G. Libey, H.P. Veit. (1994). A Comparison of the Hematologic Reference Intervals for Hybrid Striped Bass. Presented at the Eastern Fish Health Workshop, May 25 - 28, 1994. Blacksburg, Virginia.

T.C. Hrubec, S.A. Smith, J.L. Robertson, B. Feldman, M. Tinker, G. Libey, H.P. Veit. (1994). Blood Chemistry Reference intervals for Hybrid Striped Bass Under Different Culture Conditions. Presented at the Eastern Fish Health Workshop, May 25 - 28, 1994. Blacksburg, Virginia.

S.A. Smith, P.E. McAllister, T.C. Hrubec, H.P. Veit. (1993). Survey of Endemic Diseases of Cultured Rainbow Trout in Virginia, U.S.A. Presented at the Aquaculture Canada 93 meeting August 1993, Prince Edward Island, Canada.

T.C. Hrubec, S.A. Smith, G. Libey, H.P. Veit. (1993). Serum Chemistry Values for Hybrid Striped Bass Raised in a Recirculating Culture System. Presented at the Fifth Annual Research Day Symposium. May 24 - 25, 1993. VA-MD Regional College of Veterinary Medicine, Blacksburg, Virginia.

T.C. Hrubec, S.A. Smith, J.L. Robertson. (1993). The Ontogeny of the Immune System in Hybrid Striped Bass. Presented at the 9th Annual Graduate Student Research Symposium. March 30, 1993. Virginia Tech, Blacksburg Virginia.

T. Caceci, T.C. Hrubec. (1992). Histology and Ultrastructure of the Gall Bladder of the Black Mollie, *Poecilia* spp. Presented at the American Association of Veterinary Anatomists. August 5 - 7, 1992. Boston Massachusetts.

T.C. Hrubec, S.A. Smith, H. Suliman, H.P. Veit, G. Libey. (1992). Normal Hematological Values for Hybrid Striped Bass Raised in Recirculating Systems. Presented at the Eastern Fish Health and American Fisheries Society Fish Health Section Workshop. June 16 - 19, 1992. Auburn, Alabama.

T.C. Hrubec, J.M. Robinson, R.P. Donaldson (1984). Effect of CO₂ Enrichment on Soybean Leaf and Mitochondrial Respiration. Presented at the Annual Meeting of the American Society of Plant Physiologists August 12-17, 1984. Plant Physiology Supplement 75, 158.

Professional Societies:

American Association for the Advancement of Science
American Veterinary Medical Association
American Fisheries Society - fish health section
International Association of Aquatic Animal Medicine
Sigma Xi
Phi Zeta

Awards and Scholarships:

Best Graduate Student Presentation-Basic Science Category,
Fifth Annual Research Day 1993.
Elanor Watt Scholarship: Welfare of Animals in Disease
Research and Surgery 1990.
Board of Trustees Scholarship, GW University, 1980.
David Lloyd Kreeger Prize for Sculpture 1979.