ABSTRACT

Johnson, Andrea Karen. Assessment of the Health Status of Atlantic Menhaden (Brevoortia tyrannus). (Under the direction of Jay Levine and J McHugh Law.)

In 2002, Atlantic menhaden were collected from the White Oak, Neuse and Pamlico river estuaries in North Carolina and the seasonal changes in health indices were assessed during that period. Indicators of tissue damage (histopathological analyses of gills, heart, liver, intestine, and anterior kidney), nutritional status and exposure to environmental stressors (liver-somatic index), immune status and disease (TGF-β mRNA production, lymphocyte mitogenesis, differential counts, hematocrit, and spleno-somatic index) were compared in Atlantic menhaden from all three river systems. The bioindicators used to assess the health of Atlantic menhaden in the estuaries of North Carolina showed that there is seasonal variability in most health indices and that this pattern is strongly influenced by temperature.

During the fall seasons, 2001 and 2002, Atlantic menhaden were collected from several creeks in the Pamlico River estuary. High lesion prevalence was associated with the Atlantic menhaden kills in the fall of 2001, while there were no fish kills in the fall of 2002, and lesion prevalence was very low. Indicators of tissue damage (histopathological analyses of gills, heart, liver, intestine, and anterior kidney), nutritional status and exposure to environmental stressors (liver-somatic index), immune status and disease (TGF-β mRNA production, lymphocyte mitogenesis, hematology, plasma chemistry, and spleno-somatic index) were compared between menhaden with and without lesions over the two years. Atlantic menhaden with ulcerative lesions had significantly higher splenic
mononuclear cell TGF-β mRNA levels, spleno-somatic indices, liver-somatic indices, neutrophil and monocyte counts, and significantly lower lymphocyte counts, thrombocyte counts, hematocrit values, plasma proteins and calcium than those without. The health indicators used in this study provided information on the non-specific and specific responses of the Atlantic menhaden immune system and overall health, thus increasing our knowledge of the changes in the health of Atlantic menhaden with and without lesions.

The immune-endocrine interaction was examined in captive Atlantic menhaden administered the synthetic glucocorticoid, triamcinolone acetonide. Its effects on Atlantic menhaden liver-somatic index, spleno-somatic index, hematology, plasma chemistry, lymphocyte mitogenesis, and splenic mononuclear cell TGF-β mRNA transcription were measured and compared to untreated fish at 48 and 96 hr post-treatment. Triamcinolone-treated Atlantic menhaden showed suppression of TGF-β mRNA production, neutrophilia, moncytosis, lymphopenia, and an increase in blood glucose levels. Knowledge of the interactions of the immune and endocrine systems provided by this study will improve our understanding of the immunodefense mechanisms of Atlantic menhaden and help us interpret some of the changes observed during the development of ulcerative lesions in wild caught Atlantic menhaden and other aquatic species.

Low dissolved oxygen (DO) induced mass mortality of Atlantic menhaden (Brevoortia tyrannus) is relatively common in North Carolina estuaries. The effects of acute and subacute exposure to low DO were evaluated in Atlantic menhaden under controlled laboratory conditions. Hematology, plasma chemistry, and spleno-somatic
indices were measured at five different oxygen saturations (5, 10, 15, 20, and 84%) in the acute study. Splenic TGF-β mRNA, lymphocyte mitogenesis, and blood parameters were measured at 20% and 84% oxygen saturation in the subacute exposure study. In both experiments glucose and electrolytes were the blood parameters most affected by hypoxic conditions. Blood glucose concentrations were elevated in both studies. Fish exposed to 5% oxygen saturation in the acute study showed signs of blood acidosis while those exposed to 20% oxygen saturation in the subacute exposure study showed signs of blood alkalosis. These effects may have consequences in estuarine systems where fish are exposed to multiple stressors, which may cumulatively affect their ability to withstand additional stressful events and resist disease.
Assessment of the Health Status of Atlantic Menhaden (*Brevoortia tyrannus*)

By

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BIOGRAPHY

Andrea Karen Johnson was born in Kingston, Jamaica. She attended Wolmer’s Girls’ School in Kingston. After graduating in 1982, she worked at the Bank of Jamaica for several years before migrating to the United States in 1988. She graduated from the Community College of Philadelphia in 1989 and received a Bachelor of Science degree in Marine Science at the University of Miami in 1991. After working at the National Marine Fisheries Service in Beaufort, North Carolina, she attended the University of South Florida in 1992 where she received a Master of Science degree in Marine Science in 1995. She received her training in fish endocrinology under the tutelage of Dr. Peter Thomas at the University of Texas in 1994 and 1995. She worked as a Marine Biologist for the Florida Marine Research Institute in St. Petersburg, Florida and the South Carolina Department of Natural Resources in Charleston, South Carolina before starting her Doctorate Degree at North Carolina State University in 1997. She transferred from the Physiology program in the Department of Zoology in 1998 to the Comparative Biomedical Sciences Program at College of Veterinary Medicine. Her graduate studies in Population Medicine have been under the direction of Drs. Jay Levine and Mac Law.
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LITERATURE REVIEW

I. THE TELEOST IMMUNE SYSTEM

Fishes make up more than half the species of living vertebrates. The Agnathans (e.g. hagfish and lampreys) are considered the least developed of fishes and the Osteichthyes (bony fishes), the most complex (Zapata et al., 1996). Like other animals, fishes have developed mechanisms for identifying and eliminating organisms that may cause disease or death. Teleosts, the largest phylogenetic group, consist of over 23,000 extant species (Helfman et al., 1997). The immune system of teleosts shares many similarities and differences to that of higher order vertebrates.

Lymphoid organs

The lymphoid organs of the fish immune system include the thymus (primary lymphoid organ), spleen, kidney and mucosa-associated lymphoid tissues (secondary lymphoid organs; Press and Evensen, 1999). The thymus is similar structurally to that of mammals (Ellis, 1989). In fish, the thymus is a paired organ that develops before other lymphoid tissues (Kennedy-Stoskopf, 1993; Press and Evensen, 1999). It is composed primarily of lymphocytes with a few macrophages and epithelial-type cells (Ellis, 1989). The spleen consists of red and white pulp as well as macrophage aggregates, important for storage, destruction, detoxification and recycling of both endogenous and exogenous materials (Vogelbein et al., 1987; Wolke, 1992; Press and Evensen, 1999). In teleosts, the anterior and posterior kidney may be separate or continuous. The posterior kidney is an excretory organ functionally similar to the mammalian kidney (Tizard, 1996). The head (anterior or cephalic) kidney lacks renal function and is an important hematopoietic organ.
It is phylogenetically related to the bone marrow of higher vertebrates (Zapata et al., 1996) and considered a lymph node analog important in the induction and elaboration of immune responses (Kaattari and Irwin, 1985). The head kidney is a major producer of antibodies and it also contains macrophage aggregates (Press and Evensen, 1999; Wolke, 1992; Ellis, 1989). Mucosa-associated lymphoid tissues of fish include the gut, skin and gills. They are exposed to the external environment and function as initial barriers to pathogens (Press and Evensen, 1999).

Fish display both innate and adaptive immune responses. The innate or non-specific immune system is highly conserved and apparent in invertebrates, while the antigen-specific adaptive immune system is more evolutionarily advanced, appearing in fishes and higher vertebrates (Ellis, 1989; Tizard, 1996). Where the innate immune response is non-specific, responding to a wide variety of agents, the adaptive immune response produces antibodies against specific pathogens and can provide life-long immunity to some pathogens (Ellis, 1989; Kennedy-Stoskopf, 1993; Secombes, 1996).

**Innate Immunity**

Elements of an innate immune system, also defined as the natural or non-specific immune response, are found in all living organisms (Tizard, 1996). Skin and mucus provide physical and chemical barriers against microbial infection as the first line of defense in teleosts (Ellis, 1989; Heath, 1995; Robertsen, 1999). Epithelia covering the gills, skin and gut secrete mucus that contains non-specific factors (Ellis, 1989; Secombes, 1996). These include inhibitors such as lectins and antiproteases, and lysins such as proteases,
C-reactive proteins, lysozyme and complement (Ellis, 1989; Ellis, 1999; Yano, 1996; Robertsen, 1999). If this first line of defense is breached, cellular mechanisms and other immunoregulatory factors are activated.

As in mammals, the entry of pathogens in the tissue of fish results in an inflammatory response. The innate cellular response in fish is driven by granulocytes (neutrophils, eosinophils, and basophils), monocytes/macrophages and nonspecific cytotoxic cells (NCC; Secombes, 1996). Granulocytes, macrophages and nonspecific cytotoxic cells are found in lymphoid tissues, blood, and the peritoneal cavity (Secombes, 1996; Zapata et al., 1996). Both granulocytes and macrophages/monocytes of fish are highly phagocytic and produce reactive oxygen species (Secombes, 1996). Nonspecific cytotoxic cells in fish are considered equivalent to mammalian natural killer (NK) cells. They play an important role in resistance to viral and parasitic infection (Secombes, 1996). Teleost eosinophilic granule cells (EGC) are considered the homolog of the mammalian mast cells. These cells protect against bacterial infection (Wedemeyer et al., 2000) and parasites (Reite, 1998). They kill pathogens directly or indirectly by activating effector cells such as neutrophils (Silphaduang and Noga, 2001).

**Adaptive Immunity**

Lymphocytes (B- and T- like lymphocytes), found in the thymus, kidney, spleen and blood of teleosts, are the main cells of the adaptive immune system in fish and their properties are similar to those of mammals (Tizard, 1996). Macrophages are involved in
antigen presentation, processing and presenting the antigen to lymphocytes (Secombes, 1996).

T lymphocytes (T cells) perform functions as cytotoxic T cells and helper T cells (Tizard, 1996). Helper T cells are activated by the interaction with the antigen and cytokines (interleukins) secreted by the macrophages (Secombes, 1996; Manning and Nakanishi, 1996). When activated T helper cells can stimulate the differentiation and proliferation of B lymphocytes (Tizard, 1996). When activated B lymphocytes (B cells) differentiate into plasma cells capable of producing specific antibodies that will kill invading micro-organisms (Tizard, 1996). Teleosts produce only one class of antibody (IgM) capable of performing functions attributed to numerous mammalian antibody classes. Fish blood vessel walls are permeable to immunoglobulins and these molecules are found in most tissue fluids, plasma, lymph and skin mucus (Tizard, 1996).

The fish immune system is regulated by exogenous factors and endogenous immunoregulatory factors such as cytokines and the endocrine system (Yano, 1996; Manning and Nakanishi, 1996). Cytokines are soluble factors that play a regulatory or enhancing role within the immune system (Manning and Nakanishi, 1996). The cytokines characterized in fish include: interleukin (IL-1β), interferons (IFN), macrophage migration-inhibition factor (MIF), macrophage-activating factor (MAF), tumor necrosis factor (TNF-α), chemokines and TGF-β (Secombes et al., 1996; Harms et al., 2000a). The interaction between the immune and neuroendocrine systems is important for maintaining homeostasis. The activation of the hypothalamus-pituitary-
interrenal axis (HPI) due to stressors (stimuli) initiates the adaptive response in organisms (Pickering, 1989). This results in a complex interaction between the immune and neuroendocrine systems and factors such as the health status of a species and its environment can influence the outcome of this interaction (Balm, 1997).

Both innate and adaptive immune systems appear to be integrated as in mammals. The defense system of teleosts includes protection by skin and mucus, destruction of errant cells and pathogens by the innate and adaptive immune systems and regulation by both endogenous and exogenous factors. These factors along with the organism’s physiology (e.g. sex, maturational status, genetics) and nutritional status are important factors affecting its health and ability to resist disease (Schreck, 1996).

II. ATLANTIC MENHADEN BIOLOGY

Atlantic menhaden (*Brevoortia tyrannus* Latrobe) (Clupeidae) are found along the Atlantic coast of the United States, from the Gulf of Maine to Florida (Reintjes, 1969). The genus *Brevoortia* consists of four North American species *B. tyrannus* (Atlantic menhaden), *B. patronus* (Gulf menhaden), *B. smithi* (yellowfin menhaden) and *B. gunteri* (finescale menhaden) (Reintjes, 1969). Members of this genus can be distinguished from other clupeids by their pectinated scales, absence of teeth (post-juvenile stage), relatively large heads and placement of the dorsal fin (between pelvic and anal fins) (Ahrenholz, 1991, Reintjes, 1969, Hildebrand, 1963). Atlantic menhaden are thought to be the most long lived (10-12 years) and largest of the genus *Brevoortia* (Ahrenholz, 1991). The menhaden fishery, which consists mainly of the large-scaled menhaden (*B. tyrannus* and
B. patronus), is one of the most economically important commercial fisheries in the United States (Rulifson and Cooper, 1986; Dryfoos et al., 1973). The menhaden fishery is North Carolina’s oldest fishery and has been in continuous operation since 1870 (Goode and Atwater, 1880, Frye, 1978). Menhaden are important to the fishing industry as a source of fish oil, fish meal and food fish for other commercially important species and as edible oil in Europe and Canada (Vaughan and Smith, 1991).

The Atlantic menhaden is an estuarine-dependent marine migratory species that forms large, dense schools (Smith, 1999a). Differential distribution of age and size groups, seasonal migration and population structure result in variation in the times and places of spawning (Higham and Nicholson, 1964). Adult Atlantic menhaden are distributed by size and age from northern Florida to Maine during the summer months. The oldest and largest fish are found as far north as southern Maine (up to age 7), while the younger and smaller fish are found in the southern half of the species range (Smith, 1999a). The northern portion of the population migrates southward in September. By December, a large portion of this northern population (north of the Chesapeake Bay) has moved into waters off the coast of North Carolina along with large numbers of juvenile menhaden. By late January, the schools of adult menhaden move from North Carolina coastal waters into oceanic waters. They reassemble in March or early April off North Carolina’s coast before moving northwards. By June, the population is redistributed from Florida to Maine (Ahrenholz, 1991).
Larval Atlantic menhaden (14-34 mm FL) are particulate omnivores (Friedland et al., 1996). In the estuaries, they metamorphose into juveniles or young-of-the year (YØY; 38-171 mm FL), obligate filter-feeding phytoplanktivores, and as adults (> 171 mm FL) they continue to filter feed but on larger food particles than those utilized by juveniles. Menhaden distribution in the estuary is thought to be defined by phytoplankton gradients (Friedland et al., 1996). The concentration of food (i.e. the distribution of phytoplankton) is the dominant factor that defines the movement and location of juvenile menhaden in the estuary while temperature is assumed to play a role when fish emigrate to the sea (Friedland et al., 1996).

Atlantic menhaden are prolific spawners. Females mature at age 2 (195-320 mm FL) and all mature by age 3 (over 200 mm FL). Atlantic menhaden can produce more than one group of ova during a single season (Higham and Nicholson, 1964). In the North Atlantic, spawning is believed to occur in the late summer and fall. There is little evidence of spawning in the Chesapeake Bay. In the South Atlantic spawning persists throughout the fall and winter months (Checkley et al., 1999). Studies in the 1880s suggested that certain schools of menhaden spawn from Florida to North Carolina (South Atlantic) and that spawning probably took place as temperature declined (Goode and Atwater, 1880). Spawning off North Carolina’s coast is believed to occur during cooler months in warm waters near the western edge of the Gulf Stream that is rich in plankton (Warlen et al., 1994). Water temperatures in these areas are usually 18°C or above (Warlen et al., 1994). Larval migration into estuaries is determined by spatial/temporal distribution of spawning and/or transport dynamics (Rice et al., 1999). In the estuaries,
juveniles move upstream and, as they mature, they move downstream and back into the open ocean. During the period spent in the estuaries, menhaden are exposed to both environmental and anthropogenic factors that potentially impact their immune function and affect their susceptibility to infection by opportunistic pathogens (e.g. fungi).

III. ULCERATIVE SKIN LESIONS IN ATLANTIC MENHADEN

Ulcerative lesions have been observed in fish in estuarine and coastal areas worldwide. In Australia (Callinan et al., 1995), Southeast Asia (Roberts et al., 1993), and Japan (Hatai et al., 1977) a variety of viral, fungal, bacterial and parasitic agents (Sindermann, 1988) have been associated with ulcerative diseases. In the United States, ulcerative lesions have been documented for many years in estuaries from Maryland to Florida (Noga and Dykstra, 1986; Noga et al., 1988; Dykstra et al., 1989; Levine et al., 1990b, Levine et al., 1990a; Kane et al., 1998; Blazer et al., 1999) and these lesions have been found in many economically important estuarine species, including Atlantic menhaden (Levine et al., 1990b). Although ulcerative lesions are found in other commercially important estuarine species and on adult Atlantic menhaden, juvenile Atlantic menhaden are the most frequently affected (Levine et al., 1990b).

Ulcerative lesions in Atlantic menhaden and other species have been observed from the Chesapeake Bay to Florida as early as 1978 (Sindermann, 1988). In 1984, outbreaks of ulcerative disease were reported in Atlantic menhaden from the Pamlico River and Albermarle Sound, North Carolina (Noga and Dykstra, 1986; Dykstra et al., 1986). Atlantic menhaden were observed with deep, necrotic ulcers and the prevalence of the
disease incidence increased during the season with a large fish kill in November 1984 (Noga and Dykstra, 1986). Lesions similar to those found in fish in North Carolina were also observed in Atlantic menhaden collected from the Peconic River, NY in 1982 (Ahrenholz et al., 1987), the Rappahannock River, Virginia in 1984 (Hargis, 1985) and St. Johns River estuary in Florida (Grier and Quintero, 1987; TeStrake and Lim, 1987).

These ulcerative lesions found in Atlantic menhaden were termed ulcerative mycosis (UM), because of the consistent presence of fungal hyphae within the lesions. These lesions were also called epizootic ulcerative syndrome (EUS), and are similar to lesions in species such as striped mullet (*Mugil cephalus*) and chevon snakehead (*Channa striata*) caused by *Aphanomyces invadans* in South Asia, Japan and Australia. Both *Aphanomyces* sp. and *Saprolegnia* sp. were isolated from the lesions in Atlantic menhaden collected from the Pamlico River, but *Aphanomyces* sp. was most common in lesions of Atlantic menhaden with optimum growth and sporulation within salinity ranging from 2-6 ppt (Dykstra et al., 1986; Dykstra et al., 1989). Detailed description of the development of ulcerative mycosis in Atlantic menhaden was reported by Noga et al., (1988). Ulcerative lesions termed ulcerative mycosis were described as focal ulcers (2-20 mm diameter) that were usually singular and found mainly on the ventrum of the fish near the anus. Lesions were described as early type 1 and type 2, advanced, end-stage and healed lesions. The early stages are very uncommon, consisting of a red area that may be flat in the type 1 lesion and raised in the type 2 lesion. In the advanced stage, lesions penetrate deep into the peritoneal cavity and adjacent organs. The necrotic mass of tissue becomes sloughed leaving a crater-shaped cavity in the end-stage (Noga et al.,
1988). Apparently healed lesions were uncommon and small (< 5 mm diameter) appearing as smooth, non-ulcerated areas of tissue loss (Noga et al., 1988).

Pound-net and trawl surveys in the mid 1980s showed that UM is most prevalent in the upper areas of the Pamlico River but there was no relationship observed between water quality parameters, temperature, dissolved oxygen, and pH (Noga et al., 1989). Studies in the late 1980s and early 1990s showed a correlation of low salinity with the development of UM (Noga et al., 1989; Levine et al., 1990). There was also evidence for an association with fluctuations in dissolved oxygen and pH (Noga et al., 1993a).

Many pathogens have been implicated as possible components of the causal pathway leading to the development of ulcerative lesions in Atlantic menhaden. The dinoflagellate, *Pfiesteria piscicida* Burkholder and Steidinger, first discovered unintentionally after the sudden death of tilapia (*Orechromis aureus* and *O. mossambica*) in water collected from the Pamlico River (Smith et al., 1988), was associated with a large portion of the ulcerative lesions and fish kills in Atlantic menhaden observed in the Neuse and Pamlico river estuaries (Burkholder et al., 1992; Burkholder et al., 1993) as well as the Chesapeake Bay (Lewitus et al., 1995). The ulcerative lesions in Atlantic menhaden and other estuarine species were assumed to be associated with an unidentified neurotoxin released from the dinoflagellate that is stimulated by a substance from fresh fish excreta (Burkholder et al., 1992). Fungal infections were considered secondary, opportunistic infections in the lesions formed from *Pfiesteria* toxins (Noga et al., 1996). As described by Burkholder and co-workers (1995a) the dinoflagellate is an “ambush
A "predator" that releases a toxin that kills fish and then phagocytizes the sloughed tissue of the dead or moribund fish (Burkholder et al., 1995a).

*Pfiesteria piscicida* is believed to be a generalist affecting finfish, shellfish, bacteria, small algae, microfauna, and mammalian tissue (Burkholder et al., 1993; Landsberg et al., 1995; Burkholder et al., 1995a; Burkholder et al., 1995b; Burkholder and Glasgow, 1997b; Burkholder and Glasgow, 1997a) as well as fish in aquaria and pond aquaculture systems (Noga et al., 1993b; Noga, 1998). Its life cycle is believed to be complex, consisting of at least 24 distinct forms, with flagellated, amoeboid, and encysted stages; some of these stages are toxic to both fish and mammals (Burkholder and Glasgow, 1995; Steidinger et al., 1996; Burkholder, 1999). Transformations between life history stages are believed to be controlled by the availability of fresh fish secretions or fish tissues and the availability of alternate prey (Burkholder and Glasgow, 1997b). A more recent study on the phylogeny of *P. piscicida* has found no evidence for the alleged 24 life cycle stages and transformations to amoebae (Litaker et al., 2002). This study concluded that the life cycle of *P. piscicida* is typical of free-living marine dinoflagellates (Litaker et al., 2002). Other toxic *Pfiesteria*-like dinoflagellates have also been associated with the ulcerative lesions in fish (Steidinger et al., 1995; Burkholder and Glasgow, 1997a).

The Oomycete, *Aphanomyces invadans* and the myxosporean protozoal organism, *Kudoa clupeidae* have also been identified as agents that can contribute to the development of lesions in Atlantic menhaden. Blazer et al., (1999) reported that the ulcerative lesions observed in Atlantic menhaden from the Chesapeake Bay tributaries were similar both
grossly and histologically to those associated with UM. *Aphanomyces invadans* was later identified in lesions from Atlantic menhaden collected in estuarine waters along the eastern United States (Blazer et al., 2002). These lesions observed in Atlantic menhaden have been reproduced by injection and bath exposure to fungal zoospores (Kiryu et al., 2002), thus fulfilling Koch’s postulates (Koch, 1884). The myxosporean protozoal organism, *Kudoa clupeidae* has been associated with the development of raised lesions and skin ulcerations in juvenile Atlantic menhaden from the Chesapeake Bay tributaries (Reimschuessel et al., 2003).

Host, pathogen and environmental factors collectively define the disease process (Snieszko, 1974). Although fish possess behavioral and physiologic defenses that support their survival, abiotic and biotic factors potentially alter the immune system in a manner that predisposes fish to infections by a variety of pathogens. Atlantic menhaden are exposed to environmental factors that can adversely alter their immune function and overall health in the estuaries. A clear understanding of the immunologic response of Atlantic menhaden to changes in their environment and during their residency in estuaries would support the efforts of resource managers to sustain the economically important menhaden fishery.

**IV. Environmental factors and their role in fish health**

Fish health is determined by many factors, both extrinsic and intrinsic. Since fish live immersed in their environment, natural and anthropogenic environmental changes may have both direct and indirect consequences for fish health and disease resistance
Environmental factors such as toxic chemicals, low dissolved oxygen (DO) and abnormal pH may reduce the resistance of aquatic animals to pathogens. Seasonal fluctuations in environmental variables such as changes in salinity or temperature have been shown to affect the physiology of most teleosts. Behavioral changes (e.g. rapid dispersal) and altered metabolism and hormone secretions help fish adapt to these changes in their environment (Billard et al., 1981; Schreck, 1981).

As poikilotherms, fish are affected by water temperature, an important environmental factor regulating many physiological processes such as oxygen demand, metabolism, immunological response, reproduction and growth (Snieszko, 1974; Ellis, 1981; Nikinmaa, 2002). There have been many reports on the effects of temperature on the immune system (Avtalion et al., 1976; Ellis, 1981; Snieszko, 1974; Bly and Clem, 1992; Kennedy-Stoskopf, 1993). Most studies on the influence of temperature on fish immune systems have noted that low temperatures or abrupt changes in temperature suppressed the immune system and predisposed fish to disease (reviewed by Bly and Clem, 1992, Ellis, 1981). Winter saprolegniasis in channel catfish (*Ictalurus punctatus*) and a decrease in skin mucus cell density are associated with an acute drop in temperature (Bly and Clem, 1992; Quiniou et al., 1998), and spring viraemia in carp is associated with stress caused by overwintering (Snieszko, 1974). Hyperthermia has also been noted as a stressor in teleosts. Lymphopenia was observed in coho salmon, *Oncorhynchus kisutch*, exposed to high temperatures (McLeay, 1975). Hybrid striped bass subjected to an increase in water temperature within their preferred range of 24-30°C showed stress-related lymphopenia (Carlson et al., 1995). Also, largemouth bass (*Micropterus*
*salmoides*) stressed by elevated water temperature became susceptible to red-sore disease (*Aeromonas hydrophilia*; Huizinga et al., 1979) and hyperthermia has been associated with epithelial thinning in trout (Iger et al., 1994).

Low DO is considered one of the most common environmental factors that fish encounter and low DO has been associated with infection (Plumb et al., 1976). Hypoxia may result in alteration of cellular components such as enzymes, cell membranes, or impairment of vital functions such as respiration, circulation, immune response, osmoregulation, and hormonal regulation (Adams, 1990). Nutrient loading may cause an increase in algal biomass, increased organic loading. When oxygen needed for the degradation of organic material is depleted fish health can be affected (Plumb, 1984). Skin damage, muscle necrosis and increased prevalence of viral skin lesions have been observed in fish exposed to chronic hypoxic events (Scott and Rogers, 1981; Plumb et al., 1976; Mellergaard and Nielsen, 1995).

Other factors such as salinity, pH, and organic pollutants, and heavy metals (e.g. cadmium, lead, copper, zinc) can also modulate the immune system of teleosts (Heath, 1995). Salinity tolerance in teleosts is controlled by cortisol (Wendelaar Bonga, 1997). Cortisol is essential for hydromineral balance in teleosts and its mineralocorticoid activity includes stimulation of branchial Na\(^+\) and Cl\(^-\) extrusion (Wendelaar Bonga, 1997). Elevation of cortisol levels have been associated with abrupt changes in salinity in many teleosts (Kelly and Woo, 1999; Morgan et al., 1997; Madsen et al., 1994). Suppression of antibody responses to *Yersinia ruckeri* has been observed in rainbow trout exposed to
hyperosmotic saline (Wendelaar Bonga, 1997). Both low and high pH have been associated with skin damage in teleosts (Noga, 2000). Low pH has been associated with epizootic ulcerative syndrome in Australian estuaries (Callinan et al., 1996). Exposure to low environmental pH caused decreases in fecundity and egg viability in fathead minnow, *Pimphales promelas* and brook trout, *Salvelinus fontinalis* (Billard et al., 1981). Low pH also caused prolonged elevation of blood cortisol levels in char, *Salvelinus alpinus*, rainbow trout and brook trout (Pickering, 1989). Prolonged stimulation of hypothalamus-pituitary-interrenal (HPI) axis may predispose fish to disease and suppress sexual reproduction (Pickering, 1989).

Anthropogenic discharges of chemicals such as pesticides, municipal sewage, pulp mill effluent, polynuclear aromatic hydrocarbons, polychlorinated biphenyls, heavy metals and detergents have all been shown to affect the immune system of teleosts (Heath, 1995). Numerous pollutants have been associated with skin damage in fish (Noga, 2000). Other environmental factors such as food availability, water flow, turbidity, and water chemistry and biological content can also have an effect on fish health.

Environmental factors can have population level effects. The higher energy cost of maintaining homeostasis may result in reductions in 1) intrinsic growth rate 2) recruitment and 3) compensatory reserve; and in altered species abundance and diversity (Ellis, 1981; Adams, 1990). Environmental factors and the innate and adaptive immune response contribute to the overall health of all aquatic species. Enhanced understanding
of the response of fish to environmental factors and pathogens will assist fisheries
biologists in the long-term management of coastal fisheries and estuarine health.

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A Comparative Study of Health Indices of Atlantic menhaden (*Brevoortia tyrannus*) during residency in three estuaries in North Carolina

ABSTRACT

Atlantic menhaden (*Brevoortia tyrannus*) are exposed to environmental factors that may adversely impact their immune function and overall health during residency in estuaries. Due to the recent increases in large fish kills as well as outbreaks of ulcerative skin lesions involving this species, Atlantic menhaden were collected during 2002 from the White Oak, Neuse and Pamlico river estuaries in North Carolina and the seasonal changes in health indices were assessed during the same period. Indicators of tissue damage (histopathology of gills, heart, liver, intestine, and anterior kidney), nutritional status and exposure to environmental stressors (liver-somatic index), immune status and disease (TGF-β mRNA production, lymphocyte mitogenesis, white blood cell differential counts, hematocrit, and spleno-somatic index) were compared in Atlantic menhaden from all three river systems. The bioindicators used to assess the health of Atlantic menhaden in the estuaries of North Carolina showed that there is seasonal variability in liver-somatic and spleno-somatic indices, blood parameters (hematocrit, lymphocytes, glucose, plasma proteins, calcium, and muscle enzymes), and TGF-β, and that this pattern is strongly influenced by temperature. Recognition of the influence that water quality
parameters have on the health of menhaden and other estuarine species will provide additional information that can be used by resource managers to improve our coastal ecosystems and to help provide clues to the apparently complex pathogenesis behind outbreaks of ulcerative lesions in fish.

**INTRODUCTION**

Estuaries serve as nursery areas for many species important to both the commercial and recreational fisheries. They are dynamic, complex, highly productive ecosystems where environmental variables may fluctuate on both a diel and seasonal scale. Estuarine environments are constantly changing and challenging the adaptive and compensatory strategies of teleosts (Wedemeyer *et al.*, 1984; Whitfield and Elliott, 2002). Since fish live in close contact with their environment, environmental changes, natural and anthropogenic, may have both direct and indirect consequences on fish health and disease resistance (Anderson, 1990; Schreck, 1996). Seasonal fluctuations in environmental variables such as changes in salinity or temperature have been shown to affect the physiology of most teleosts and modification of behavior, metabolism and hormone secretions are some of the ways fish adapt to these changes in their environment (Billard *et al.*, 1981; Schreck, 1981).

Atlantic menhaden (*Brevoortia tyrannus*) are estuarine-dependent marine migratory species that spawn in the open ocean off North Carolina. Larvae are transported into the estuaries where they metamorphose into juveniles (Nelson *et al.*, 1977; Smith, 1999a; Ahrenholz, 1991). In the estuaries, Atlantic menhaden are affected by seasonal changes
in environmental variables that may impact their development. The life history of Atlantic menhaden reflects adaptive responses to seasonal changes in temperature and photoperiod (Lewis, 1966; Quinlan et al., 1999) and both temperature and salinity affect the distribution of Atlantic menhaden in estuaries (Hettler, 1976). Despite the abundance of Atlantic menhaden in estuaries of North Carolina, and their importance to the commercial fishery as a source of fish meal, fish oil and food fish (Vaughan and Smith, 1991), very little is known about the seasonal changes in their health indices during their residency in these estuaries.

Fish health assessment using various health indicators, biochemical, physiological and histopathological, have been widely studied, and applied to both laboratory and field studies (Zelikoff et al., 2000; Adams, 1990; Hugget et al., 1992; Mayer et al., 1992; Bernet et al., 2000; Rice et al., 1996; Adams et al., 2003). These health indicators may not be able to distinguish individual factors that are potential stressors in the aquatic environment, but an integrated approach of using multiple health indicators can be used to detect the effects or exposure to multiple environmental factors, natural and/or anthropogenic, before effects are observed at the population, community and ecosystem levels (Mayer et al., 1992).

This study describes the seasonal changes in health indices in Atlantic menhaden during residency in the White Oak, Neuse and Pamlico River estuaries in North Carolina. The White Oak River was chosen as a reference river system because ulcerative skin lesions found in Atlantic menhaden from both the Neuse and Pamlico Rivers have not been
observed in that system. Health indicators included immune function assays (TGF-β mRNA and lymphocyte mutagenesis), liver-somatic and spleno-somatic indices, histopathology (liver, gill, heart, kidney and intestines), hematology, and plasma chemistry. Water quality parameters were dissolved oxygen, temperature, pH and salinity. These health indicators along with quality measurements were used to assess the health of Atlantic menhaden during residency in these three estuaries in North Carolina.

MATERIALS AND METHODS

Study Sites

Atlantic menhaden were collected by cast net in the White Oak, Neuse, and Pamlico Rivers of North Carolina in 2002 (Fig. 1 and 2). Only Atlantic menhaden without visible ulcerative skin lesions were used in this study, although fish with ulcerative skin lesions were caught in the Neuse and Pamlico Rivers during the collection period. Ulcerative skin lesions and fish kill events involving large numbers of Atlantic menhaden in the Neuse and Pamlico Rivers have been absent so far in the White Oak River. Fish were collected once a month from June to October near Swansboro, NC in the White Oak River estuary, a shallow, estuarine system that flows into Bogue Sound (Fig. 1; Table 1). The Neuse and Pamlico Rivers are similar in length and both flow into the Pamlico Sound (Fig. 2). In the Neuse and Pamlico Rivers, fish were collected from several creeks beginning in June and July, respectively (Fig. 2; Table 1). Bimonthly sample collections were planned for both the Neuse and Pamlico Rivers to include both upper and lower
locations, but the actual sampling schedule was determined by the weather conditions and vessel availability while the presence of Atlantic menhaden determined the locations sampled (Table 1). Sampling was terminated in October when it became difficult to find fish in the estuaries. Water quality parameters, DO, temperature, salinity, and pH were measured at the time of each sampling.

**Blood Collection**

Menhaden were euthanized with an overdose of tricaine methanesulfonate (MS-222; Argent Chemical Laboratories, Redmond, Washington). Blood samples were collected from fish in each river system for the following assays: lymphocyte mitogenesis, hematology and plasma chemistry. For the lymphocyte mitogenesis assay, fish were bled from the caudal vein with sterile heparinized syringes (1 ml syringe, 22 gauge needle) and samples were placed in 10 ml vacutainers. Samples for hematology were collected similarly but syringes were flushed with the heparinized saline solution and samples were stored in 5 ml vacutainers. Both individual and pooled whole blood samples were used for hematological analysis and lymphocyte mitogenesis. Two to three fish were pooled for hematological analysis and three fish for lymphocyte mitogenesis. Pooled blood samples have also been used in other studies with Atlantic menhaden to obtain the necessary amount of blood required for blood chemistry (Engel et al., 1987). Similar numbers of Due to the small size of fish caught in the Neuse and Pamlico river estuaries most of the samples collected had to be pooled to obtain the required quantity of blood necessary for each assay. Fish collected in the White Oak were larger and, therefore, pooling was not necessary.
**Sample Processing**

For each fish, fork length (FL), body weight, liver weight, and spleen weight were measured. Samples of gill, heart, liver, intestine, gonads, spleen, and kidney were collected and fixed in 10% neutral buffered formalin for histopathology. Spleen samples collected from fish in the White Oak river were excised, weighed and stored in sterile cell culture medium, complete RPMI (RPMI-1640 plus 10% heat-inactivated fetal bovine serum, 100 U ml\(^{-1}\) penicillin, 100 µg ml\(^{-1}\) streptomycin, and 2 mM EDTA) for TGF-β mRNA assay. Spleen samples placed in complete RPMI were stored at 4°C and processed within 24 h for isolation of mononuclear cells to be used in a real-time RT-PCR assay developed for Atlantic menhaden TGF-β mRNA.

**Organosomatic Indices**

Liver-somatic indices (LSI) and spleno-somatic indices (SSI) were calculated as the organ weight (in mg) divided by the body weight (in mg; Goede and Barton, 1990).

**Histopathology**

Fixed tissue specimens were routinely processed, embedded in paraffin, sectioned at 5 µm and stained with hematoxylin and eosin (H&E). All samples were evaluated by light microscopy using the following grading system: Grade 0 = no remarkable microscopic abnormalities, Grade 1 = very mild changes; Grade 2 = mild changes; Grade 3 = moderate changes; Grade 4 = moderately severe changes; and Grade 5 = severe, at the extreme range of pathology for the given lesion type (Hurty *et al.*, 2002). No spleen
samples were used for histopathology from fish caught in the White Oak as these samples were used for TGF-β mRNA analysis. Gonads were collected if visible and used to determine sex.

**Hematology**

Blood smears for differential leukocyte counts and whole blood samples were analyzed by the Clinical Pathology Laboratory at the North Carolina State University College of Veterinary Medicine (NCSU-CVM). Blood smears were stained with Wright-Giemsa stain (Volu-Sol, Inc., Salt Lake City, UT) and 100 leukocytes on each slide were identified. To determine white blood cell and thrombocyte (WBC/thrombocyte) counts, cells were stained and diluted in Natt and Herrick’s solution and counted on a hemacytometer. The packed cell volume (PCV) or hematocrit values were determined by reading the packed cell percentages using microhematocrit tubes. Plasma total solids were measured with a refractometer (Leica Microsystems, Germany). The following substances were measured with a Hitachi 912 chemical analyzer (Roche Diagnostics, Indianapolis, IN): glucose, creatinine, phosphorus, calcium, total protein, albumin, aspartate aminotransferase (AST), creatinine kinase (CK), lactate dehydrogenase (LDH), sodium (Na⁺), potassium (K⁺), chloride (Cl⁻), and bicarbonate (HCO₃⁻). The analyzer calculated albumin/globulin ratio, sodium/potassium ratio, and anion gap (AGAP). Anion gap was determined as follows: [(Na⁺ + K⁺)-(Cl⁻ + HCO₃⁻)].
Lymphocyte Mitogenesis Assay

Isolation of Peripheral Blood Leukocytes (PBL): Blood samples were diluted 1:1 in PBS and centrifuged at 45 x g for 10 min in a swing-out rotor. The upper leukocyte layer was removed from the top of the red blood cell layer with a 1 ml pipette, diluted 1:1 in PBS and carefully layered over 2 ml of a 55% Percoll gradient (specific gravity 1.070 g ml\(^{-1}\)) in 0.15 M NaCl. Samples were centrifuged at 400 x g for 30 min at 22\(^\circ\)C, the leukocyte rich interphase was collected and washed twice in 10 ml complete RPMI by centrifuging for 5 min at 300 x g. Viable cell counts were performed with cells suspended in 0.2% trypan blue (Gibco Laboratories, Grand Island, NY). Cell suspensions were diluted in complete RPMI to a concentration of 1.0 x 10\(^6\) cells ml\(^{-1}\) (5.0x10\(^4\) cells/50\(\mu\)l).

Colorimetric MTT (tetrazolium) assay: The MTT assay used in this study was based on a modification of several techniques (Mosmann, 1983, Hansen et al., 1989 and Daly et al., 1995). MTT (3, (4,5-dimethylthiazol-2-yl) 2,5-dipheyl-tetrazolium bromide) (M2128, Sigma, St. Louis, MO) was dissolved in sterile PBS at 5 mg/ml, sterilized by filtration and stored in a dark bottle at 4\(^\circ\)C. Concanavalin A (Con A; C-0412, Sigma) and lipopolysaccharide (LPS; L-5262 from Vibrio cholerae, Sigma) were resuspended in PBS (1 mg/ml stock) and stored at –20\(^\circ\)C. Both mitogens were diluted in complete RPMI to a final concentration of 10 \(\mu\)g/ml (LPS) and 2.5 \(\mu\)g/ml (Con A) before use. The mitogen doses and incubation times were optimized for Atlantic menhaden in preliminary
experiments. Stimulation indices ranged from 1.09-1.7 for Con A and 1.15-1.4 for LPS. These stimulation indices were similar to those obtained by Espelid et al., 2003).

All mitogenesis assays were performed in quadruplicate. Cell suspensions (1.0 x 10^6 cells ml\(^{-1}\)) were loaded into 96 well round-bottomed tissue culture plates at 50 µl/well. Immediately after leukocyte plating, 50 µl of complete RPMI with or without mitogen (control) was added to each well and samples were incubated in a humidified container at 27°C for 5 days. After the incubation period, 10 µl stock MTT (5 mg/ml PBS) was added to each well and the samples were incubated at 27°C for 4 h for MTT cleavage. After incubation, formazan precipitate was dissolved by adding 120 µl of a solubilization buffer (pH = 4.7) consisting of 20% sodium dodecyl sulfate (SDS; Sigma, St. Louis, MO) in 50% N, N-dimethylformamide (DMF; Fisher Scientific, Suwannee, GA) per well and plates were incubated overnight. Plates were read at OD\(_{570}\) with reference wavelength of OD\(_{630}\) using a microplate reader. Stimulation indices were calculated using the formula:

\[
SI = \frac{\text{mean OD of stimulated culture}}{\text{mean OD of non-stimulated control culture}}
\]

**TGF-β real-time RT-PCR**

Splenic mononuclear cell TGF-β transcription was determined for fish collected in the White Oak river by real-time RT-PCR procedures developed for Atlantic menhaden (in press). Spleen samples collected from fish in complete RPMI were minced finely, resuspended in complete RPMI and centrifuged on two-step Percoll gradients (35% and
55% stock solution in 0.15 M saline; specific gravity 1.046 and 1.070 g ml\(^{-1}\)). Splenic mononuclear cells were harvested from the 35/55% interface, viable cell counts were performed with cells suspended in 0.2% trypan blue. Cell pellets were lysed in Tri Reagent (Molecular Research Center, Cincinnati, OH) at 1ml per 5-10 x 10\(^6\) cells and stored at –80°C.

RNA isolation and reverse transcription were performed following published procedures (Harms et al., 2000a). Total RNA was isolated by the guanidine thiocyanate method (Tri Reagent) following kit instructions. The RNA pellet was washed with 75% ethanol and resuspended in sterile diethyl pyrocarbonate (DEPC)-treated water. Splenic mononuclear cell RNA was resuspended to a concentration of 5 x 10\(^4\) cell equivalents µl\(^{-1}\). Messenger RNA was reverse transcribed using Superscript II RT (Gibco BRL, Gaithersburg, Maryland) to cDNA with oligo dT\(_{15}\) priming of 3 x 10\(^6\) cells equivalent. Samples of cDNA were stored at –20°C. Negative RT controls were run in parallel.

Real-time PCR was performed using primers developed specifically for Atlantic menhaden and procedures described previously (Johnson et al., in press). Amplification of reverse transcribed cDNA samples and standards was performed in parallel. PCR efficiencies were above 90% and correlation coefficient above 0.996. Results are reported as the ratio of TGF-β/β-actin.
Statistical Analyses

Monthly differences in blood parameters were analyzed first for each river system. Comparisons of blood parameters among the three river systems were performed using analysis of variance (ANOVA) with river and month as main effects and included their interaction (Neter et al., 1996). Body weight was added to the model to determine whether the blood parameters were affected by weight. Comparisons among the three rivers were also performed on the organosomatic indices and lymphocyte mitogenesis data. Monthly comparisons were evaluated for TGF-β from fish collected in the White Oak River. The Statistical Analysis System (SAS; SAS Institute Inc, Cary, NC) was used for all analyses. Significant ANOVAs ($P < 0.05$) were followed by Tukey’s multiple comparison tests (Neter et al., 1996). A visual assessment of residual plots was used to determine homogeneity of variance. To reduce variance heterogeneity, neutrophils, thrombocytes, WBC/thrombocyte counts, plasma protein, albumin/globulin, $\text{Na}^+$, $\text{K}^+$, $\text{Na}^+/\text{K}^+$, AST, and CK were log transformed, and monocyte and eosinophil counts were square root transformed (Neter et al., 1996).

RESULTS

River Systems

Atlantic menhaden collected in the White Oak River estuary were larger than those collected in the Neuse and Pamlico River estuaries during this study except for fish collected in July in the Pamlico River estuary (Fig. 3). Atlantic menhaden are very
prevalent in the White Oak, but the outbreaks of ulcerative skin lesions and fish kill events observed in the Neuse and Pamlico have not been observed so far in this estuary.

**Water Quality**

Water quality parameters varied depending on the river system (Fig. 4), time of collection and locations within each river system (Table 1). In the Pamlico River, salinity, pH, and temperature were highest in August and dissolved oxygen levels were highest August and September and lowest in July and October (Fig. 4). In the Neuse, salinity was highest in June and July. Most of the fish caught during June and July were from the lower parts of the estuary while those caught in August, September and October were from creeks in the upper parts of the river. The lowest pH was measured in September in the Neuse. There was very little fluctuation in DO levels during the study period in the Neuse. In the White Oak River, pH was lowest in August and salinity was lowest at the beginning and end of the study period. Salinities in the White Oak River, near Swansboro, were much higher than those found in the creeks of the Neuse and Pamlico River estuaries (Fig. 4). Similar to the Neuse and Pamlico Rivers, temperature in the White Oak River was highest in August and lowest at the beginning and end of the study period (Fig. 4).

**Organosomatic indices**

**Liver-somatic index**

Liver-somatic indices were significantly different in Atlantic menhaden over the study period and in fish collected in the different river systems (Fig.5a). In the White Oak
River, LSI were significantly lower in June than all other months except for October ($P < 0.005$) and significantly higher in August than both June and October ($P < 0.0001$ and $P = 0.0102$ respectively; Fig. 5a). Liver-somatic indices were lowest in August for fish caught in the Pamlico River and significantly higher for the month of July than all other months ($P < 0.0005$; Fig. 5a). Liver-somatic indices were not significantly different over the study period for fish caught in the Neuse River. Liver-somatic indices were highest in August and lowest at the beginning and end of the study period for fish caught in both the Neuse and White Oak rivers. Atlantic menhaden caught in the Pamlico River had significantly higher LSI than fish caught in the White Oak River in July and October ($P = 0.0005$ and 0.0118 respectively; Fig. 5a). Significantly lower LSI were measured in the Pamlico River than the Neuse in August ($P = 0.0439$; Fig. 5a). There were no significant differences in LSI between the White Oak and Neuse Rivers.

**Spleno-somatic Index**

Spleno-somatic indices were significantly different over the study period and among the three river systems (Fig. 5b). Similar to LSI, SSI were lowest in June and highest in August for Atlantic menhaden caught in the White Oak River; SSI were significantly higher in August than June and July ($P = 0.0129$ and 0.0353 respectively; Fig. 5b). In the Neuse River, SSI were significantly higher for fish caught in June than all other months ($P < 0.005$; Fig. 5b). Atlantic menhaden caught in the Pamlico River in July had significantly higher SSI than those caught in October ($P = 0.0308$; Fig. 5b). Significant differences were observed among the three river systems. Spleno-somatic indices were significantly higher in Atlantic menhaden caught in the Neuse River than those from the
White Oak in June ($P = 0.0172$; Fig. 5b) and SSI were also significantly higher in fish from the Pamlico River than the White Oak in July ($P = 0.0062$; Fig. 5b).

**Histopathology**

Remarkable microscopic lesions found in Atlantic menhaden were pigmented macrophages (PMAs) in the spleen and kidney, liver vacuolation and lymphocyte aggregates, protozoa (kidney and heart) and granulomas and *Ichthyophonus* in the kidneys (Fig. 6).

Pigmented macrophage aggregates (PMAs), protozoa, granulomas, and *Ichthyophonus* sp. were the most common abnormalities found in the kidney (Fig 6a-d). Kidney PMAs were higher in the White Oak River than the Neuse and Pamlico Rivers except for July in the Pamlico River (Fig. 6a) where fish were similar in size to those caught in the White Oak River (Fig. 3). Kidney protozoa were found in 70% of Atlantic menhaden caught in the Neuse River in August (Fig. 6b). Protozoa were not found in Atlantic menhaden in the Pamlico River and only a few fish from the White Oak (Fig. 6b). Granulomas were found in menhaden from all three river systems but were most numerous in the White Oak River (Fig. 6c). Spores of *Ichthyophonus* sp. were most prevalent in the kidneys of fish from the White Oak River in August (Fig. 6d and 7).

No spleen samples were collected for histopathology from fish caught in the White Oak River because spleens were used for TGF-β assay. Splenic PMAs were most common in fish in September for the Neuse and in October for the Pamlico River (Fig. 6e). Liver
vacuolation and hepatic lymphocyte aggregates were found in a few Atlantic menhaden (Fig. 6f and 6g). Mild to moderate vacuolation of hepatocytes was found mainly in menhaden from the Pamlico River (Fig. 6f). Lymphocyte aggregates in the liver were mild to moderate in fish caught during the study period (Fig. 6g). Aggregates were most prevalent in fish caught in August from the Pamlico River and in August and October from the White Oak River (Fig. 6g).

Protozoal organisms found in the cardiac muscle were mainly myxosporeans; these were most common in fish from the White Oak River (Fig. 6h and 8) and those caught in July in the Pamlico River. Myxosporeans were only found in large menhaden during the study period although other types of protozoa were found in menhaden caught in Neuse River in August (Fig. 6h). Protozoa found in fish from the Neuse were only associated with the epicardium.

**Hematology**

**White Oak:** Most hematological parameters varied over the study period in Atlantic menhaden collected from the White Oak River. Neutrophil counts were significantly higher in Atlantic menhaden caught in June than all other months except for August ($P < 0.05$; Table 2). The highest lymphocyte counts were found in Atlantic menhaden caught in June and the lowest in August. Lymphocyte counts were significantly higher in Atlantic menhaden caught in June than all other months except for October ($P < 0.05$)
and counts were also significantly higher in fish caught in October than August ($P = 0.0294$; Table 2). The opposite trend was observed with plasma protein concentrations. Plasma protein concentrations were lowest in Atlantic menhaden caught in June and highest in those caught in August. Concentrations were significantly lower in fish caught in June than all other months except October ($P < 0.05$; Table 2). Plasma protein concentrations were also significantly lower in Atlantic menhaden caught in October than September ($P = 0.0165$). Thrombocyte and hematocrit values were significantly lower in fish collected in June than all other months ($P < 0.05$, Table 2). Hematocrit percentages were also significantly lower in Atlantic menhaden caught in July than August ($P = 0.0387$). There were no significant differences in counts of monocytes, eosinophils, and WBC/thrombocytes over the study period (Table 2).

**Neuse:** Thrombocyte, hematocrit and plasma protein values were the only hematological parameters that were significantly different over the study period in the Neuse (Table 2). Thrombocyte counts were significantly higher in Atlantic menhaden caught in June than all other months except October ($P < 0.05$). Fish caught in October had significantly lower thrombocyte counts than those caught in August ($P = 0.009$; Table 2). Hematocrit values were lowest in Atlantic menhaden caught in June and highest in those caught in August (Table 2). Hematocrit values were significantly lower in Atlantic menhaden caught in June than all other months ($P < 0.05$). Hematocrits were significantly lower in fish collected in July and October than those caught in August and September ($P < 0.05$). Plasma protein concentrations were significantly higher in Atlantic menhaden caught in August than all other months except October ($P < 0.05$). Concentrations of plasma
protein were significantly higher in Atlantic menhaden collected in October than June and July ($P = 0.0010$ and $0.0014$ respectively; Table 2). There were no significant differences in counts of neutrophils, monocytes, eosinophils, lymphocytes, and WBC/thrombocytes over the study period (Table 2).

**Pamlico:** Atlantic menhaden collected in the Pamlico River showed significant differences in hematological parameters: lymphocytes, thrombocytes and hematocrit values over the study period (Table 2). Lymphocyte counts were significantly lower in Atlantic menhaden caught in August than all other months ($P < 0.05$) while thrombocyte counts were significantly higher in fish caught in August than all other months ($P < 0.05$). Hematocrit values were significantly higher in September than July and October ($P = 0.0063$ and $0.013$ respectively; Table 2).

All hematological parameters, except for monocytes and plasma protein values, were significantly different in Atlantic menhaden from the three river systems (Fig. 9; Table 2). Neutrophil counts in Atlantic menhaden from the three river systems differed significantly only in June and September. Neutrophil counts were significantly higher in Atlantic menhaden from the White Oak River than the Neuse in June ($P = 0.0006$). Although neutrophil counts were lower in Atlantic menhaden from the Pamlico River than both the Neuse and White Oak rivers in September, counts were only significantly lower than that of the Neuse ($P = 0.0311$; Fig. 9a). Eosinophil counts were significantly higher in Atlantic menhaden from the White Oak than both the Neuse and Pamlico rivers in August ($P = 0.0213$ and $0.0197$ respectively; Table 2).
Counts of WBC/thrombocytes were significantly different in June and August among the three river systems. Counts were significantly higher in Atlantic menhaden collected in the White Oak River than Neuse in June ($P = 0.03$) and counts were significantly lower in fish from the Pamlico River than the Neuse in August ($P = 0.0334$; Fig. 9b). Thrombocyte counts were significantly higher in Atlantic menhaden from the Neuse than the White Oak River in June ($P < 0.0001$; Fig. 9c). In August, lymphocyte counts were higher in Atlantic menhaden from the Neuse than both the Pamlico and White Oak rivers but counts were only significantly higher than that of the Pamlico ($P < 0.05$; Fig. 9d). Hematocrits were significantly lower in fish from the White Oak River than both the Neuse and Pamlico rivers in September ($P = 0.039$ and 0.0012 respectively; Fig. 9e).

**Plasma chemistry**

**White Oak:** In the White Oak River, most blood chemistry parameters were lowest in Atlantic menhaden caught at the beginning and end of the study period, June and October, and highest in August (Table 3). Glucose concentrations were significantly higher in Atlantic menhaden collected in August than June and October ($P = 0.0006$ and 0.0044 respectively) and concentrations were significantly lower in June than September ($P = 0.015$; Table 3). Total protein concentrations were significantly lower in fish caught in June than all other months ($P < 0.05$) and concentrations were lower in October than August ($P = 0.045$; Table 3). A similar trend was observed with albumin and calcium.
Concentrations of albumin were significantly lower in Atlantic menhaden caught in June and July than all other months ($P = 0.005$) and calcium concentrations were significantly lower in fish caught in June than all other months ($P < 0.05$; Table 3).

There were similar trends in muscle enzymes, AST and LD. Concentrations of AST were significantly higher in Atlantic menhaden collected in August than September and October ($P = 0.0128$ and 0.0026 respectively; Table 3) and concentrations were also lower in fish collected in October than July ($P = 0.0148$). Lactate dehydrogenase concentrations were significantly higher in Atlantic menhaden collected in August than June and September ($P = 0.0038$ and 0.0215 respectively; Table 3). Creatine kinase concentrations were lowest in June and September; concentrations were lower in June than all other months except for September ($P < 0.05$). Creatine kinase concentrations were also lower in Atlantic menhaden collected in September than July and October ($P = 0.0060$ and 0.0084 respectively; Table 3).

Plasma electrolytes also varied over the study period (Table 3). Atlantic menhaden collected in October had significantly lower sodium concentrations than those collected in August and September ($P = 0.0097$ and 0.0021 respectively) and concentrations were also lower in July than September ($P = 0.0344$; Table 3). Potassium concentrations were significantly higher in fish caught in June than all other months ($P < 0.02$) and higher in fish collected in October than August and September ($P = 0.0243$ and 0.0028 respectively; Table 3). Also, Atlantic menhaden collected in July had significantly higher levels of potassium than those collected in September ($P < 0.048$; Table 3). Chloride
concentrations were significantly lower in fish collected in July and October than all other months \((P < 0.05)\) and concentrations were also lower in June than September \((P = 0.0357; \text{Table 3})\). Bicarbonate concentrations were significantly lower in Atlantic menhaden collected in both July and August than September and October \((P < 0.05)\) and concentrations were significantly higher in June than both July and August \((P = 0.0244\) and \(0.0463\) respectively; \text{Table 3}).

**Neuse:** Similar trends in plasma chemistry observed in the White Oak River were also observed in the Neuse. Glucose concentrations were significantly lower in Atlantic menhaden collected in June than all other months except for July \((P < 0.05; \text{Table 3})\) while phosphorus concentrations were significantly higher in Atlantic menhaden caught in June than all other months except for August \((P < 0.05)\). Albumin concentrations were significantly lower in fish in June than all other months \((P < 0.02; \text{Table 3})\) whereas globulin concentrations were significantly higher in June than July and October \((P = 0.0049\) and \(0.0327\) respectively; \text{Table 3}).

Concentrations of AST were significantly lower in Atlantic menhaden collected in October than all other months \((P < 0.02; \text{Table 3})\). Creatine kinase concentrations were significantly lower in June and July than all other months \((P < 0.05; \text{Table 3})\). Lactate dehydrogenase concentrations were significantly lower in Atlantic menhaden collected in June, July and October than August and September \((P < 0.05; \text{Table 3})\).

Sodium concentrations were significantly lower in fish collected in September than all other months \((P < 0.05; \text{Table 3})\). Potassium concentrations were significantly lower in
fish in July than all other months except for October and significantly higher in September than all other months ($P < 0.05$; Table 3). Chloride concentrations were significantly higher in Atlantic menhaden collected in June and July than August and September ($P < 0.05$; Table 3). Bicarbonate was significantly higher in Atlantic menhaden collected in September than all other months ($P < 0.0006$; Table 3). Anion gap was significantly higher in Atlantic menhaden collected in August than all other months except for October ($P < 0.05$) and fish caught in October had significantly higher anion gap than those caught in July ($P = 0.0249$; Table 3).

Pamlico: Similar to the White Oak and Neuse, plasma chemistry values tended to be lowest in Atlantic menhaden collected at the beginning and end of the study period in the Pamlico River (July and October respectively). Calcium levels were lowest in fish caught in July and October and highest in August (Table 3). Calcium levels were significantly higher in Atlantic menhaden collected in August than all other months ($P < 0.003$; Table 3). Phosphorus, CK, AST and LD concentrations were significantly lower in Atlantic menhaden caught in July than all other months ($P < 0.05$; Table 3) while glucose concentrations were significantly higher in fish caught in July than all other months ($P < 0.003$; Table 3). Sodium and chloride concentrations were significantly lower in Atlantic menhaden collected in October than all other months ($P < 0.05$). Sodium concentrations were also significantly lower in fish caught in September than August ($P < 0.0106$; Table 3) while chloride concentrations were significantly higher in fish caught in July than all other months ($P < 0.05$; Table 3). Potassium concentrations were significantly lower in Atlantic menhaden collected in July than all other months.
except for August ($P < 0.02$) and bicarbonate concentrations were significantly higher in fish caught in September than all other months ($P < 0.02$). Anion gap was significantly lower in Atlantic menhaden collected in July than all other months and significantly higher in fish caught in August than all other months ($P < 0.05$; Table 3).

Some plasma chemistry parameters measured over the study period were significantly different among the three rivers (Table 3). Glucose concentrations were significantly lower in Atlantic menhaden caught in the Neuse than those from the White Oak and Pamlico rivers in July ($P = 0.0362$ and $0.0024$ respectively; Table 3; Fig. 9f). Phosphorus concentrations were significantly influenced by the body weight of Atlantic menhaden. Phosphorus concentrations were significantly lower in Atlantic menhaden collected in the Pamlico River than in those from the Neuse and White Oak in July ($P = 0.0421$ and $0.0009$ respectively; Table 3). In contrast, phosphorus concentrations were significantly higher in the White Oak River than the Neuse and Pamlico in October ($P = 0.0429$ and $0.0182$ respectively; Table 3). Globulin concentrations were significantly higher in Atlantic menhaden caught in the White Oak than the Neuse in October ($P = 0.0096$; Fig. 9g) and calcium concentrations were significantly lower in fish from the Pamlico River than the White Oak in October ($P = 0.0188$; Table 3).

Muscle enzymes, AST, LD, and CK were significantly influenced by the body weight of Atlantic menhaden. Atlantic menhaden caught in the White Oak had significantly higher AST concentrations than fish from both the Neuse and Pamlico rivers in July ($P = 0.0103$ and $0.0004$ respectively; Table 3). Concentrations of AST were significantly lower in
Atlantic menhaden collected in the Pamlico River than both the Neuse and White Oak in August ($P = 0.0336$ and $0.0266$ respectively; Table 3). Concentrations of AST were significantly lower in Atlantic menhaden caught in the Pamlico than the Neuse in September ($P = 0.0132$; Table 3). Creatine kinase concentrations were significantly lower in Atlantic menhaden caught in the White Oak than the Neuse and Pamlico in October ($P = 0.0067$ and $0.0151$ respectively; Table 3).

There were significant differences in plasma electrolytes in Atlantic menhaden from the three rivers. Sodium concentrations were significantly higher in Atlantic menhaden from the White Oak than those from the Pamlico River in July ($P < 0.0001$; Fig. 9h). Atlantic menhaden collected from the three river systems had significantly different potassium concentrations in September ($P < 0.05$; Table 3). Both sodium and chloride concentrations were significantly lower in Atlantic menhaden caught in the Neuse than those caught in the Pamlico River in September ($P < 0.05$; Fig. 9h and 9i respectively). Concentrations of both sodium and chloride were also significantly different in Atlantic menhaden from all three river systems in October ($P < 0.02$; Fig. 9h and 9i). Bicarbonate concentrations were significantly higher in fish from the Neuse than the Pamlico in September ($P = 0.0001$; Fig. 9j).

**Lymphocyte mitogenesis**

Neither ConA nor LPS stimulation of leukocytes showed significant differences over the study period for any of the river systems except for the Neuse (Table 2). Both ConA and
LPS responses were significantly higher in August than all other months ($P < 0.05$). In the White Oak River, both ConA and LPS responses were lowest in August (Table 2), but stimulation indices were not significantly different.

**TGF-β**

Splenic mononuclear cell TGF-β mRNA production varied over the study period in the White Oak river. TGF-β transcription was lowest in June and September while production was significantly higher in August than June ($P = 0.0249$; Fig. 10).

**DISCUSSION**

In this study, significant seasonal variability was observed in the bioindicators used to assess the health of Atlantic menhaden from the three estuarine systems. In the aquatic environment it is usually not possible to distinguish the individual factors that are potential stressors, but an integrative approach of using multiple health indicators can help determine the cumulative effect or exposure to natural and anthropogenic environmental conditions that can be stressful to aquatic organisms (Adams, 1990).

**Organosomatic indices**

Organosomatic indices have been reported to vary naturally with food availability, sexual maturation and life history (Goede and Barton, 1990). In the White Oak River, we observed a similar trend in both LSI and SSI. These organosomatic indices were highest
in August and lowest at the beginning and the end of the collection period. This trend was also observed in several blood parameters (hematocrit, thrombocytes, glucose, plasma proteins, calcium, AST, LDH, CK) and TGF-β production measured in the White Oak River. The trend observed was similar to the temperature profile for that river system, high temperatures in the summer and low temperatures at the beginning and end of the season.

It has been proposed that a decrease in LSI may be observed in fish limited by food or exposed for a short period to pollutant stress, while an increase in LSI may be due to chronic exposure to stress when food is not limited (Heath, 1995). It is possible that these fish caught at the beginning and end of the season were either food limited or decreased their feeding, and the decline in organosomatic indices may reflect the depletion of energy reserves such as stored glycogen or body fat (Goede and Barton, 1990). The increase in LSI observed in Atlantic menhaden caught in the summer may reflect hyperplasia or hypertrophy of the hepatocytes. This may be an adaptive response to increase the capacity of the liver to detoxify contaminants (Heath, 1995).

The high SSI observed in the Neuse River in June reflects SSI from some of the fish caught in Broad Creek. Although fish collected in that creek did not show signs of gross ulcerative skin lesions, fish with ulcerative skin lesions were present during the time of collection. Therefore, the high SSI may be an indicator of existing environmental conditions, disease and/or immune functional response (Anderson et al., 1982; Goede and Barton, 1990). Splenomegaly is common in diseased fish (Goede and Barton, 1990) and
splenic dysfunction due to adverse environmental conditions have been observed in teleosts exposed to contaminants (Adams et al., 1992).

Both LSI and SSI were high in Atlantic menhaden caught in July in the Pamlico River. These fish were caught in an area with a school of dolphins that were apparently feeding on schools of Atlantic menhaden. There was also one fish with an ulcerative lesion caught in our samples. The high organosomatic indices measured during July may reflect the stressful situation encountered by these fish and their nutritional status, or a combination of factors. High plasma glucose concentrations were also observed in these fish.

Fluctuations in organosomatic indices in these estuaries may be influenced by seasonal fluctuations in water quality, and food availability. Some of the increases in both LSI and SSI observed in the Neuse and Pamlico river estuaries may indicate disease events, and existing environmental conditions at the specific locations in the estuaries. The organosomatic indices used in this study can be considered sensitive to the changes in environmental conditions in these estuaries and therefore good indicators of the general condition of Atlantic menhaden.

**Histopathology**

The main difference in histopathology among the three river systems was the absence of myxosporeans in the hearts of juvenile Atlantic menhaden (<120mm FL) in the Neuse and Pamlico rivers during the study period. There were few histopathological
abnormalities observed in Atlantic menhaden collected in the Neuse River, although fish caught in August had protozoa in the gills, heart and kidney. These fish caught in the Neuse in August were found in an area where Atlantic menhaden with ulcerative skin lesions were present. Most of the protozoal organisms seen in menhaden tissues were encysted, with no apparent inflammatory response. Therefore, we do not know the possible health impacts of these *Kudoa*-like parasites. Occasional fish had a more severe parasitic infection, characterized by a mild surrounding granulomatous inflammatory response, and it is possible that a heavy protozoal burden within the cardiac interstitium could lead to increased stress and susceptibility to other diseases. *Kudoa*-like myxosporeans were recently associated with ulcerative lesions in Atlantic menhaden in the Chesapeake Bay area (Reimschuessel *et al.*, 2003).

Splenic PMAs increased over the study period in fish from the Neuse and Pamlico Rivers, and kidney PMAs were more prevalent in the larger Atlantic menhaden from the White Oak River and those caught in the Pamlico River in July. Pigmented macrophages are important for storage and destruction of both exogenous and endogenous material (Wolke, 1992) and are very common non-specific lesions found in Atlantic menhaden spleen and kidney (Cahn, 1975; Johnson *et al.*, in prep). Since the presence of PMAs in lymphoid organs is age-dependent (Agius, 1981), the increase in kidney PMAs in the larger Atlantic menhaden is expected.

**Blood parameters**

In this study, most blood parameters showed a similar seasonal pattern. Seasonal variability in blood parameters was most pronounced in the White Oak River estuary than
in the Neuse and Pamlico Rivers. Water temperature is an important environmental factor affecting many physiological processes in fish such as oxygen demand, metabolism, immunological response, reproduction and growth (Snieszko, 1974; Nikinmaa, 2002). The amount of oxygen dissolved in water, toxicity of pollutants and growth of pathogens are also influenced by temperature (Snieszko, 1974). Unusually high or low temperatures, insufficient food or decrease in dissolved oxygen concentrations in water have been reported to have adverse effects on the values of blood indices (Hille, 1982).

Both hematocrit and thrombocytes were lowest in June while neutrophil and lymphocyte counts were highest at that time in the White Oak River. The low hematocrit values observed in Atlantic menhaden at the beginning of the study period have also been detected in other species. In striped mullet, _mugil cephalus_, hematocrit values increased from spring to summer (Hardig and Hoglund, 1984). Nutrition is considered environmental factor that influences the immune defense mechanisms and food availability may vary seasonally (Jokinen _et al._, 2003). Hematocrit values have been reported to decrease in opaleye, _Girella nigricans_ with the lost of appetite or disease (Blaxhall, 1972). Northern pike, _Esox lucius_ showed a decrease in hematocrit and serum glucose during starvation (Bullis, 1993), and long-term starvation caused a significant decrease in plasma glucose concentration in rainbow trout, _Salmo gairdneri_ (Vosyliene and Kazlauskiene, 1999). High concentrations of blood glucose in the summer and low values in the winter have also been reported in rainbow trout and factors such as starvation and temperature fluctuations have been reported to cause neutrophilia (Hine, 1992).
Plasma proteins, total protein, albumin, calcium, muscle enzymes (LDH, CK and AST) were all lowest at the beginning and the end of the study period in the White Oak River. The low concentrations of plasma proteins and muscle enzymes observed at the beginning and end of the season could result from nutritional imbalance, starvation, protein catabolism or protein degradation associated with immune function responses (Pages et al., 1995; Stoskopf, 1993; Wedemeyer et al., 1984) during these periods. Most of the calcium in plasma is bound to albumin therefore low concentrations of calcium at the beginning and end of the study period are also expected. Metabolic activities are enhanced by increasing water temperature and this is reflected in most plasma constituents (Hille, 1982). Therefore, the low concentrations of plasma components at the beginning and end of the study period and the high levels during the summer are likely due to the effects of temperature on the metabolic activities of Atlantic menhaden.

Most studies on the influence of temperature on fish immune systems have noted that low temperatures or abrupt changes in temperature suppressed the immune system and predisposed fish to disease (reviewed by Bly and Clem, 1992, Ellis, 1981). The decrease in lymphocytes observed during the summer in Atlantic menhaden from both the White Oak and the Pamlico Rivers have been observed in other teleosts. Hyperthermia has been associated with changes in immune function such as lymphopenia in coho salmon, Oncorhynchus kisutch, exposed to high temperatures (McLeay, 1975). Hybrid striped bass subjected to an increase in water temperature within their preferred range of 24-30°C, showed stress-related lymphopenia (Carlson et al., 1995) and hyperthermia was
associated with red sore disease (*Aeromonas hydrophilia*) in largemouth bass, *Micropterus salmoides* (Huizinga *et al.*, 1979). Winter saprolegniiasis in channel catfish (*Ictalurus punctatus*) and a decrease in skin mucus cell density are associated with an acute drop in temperature (Bly and Clem, 1992; Quiniou *et al.*, 1998), and spring viraemia in carp is associated with stress caused by overwintering (Snieszko, 1974).

The observed seasonal variability in blood parameters measured in Atlantic menhaden may be influenced by a combination of environmental factors, some of which may vary both temporally and spatially in the estuaries. The variability in blood parameters measured suggests that Atlantic menhaden, like other teleosts may adjust their immune function responses and metabolic activities during periods of high and low temperatures. The prevailing environmental conditions, presence or absence of infectious agents at the different locations, and food availability, are some of the factors that may influence the immune response of Atlantic menhaden and other teleosts.

**Immune Function Assays**

In fish, TGF-β is primarily immunosuppressive and both T and B cell proliferation are down regulated by this cytokine (Ruscetti and Palladino, 1991). The high TGF-β production, low lymphocyte proliferation with mitogens ConA and LPS, and low circulating lymphocytes measured in Atlantic menhaden in August in the White Oak River suggest that the immune systems of these fish were downregulated. In contrast, the significantly higher lymphocyte proliferation observed in Atlantic menhaden caught in
August in the Neuse River and the extremely high lymphocyte counts in these fish indicated possible immune system activation.

Although these fish caught in the Neuse River in August did not show signs of ulcerative skin lesions, the high prevalence of protozoa in the kidney and other organs may be responsible for the activation of lymphocytes in these fish. Increased lymphocyte proliferation has been reported in Atlantic menhaden with ulcerative skin lesions (Faisal and Hargis, 1992). Therefore, the protozoal infection in these fish caught in August may be responsible for activation of the immune system.

The seasonal variability observed in TGF-β production in the White Oak River estuary was similar to that observed in the other health indices and also reflected the temperature profile. Temperature does affect the immune system of teleosts, therefore it is possible that the variability in temperature over the study period in the White Oak River may have influenced the seasonal variability observed in TGF-β. Temporal variability in TGF-β production was also observed in white perch (Morone americana) in the Chesapeake Bay tributaries (Harms et al., 2000b).

This study highlights the utility of monitoring a variety of health parameters for assessing the health of free-ranging estuarine fish species. Seasonal variations in Atlantic menhaden health indices reflected changes in environmental conditions. Temperature seemed to be a very important environmental factor affecting health indices in Atlantic menhaden during residency in the three estuaries of North Carolina. In the
White Oak River, where the influence of ulcerative skin lesions is absent, there were clear seasonal patterns in most health indices. Most health indicators measured were low at the beginning and end of the study period, indicating poor health at these times, and levels peaked in August except for lymphocyte counts and lymphocyte stimulation by ConA and LPS. The decrease in lymphocyte counts and lymphocyte proliferation as well as the increase TGF-β in Atlantic menhaden caught in August in the White Oak together suggest that these fish were immunocompromised when temperature was highest. Similar findings in Atlantic menhaden collected from the Pamlico River suggest that many of the fish were also immunocompromised.

Since only water quality parameters were measured during this study, it possible that some of the changes observed were also influenced by other environmental factors. The presence of diseased Atlantic menhaden in the Neuse and Pamlico river estuaries during our collection was associated with the observed changes in health indices measured. Many of the fish caught in the Neuse River in August had numerous protozoa in the gills, kidney and heart. The protozoal infection observed histologically potentially explains some of the differences in health indices observed in these fish caught in August when compared with fish caught at other times of the year. The increase in lymphocyte proliferation and lymphopenia indicate that these fish caught in the Neuse were able to elicit an immune response to the protozoal and any other infection. Therefore, some of the variability in health indices observed during the study period was influenced by other environmental conditions, besides temperature.
Summary

Biochemical, physiological, and histopathological indicators have been used widely as bioindicators in assessing the health of organisms in response to environmental pollutants (Zelikoff et al., 2000; Mayer et al., 1992b) in the laboratory. But their application in field settings has been limited because of the difficulty in assigning causality to specific environmental factors.

The use of multiple bioindicators to assess the health of free-ranging fish ensures that the physiologic effects of numerous factors can be detected before effects are observed at the population, community and ecosystem levels. Active monitoring of water quality parameters and other environmental variables provide the potential exposure history needed to understand fluctuations in health indices. In this manner, disease surveillance in free-ranging fish species can serve as a valuable bioindicator for monitoring the health of our estuaries as well as an indicator of the health of commercially relevant fish species.

The suborganismal level bioindicators used in this study can be great assets to regulatory agencies because they can make predictions on fish health and the health of the aquatic ecosystem before detrimental effects on growth, reproduction and survival are detected. Therefore, unlike population, community, and ecosystem level research, these bioindicators can serve as early warning indicators of fish health and health of the aquatic ecosystem. However, extensive additional work is needed to identify the most appropriate species for estuarine health monitoring, as well as develop the baseline clinical values needed to monitor the health of individual fish species. The information
presented in this study is not available for many aquatic species, and rare for migratory estuarine-dependent species. The authors realize that the information provided in this study is not comprehensive in itself because there are many other environmental variables besides water quality that influence the health of aquatic species. But, we hope that the information provided in this study will augment the sparse information available on free-ranging estuarine species.

ACKNOWLEDGEMENTS

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Table 1. Sampling schedule for Atlantic menhaden for 2002. \( n \) = number of pooled samples for the Neuse and Pamlico River estuaries. \( n \) = number of fish for the White Oak River estuary.

<table>
<thead>
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<th>Collection Dates</th>
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<th>Estuarine System</th>
<th>( n )</th>
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<td>June 8</td>
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Table 2. Morphometric, organosomatic, hematological, and immune function parameters for Atlantic menhaden from the White Oak, Neuse and Pamlico river estuaries in 2002.

<table>
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<th>August</th>
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<th>October</th>
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<td>Neuse</td>
<td>Pamlico</td>
<td>White Oak</td>
<td>Neuse</td>
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<td>Fork Length (mm)</td>
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<td>109.4 ± 30.8</td>
<td>158.3 ± 6.9</td>
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<td>Body Weight (g)</td>
<td>43.3 ± 6.0</td>
<td>23.6 ± 20.4</td>
<td>56.4 ± 8.2</td>
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<td>5.2 ± 0.5</td>
<td>6.4 ± 0.4</td>
<td>6.8 ± 0.8</td>
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<td>Spleno-somatic index</td>
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<td>0.9 ± 0.1</td>
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<tr>
<td>PCV (%)</td>
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<td>PCV (%)</td>
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<td>PCV (%)</td>
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<td>WBC/Thrombo (µl)</td>
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Note: Mean ± standard deviation.
Table 3. Monthly plasma chemistry values for Atlantic menhaden collected from the White Oak, Neuse and Pamlico River estuaries in 2002.

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<td>White Oak</td>
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<td>Pamlico</td>
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<td>282</td>
<td>52</td>
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Fig. 1. Map of the White Oak River estuary. Atlantic menhaden were collected near the Highway 24 bridge in Swansboro from June to October in 2002.
Fig. 2. Map of the Pamlico and Neuse River estuaries showing creeks sampled in 2002.
Fig. 3. Mean fork length (mm) for Atlantic menhaden collected by cast net in the White Oak (▲), Neuse (●), and Pamlico (■) River estuaries during 2002. Values are means ± standard error.
Fig. 4. Water quality variables (salinity, pH, temperature and dissolved oxygen) measured in the White Oak, Neuse, and Pamlico River estuaries in 2002. Surface (<1m) water quality measurements were obtained at the time of sampling from creeks in Table 1.
Fig. 5. Comparison of (a) liver-somatic and (b) spleno-somatic indices for Atlantic menhaden collected in the White Oak (□), Neuse (■), and Pamlico (■) River estuaries. Significant differences among the river systems for each month are indicated by different letters. In July, both SSI and LSI were significantly higher in the Pamlico River than the White Oak River ($P < 0.05$). Sample sizes for each river are in Table 1. Values are means ± standard error.
Fig. 6. Mean monthly lesion scores for Atlantic menhaden collected in the White Oak (□), Neuse (■), and Pamlico (▲) Rivers from June to October 2002. Sample sizes (n) are the same as in Table 1 except for the Pamlico River where n = 11 for July.
Fig. 7. Section of kidney showing a multinucleate spore, consistent with *Ichthyophonus* sp. A focal area of renal tubules and intervening hematopoietic tissue has been expanded and replaced by this large parasite granuloma. The multinucleate spore, consistent with *Ichthyophonus* sp., has a thick wall containing multiple 1 to 5 micron diameter bodies with pale, vacuolar cytoplasm and deeply staining nuclei. This fungus-like parasite, found primarily in kidney tissue in Atlantic menhaden caught in this study, was nearly always well encapsulated by variably thick layers of fibroblasts and epithelioid macrophages admixed with smaller numbers of lymphocytes and eosinophilic granule leukocytes. H & E.
Fig. 8. Section of heart tissue showing myxosporean spores consistent with *Kudoa* sp. from Atlantic menhaden caught in the White Oak River. (A) The myocardial interstitium is expanded and replaced by aggregates of 1 to 2µm protozoal spores. These spores incited little to no surrounding host inflammatory response. (B) Heart myocardium showing intense granulomatous inflammatory response to the *Kudoa*-like spores. The aggregate of spores occupied a substantial proportion of the myocardium. H & E.
Fig. 9. Blood parameter measurements for Atlantic menhaden collected in the White Oak (□), Neuse (■), and Pamlico (■) River estuaries during 2002. Months for which significant differences ($P < 0.05$) among the three river estuaries were observed are denoted by different letters. Values are means ± standard error.
Fig. 10. Monthly variability in TGF-β:β-actin ratios for Atlantic menhaden caught in the White Oak River estuary. Means that are significantly different (p < 0.05) have different letters. Sample sizes are the same for each month (n = 8). Values are means ± standard error.
Multi-tiered Health Assessment of Atlantic menhaden (*Brevoortia tyrannus*) in the Pamlico River, North Carolina (Fall 2001 and 2002)

**ABSTRACT**

Fish kills and ulcerative lesions in Atlantic menhaden (*Brevoortia tyrannus* Latrobe) and other estuarine species along the Atlantic coast of the United States have heightened public interest in the health of aquatic ecosystems and their relevance to human health. During the fall seasons of 2001 and 2002, Atlantic menhaden were collected from several creeks in the Pamlico River estuary, NC, to investigate recent fish kills and ulcerative skin lesions. High lesion prevalence was associated with the Atlantic menhaden kills in the fall of 2001, while there were no fish kills in the fall of 2002, and lesion prevalence was very low. Indicators of tissue damage (histopathological analyses of gills, heart, liver, intestine, and anterior kidney), nutritional status and exposure to environmental stressors (liver-somatic index), immune status and disease (TGF-β mRNA production, lymphocyte mitogenesis, hematology, plasma chemistry, and spleno-somatic index) were compared between menhaden with and without lesions over the two years. Atlantic menhaden with ulcerative lesions had significantly higher splenic mononuclear cell TGF-β mRNA levels, spleno-somatic indices, liver-somatic indices, neutrophil and monocyte counts, and significantly lower lymphocyte counts, thrombocyte counts, hematocrit values, plasma proteins and calcium than those without. The health indicators used in this study have been useful for identifying differences in the health status of Atlantic menhaden with and without ulcerative lesions in the highly impacted Pamlico River.
INTRODUCTION

Fish kills of Atlantic menhaden have been reported from Maine to Florida since the 1800s (Goode and Atwater, 1880) and ulcerative diseases have been found worldwide in many estuarine species (Sindermann, 1988). In the United States, ulcerative lesions have been documented for many years in estuaries from Maryland to Florida (Sindermann, 1988; Noga and Dykstra, 1986; Noga et al., 1988; Dykstra et al., 1989; Levine et al., 1990b; Kane et al., 1998; Blazer et al., 2002; Law, 2001) and these lesions have been found in many economically important estuarine species, including Atlantic menhaden (Levine et al., 1990a).

The Atlantic menhaden is an estuarine-dependent pelagic species that is important to the fishing industry as a source of fish oil, fish meal, and food fish for other commercially important species (Vaughan and Smith, 1991). Menhaden spawn off-shore and larvae are transported into estuaries by ocean currents where they metamorphose into juveniles (Ahrenholz, 1991). During the period spent in the estuaries, Atlantic menhaden are exposed to numerous natural and anthropogenic factors that potentially impact their immune function and alter their susceptibility to infection by opportunistic pathogens.

Large fish kills of juvenile Atlantic menhaden with ulcerative lesions were observed in 1984 in the Pamlico River estuary, North Carolina (Noga and Dykstra, 1986; Ahrenholz et al., 1987). These ulcerative lesions were described as focal ulcers that penetrate deep
beneath the basement membrane of the affected epithelial cell layers (Noga et al., 1988; Dykstra and Kane, 2000). Lesions are usually singular with a red margin and are found on the ventrum of affected fish near the anus (Noga and Dykstra, 1986; Levine et al 1990a). These lesions were termed “ulcerative mycosis” because most contained invasive fungal (Oomycete) hyphae of the genera *Aphanomyces* and *Saprolegnia*, surrounded by severe granulomatous inflammation (Dykstra et al., 1986; Noga and Dykstra, 1986).

Apart from the Oomycetes, other pathogens have been suggested to play a role in the development of ulcerative lesions in Atlantic menhaden. In the early 1990s attention focused on the potential role of the dinoflagellate *Pfiesteria piscicida* in the development of lesions (Burkholder et al., 1992; Burkholder et al., 1995a; Lewitus et al., 1995; Noga et al., 1996). Most recently the contribution of the Oomycete, *Aphanomyces invadans* to the development of the lesions has been validated (Blazer et al., 2002). The potential contribution of the myxosporean protozoal organism, *Kudoa clupeidae*, to the development of some menhaden lesions has also been suggested (Reimschuessel et al., 2003). Although these efforts have improved our understanding of the development of ulcerative lesions Atlantic menhaden, little information is available describing the health status of free-ranging Atlantic menhaden with and without ulcerative lesions and the factors that may make these fish more susceptible to such diseases.

During the fall of 2001 and 2002, Atlantic menhaden were collected from several creeks in the Pamlico River estuary, North Carolina. These fish were collected during major fish
kill events (2001) and during outbreaks of ulcerative lesions not associated with any major kill activity (2002). An integrated bioindicator approach was used to compare the health indices of Atlantic menhaden with gross ulcerative lesions to those without ulcerative lesions in the Pamlico River estuary. This was accomplished by measuring a suite of health indicators and water quality parameters.

MATERIALS AND METHODS

A three-tiered suite of bioindicators at the tissue, cellular, and molecular levels was used to contrast the health of Atlantic menhaden collected in the Pamlico River Estuary. Histopathology and two organosomatic indices (Tier I) were used to assess the general health status of Atlantic menhaden. Hematology, plasma chemistry and electrolytes provided an assessment of the biochemical status of collected fish (Tier II) and assays for transforming growth factor-beta (TGF-β) mRNA and lymphocyte mitogenesis provided a more specific assessment of menhaden immune function (Tier III). These bioindicators were then correlated with changes in water quality parameters, dissolved oxygen (DO), temperature, salinity and pH in the estuary.

Field Collection

Juvenile Atlantic menhaden were caught by cast net from October to November 2001 and September to October 2002 in several creeks in the Pamlico River estuary, NC (Fig. 1). In 2001, fish were collected from Broad Creek (October 3 and 8), Blounts Bay (October
5), Upper Goose Creek (October 8), Runyon Creek (October 18) and North Creek (November 1). In 2002, Atlantic menhaden were collected from Durham Creek (September 4 and October 23), Broad Creek (September 18 and 30) and Upper Goose Creek (October 17). Surface water quality measurements of DO, temperature, salinity, and pH were taken at the time of sampling and additional water quality data were obtained from the North Carolina Department of Environment and Natural Resources Division of Water Quality (NCDENR-DWQ) ambient monitoring stations in the river (Fig. 1).

**Blood Collection**

Menhaden were euthanized with an overdose of tricaine methanosulfonate (MS-222; Argent Chemical Laboratories, Redmond, Washington). Blood samples were collected from Atlantic menhaden at each study site for the following assays: lymphocyte mitogenesis, hematocrit, differential leukocyte counts, and plasma chemistry. For the lymphocyte mitogenesis assay, fish were bled from the caudal vein with sterile heparinized syringes (1 ml syringe, 22 gauge needle) and samples were placed in 10 ml vacutainers. Samples for hematology were collected similarly but samples were stored in 5 ml vacutainers. Both individual and pooled whole blood samples were used for hematological analysis and lymphocyte mitogenesis. Two to three fish were pooled for hematological analysis and three fish for lymphocyte mitogenesis. Fish samples were pooled to obtain the required quantity of blood necessary for each assay. For pooled samples from fish with ulcerative lesions, fish with similar stage gross lesions were combined. Both blood samples and fish were placed on ice and taken back to the lab for
further processing. Samples were processed within 24 h.

**Sample Processing**

Fork length (FL), body weight, liver weight, and spleen weight were measured and the presence of external lesions noted (Levine et al., 1990b). Fish with grossly visible skin ulcerative lesions were defined as “fish with lesions” and fish without noticeable gross lesions as “fish without lesions.” Samples of gill, heart, liver, intestine, gonads, spleen, and kidney were collected and fixed in 10% neutral buffered formalin for histopathology. In 2001, only gill and liver samples were collected for histopathology. Tissue samples of ulcerative skin lesions were also collected. Gonads were collected if visible and used to determine sex. Spleen samples were collected from fish obtained in Broad Creek, Blounts Bay, and North Creek for TGF-β quantitative RT-PCR in 2001. These samples were collected aseptically, pooled (10-20 fish) and placed in sterile cell culture media.

**Organosomatic Indices**

Liver-somatic indices (LSI) and spleno-somatic indices (SSI) were calculated as the organ weight (in mg) divided by the body weight (in mg; Goede and Barton, 1990). Only LSI were determined in 2001.

**Histopathology**

Fixed tissue samples were routinely processed, sectioned at 5 µm, stained with hematoxylin and eosin (H&E), and examined by light microscopy. Tissue sections were
examined by a single pathologist blinded to skin lesion status and evaluated using the following grading scheme: Grade 0 = no remarkable microscopic abnormalities, Grade 1 = very mild changes, Grade 2 = mild changes, Grade 3 = moderate changes, Grade 4 = moderately severe changes, and Grade 5 = severe, at the extreme range of pathology for the given lesion type (Hurty et al., 2002).

**Hematology**

Blood smears for differential leukocyte counts and whole blood samples were analyzed by the Clinical Pathology Laboratory at the North Carolina State University College of Veterinary Medicine (NCSU-CVM). Blood smears were stained with Wright-Giemsa stain (Volu-Sol, Inc., Salt Lake City, UT) and 100 leukocytes on each slide were identified. To determine white blood cells plus thrombocyte (WBC/thrombocyte) counts, cells were stained and diluted in Natt and Herrick’s solution and counted on a hemacytometer. The packed cell volume (PCV) or hematocrit values were determined by reading the packed cell percentages using microhematocrit tubes. Plasma total solids were measured with a refractometer (Leica Microsystems, Germany). The following substances were measured with a Hitachi 912 chemical analyzer (Roche Diagnostics, Indianapolis, IN): glucose, creatinine, phosphorus, calcium, total protein, albumin, aspartate aminotransferase (AST), creatinine kinase (CK), lactate dehydrogenase (LD), sodium (Na⁺), potassium (K⁺), chloride (Cl⁻), and bicarbonate (HCO₃⁻). The analyzer calculated albumin/globulin ratio, sodium/potassium ratio, and anion gap (AGAP). Anion gap was determined as follows: (Na⁺ + K⁺)-(Cl⁻ + HCO₃⁻).
**Lymphocyte Mitogenesis Assay**

The MTT assay used in this study was based on a modification of techniques described by Mosmann (1983), Hansen *et al.*, (1989) and Daly *et al.*, (1995) and published by Johnson *et al.* (2004). Blood samples were diluted 1:1 in PBS and centrifuged at 45 x g for 10 min in a swing-out rotor. The upper leukocyte layer was removed from the top of the red blood cell layer with a 1 ml pipette, diluted 1:1 in PBS and carefully layered over 2 ml of a 55% Percoll gradient (specific gravity 1.070 g ml$^{-1}$) in 0.15 M NaCl. Samples were centrifuged at 400 x g for 30 min at 22°C, the leukocyte rich interphase was collected and washed twice in 10 ml complete RPMI by centrifuging for 5 min at 300 x g. Cell suspensions were diluted in complete RPMI (RPMI-1640 plus 10% heat-inactivated fetal bovine serum, 100 U ml$^{-1}$ penicillin, 100 µg ml$^{-1}$ streptomycin, and 2 mM EDTA) to a concentration of 1.0 x 10$^6$ cells ml$^{-1}$ (5.0x10$^4$ cells/50 µl). Viable cell counts were performed with cells suspended in 0.2% trypan blue (Gibco Laboratories, Grand Island, NY).

The MTT (3, (4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide; M2128, Sigma, St. Louis, MO) was dissolved in sterile PBS at 5 mg/ml, sterilized by filtration and stored in a dark bottle at 4°C. Concanavalin A (Con A; C-0412, Sigma) and lipopolysaccharide (LPS; L-5262 from *Vibrio cholerae*, Sigma) were resuspended in PBS (1 mg/ml stock) and stored at –20°C. Both mitogens were diluted in complete RPMI to a final concentration of 10 µg/ml (LPS) and 2.5 µg/ml (Con A) before use. The mitogen doses and incubation times were optimized for Atlantic menhaden in preliminary experiments.
Stimulation indices ranged from 1.09-1.7 for Con A and 1.15-1.4 for LPS. These stimulation indices were similar to those obtained by Espelid et al., 2003).

All mitogenesis assays were performed in quadruplicate. Cell suspensions (1.0 x 10^6 cells ml⁻¹) were loaded into 96 well round-bottomed tissue culture plates at 50 µl/well. Immediately after leukocyte plating, 50 µl of complete RPMI with or without mitogen (control) was added to each well and samples were incubated in a humidified container at 27°C for 5 days. After the incubation period, 10 µl stock MTT (5 mg/ml PBS) was added to each well and the samples were incubated at 27°C for 4 h for MTT cleavage. After incubation, formazan precipitate was dissolved by adding 120 µl SDS/DMF solution (20% SDS/50% DMF, pH = 4.7) per well and plates were incubated overnight. Plates were read at OD₅₇₀ with reference wavelength of OD₆₃₀ using a microplate reader. Stimulation indices were calculated using the formula:

$$SI = \frac{\text{mean OD of stimulated culture}}{\text{mean OD of non-stimulated control culture}}$$

**TGF-β real-time RT-PCR**

Splenic mononuclear cell TGF-β transcription was determined by real-time RT-PCR procedures developed for Atlantic menhaden (Chapter 3). Spleen samples in complete RPMI were minced finely, resuspended in complete RPMI and centrifuged on two-step Percoll gradients (35% and 55% stock solution in 0.15 M saline; specific gravity 1.046 and 1.070 g ml⁻¹). Splenic mononuclear cells were harvested from the 35/55% interface, viable cell counts were performed with cells suspended in 0.2% trypan blue. Cell pellets
were lysed in Tri Reagent (Tri Reagent, Molecular Research Ctr, Cincinnati, OH) at 1ml per 5-10 \times 10^6 cells and stored at \(-80^\circ\text{C}\).

The RNA isolation and reverse transcription were performed following published procedures (Harms et al., 2000a). Total RNA was isolated by the guanidine thiocyanate method (Tri Reagent) following kit instructions. The RNA pellet was washed with 75% ethanol and resuspended in sterile diethyl pyrocarbonate (DEPC)-treated water. Splenic mononuclear cell RNA was resuspended to a concentration of 5 \times 10^4 cell equivalents/\mu l. Messenger RNA was reverse transcribed using Superscript II RT (Gibco BRL, Gaithersburg, Maryland) to cDNA with oligo dT\textsubscript{15} priming of 3 \times 10^6 cells equivalent. Samples of cDNA were stored at \(-20^\circ\text{C}\). Negative RT controls were run in parallel.

Real-time PCR was performed using primers developed specifically for Atlantic menhaden and procedures described in Chapter 3. Amplification of reverse transcribed cDNA samples and standards was performed in parallel. The PCR efficiencies were above 90% and correlation coefficient above 0.996. Results are reported as the ratio of TGF-\beta/\beta-actin.

**Statistical Analyses**

Comparisons between Atlantic menhaden with and without ulcerative lesions were analyzed using analysis of variance (ANOVA). Comparisons were performed with disease and year as main effects and included their interaction. The Statistical Analysis System (SAS; SAS Institute Inc, Cary, NC) was used for all analyses. A visual
assessment of residual plots was used to determine homogeneity of variance. To satisfy homogeneity of variance, neutrophil percentages and counts, WBC/thrombocyte counts, K, AST, and CK were log transformed, and monocyte percentages and counts were square root transformed. Significant ANOVAs ($P < 0.05$) were followed by Tukey’s multiple comparison tests (Neter et al., 1996). Figures depict sample mean ± standard error (SE).

RESULTS

Lesion Prevalence

Atlantic menhaden with ulcerative lesions were found mainly during the fall of both years (Fig. 2) but lesion prevalence was much higher in 2001 than 2002 and higher in the upper creeks than the lower creeks (Fig. 3). In October of 2001, Atlantic menhaden were collected during several fish kill events and the prevalence of fish with lesions was very high; All Atlantic menhaden collected in Broad Creek had lesions while no fish with lesions were found in North Creek. Only fish without lesions were collected in Upper Goose Creek, although lesions had been observed on a few fish collected in the creek (October 8). Both fish with and without lesions were collected in Blounts Bay and Runyon Creek. The majority of fish collected in Runyon Creek did not have lesions; however, a few fish with ulcerative lesions and fish with scars from apparently healed lesions were collected in the creek. Collections during the fall of 2002 were not associated with any major fish kills, but Atlantic menhaden with and without gross
ulcerative lesions were found in all creeks sampled during that period. Lesion prevalence was not as high in 2002 as in 2001 and severity of the lesions was not as pronounced. Most of the fish with lesions collected in 2002 had varying stages of ulcerative lesions from mild to severe. Few fish with healed lesions were collected. These lesion stages have been described previously (Noga et al., 1988).

**Organosomatic Indices**

**Liver-somatic index**

Liver-somatic indices were significantly different between Atlantic menhaden with and without gross ulcerative skin lesions, and between the two collection years (Table 1; Fig. 4). Atlantic menhaden caught in 2001 (with and without lesions) had significantly lower LSI than those caught in 2002 (Fig. 4). Although LSI were not significantly different between fish collected in 2001 with lesions (5.27 ± 0.49; n = 20) and those without (5.36 ± 0.31; n = 30; P = 0.0561; Fig. 4), LSI were significantly higher in fish with lesions (n = 5, 7.38 ± 0.86) caught in Blounts Bay than fish without lesions (n = 5; 4.55 ± 0.39; P = 0.018). Liver-somatic indices were low for Atlantic menhaden (all fish with lesions) caught in Broad Creek in 2001 (n = 15; 4.57 ± 0.47). In 2002, LSI were significantly higher for fish with ulcerative lesions (8.82 ± 0.48; n = 18) than fish without lesions (6.85 ± 0.31; n = 31; Fig. 4). Body weight was not significantly different between fish with and without lesions. Fish collected in Durham Creek were larger than fish collected from the other creeks (Table 1).
Spleno-somatic index

Spleno-somatic indices were only measured in 2002. Spleno-somatic indices were significantly higher in samples obtained from Atlantic menhaden with lesions (0.86 ± 0.06; $n = 41$) than those without lesions (0.74 ± 0.05; $n = 34$; $P = 0.0028$; Fig. 5).

Histopathology

Pamlico 2001

In the fall of 2001, a total of 51 fish were collected for histopathology. There were 21 fish with ulcerative lesions and 30 without ulcerative lesions.

Liver

Three types of lesions were present in the liver: hepatocellular vacuolation, hepatic congestion and lymphocyte aggregates. Both hepatocellular vacuolation and lymphocyte aggregates were found in menhaden with and without lesions. There were six fish with mild to moderate, diffuse, clear vacuolation of hepatocytes (Fig. 6). Fish with lesions ($n = 4$) had moderate vacuolation and fish without lesions ($n = 2$) had mild vacuolation. Three fish had scattered lymphocyte aggregates (one with lesions and two without lesions). Five fish with lesions had mild to moderate focal to multifocal areas of hepatic congestion (Fig. 7). No fish without lesions had hepatic congestion.
**Gills**

Three fish with gill lesions were collected. One fish with ulcerative lesions had a moderate infiltration of eosinophilic granule leukocytes, another fish that had ulcerative lesions had moderate chronic branchitis, and a third fish without ulcerative lesions had moderate numbers of gill protozoal parasites but little to no associated inflammatory reaction.

**Pamlico 2002**

In the fall of 2002, 50 fish were collected for histopathology, 18 with lesions and 32 without lesions. There were no remarkable, consistent microscopic lesions in the collected tissues that would distinguish Atlantic menhaden with ulcerative lesions from those without ulcerative lesions.

**Liver**

Lesions observed in liver tissue include: lymphocyte aggregates, hepatocellular vacuolation, and granulomas. Granulomas were found in one fish without ulcerative lesions. Mild to moderate vacuolation was found in a seven fish, 3 with lesions and 4 without. Six fish had mild lymphocyte aggregates, 4 with lesions and 2 without lesions. One fish without lesions had severe parasitic granulomas in the liver.
**Gills**

There were very few microscopic changes noted in the gills. Four fish without lesions had moderate lamellar thickening and one fish with lesions had a mild protozoal infection.

**Kidney**

Pigmented macrophage aggregates (PMAs) were the most common pathological changes found in the kidney of Atlantic menhaden with ulcerative lesions \((n = 4)\) and fish without lesions \((n = 7)\). One fish with lesions had severe neutrophilic inflammation of the hematopoietic tissue, and one fish without lesions had moderate granulomas. One fish with lesions and three fish without lesions had spores of *Ichthyophonus* sp. Both the renal tubules and hematopoietic tissue were expanded and replaced by these spores. The spores were visible grossly as clear spheres found in association with the kidney. *Ichthyophonus* sp. was only found in the kidneys of Atlantic menhaden in this study.

**Spleen**

Almost every spleen had PMAs. Fourteen fish with lesions (approximately 80%) and 22 fish without lesions (approximately 70%) had mild to moderate PMAs. The spleen of one fish without lesions had moderate parasitic granulomas and another had splenic congestion. A mild trematode infestation was observed in one fish with ulcerative lesions.
Hematology

Hematological differences were observed between Atlantic menhaden with and without lesions collected during the study period (Tables 2 and 3). Absolute counts of neutrophils, monocytes, eosinophils, lymphocytes, thrombocytes, as well as hematocrit percentages and total plasma protein concentrations were not included for the different creeks in 2001 (Table 2) because of the small sample sizes. Eosinophils were very rare; therefore, percentages were also not included for the different creeks in 2001 and 2002 (Table 2 and 3).

Counts of WBC/thrombocytes were significantly lower for Atlantic menhaden with lesions than those without lesions ($42.79 \pm 4.42$ and $73.08 \pm 4.52$ respectively; $P < 0.0001$). Counts were significantly lower in 2001 than 2002 ($P = 0.0003$; Table 4). Fish with lesions had higher neutrophil and monocyte percentages and counts, and lower lymphocyte percentages and counts than those without lesions (Table 4). Fish with lesions had significantly higher neutrophil counts than those without ($4.84 \pm 0.6$ and $3.15 \pm 0.72$ respectively; $P = 0.002$; Table 4). Neutrophil percentages were highest for Atlantic menhaden with lesions caught in Broad Creek and Blounts Bay in 2001 (Table 2). Monocyte counts were significantly higher in samples obtained from Atlantic menhaden with lesions than those without ($4.49 \pm 0.91$ and $1.81 \pm 0.30$ respectively; $P = 0.0068$; Table 4). Percentages were highest for fish with lesions caught in Blounts Bay and Durham Creek and lowest for those caught in Runyon Creek (Table 2 and 3).

Lymphocyte counts were significantly lower in fish with lesions than fish without lesions...
Eosinophil counts were very low in Atlantic menhaden caught in this study (Table 4).

Thrombocyte counts and percentages were significantly different for Atlantic menhaden with and without lesions as well as fish between the two years. Atlantic menhaden with lesions had lower thrombocyte counts than fish without lesions (18.76 ± 3.53 and 38.1 ± 3.74 respectively; \( P = 0.0006; \) Table 4). Percentages were also lower for both fish with and without lesions caught in Blounts Bay (2001) than all other creeks (Table 2). This significant creek difference may have contributed to the significant difference observed between the two years. Hematocrit values were significantly lower in Atlantic menhaden with lesions than menhaden without lesions (28.05 ± 2.29 and 44.61 ± 1.06 respectively; \( P < 0.0001; \) Table 4). Total plasma protein was significantly lower in fish with lesions than those without lesions (2.42 ± 0.02 and 2.77 ± 0.06 respectively; \( P < 0.0001; \) Table 4).

**Plasma chemistry**

Plasma electrolytes were the only plasma chemistries analyzed from samples collected in 2001. These were not included in the comparisons because they were limited to two locations and only fish without lesions, therefore, comparisons were only performed for samples collected in 2002 (Table 5).

Significant differences in calcium, total protein, albumin and globulin levels were observed between Atlantic menhaden with and without lesions collected in 2002.
Calcium, total protein, albumin, and globulin levels were significantly lower in fish with ulcerative lesions than fish without lesions (Table 5). No significant differences in the other plasma chemistries measured were observed between fish with and without lesions (Table 5).

**Lymphocyte mitogenesis**

Lymphocyte response to mitogens ConA and LPS were not statistically significant between Atlantic menhaden with and without lesions ($P > 0.05$; Table 1).

**TGF-β**

Splenic TGF-β: β-actin ratios were measured in three creeks, Broad Creek, Blounts Bay and North Creek in 2001 only (Table 1). Atlantic menhaden with ulcerative lesions had significantly higher levels of TGF-β mRNA than those without ($P = 0.0113$; Fig. 8). Production of TGF-β was highest in Broad Creek and lowest in North Creek (Table 1). Spleen samples from both Broad Creek ($n = 7$) and Blounts Bay ($n = 4$) were only from fish with lesions, while those from North Creek ($n = 11$) were only from fish without lesions.

**Water quality**

Water quality data were obtained from the ambient monitoring stations (Fig. 1). Although water quality data were available at 1 and 2m depth, data were only consistently collected at 1m depth for all the monitoring stations. Therefore, surface water quality data (1m depth) was used for this study. Both dissolved oxygen and pH levels were lowest in
October 2002 (site 3; Fig. 9), and salinity was lowest in September (2001) and October (2002; Fig. 9). Although there were differences in the water quality variables observed at the different monitoring sites, there were no clear patterns that would explain the ulcerative lesions found in Atlantic menhaden.

**DISCUSSION**

There were marked differences in the prevalence of lesions and fish kill events observed between the two collection years. In 2001, 48 estuarine fish kill events were reported by NCDENR-DWQ in North Carolina. Of the total, 23 events were in the Pamlico river basin, and Atlantic menhaden were associated with 14 of these kills. Atlantic menhaden with ulcerative lesions were observed in 10 of these kills, five occurred in July and five in the fall (September and October). Although comparatively less than the number of fish kills in the Neuse River estuary, NC (37 fish kills) during the same period of time, figures for 2001 represent an increase over the number of fish kill events in the system noted since 1996 (NCDENR-DWQ, 2001). In contrast, there were only 16 estuarine kills of which 6 were in the Pamlico River estuary (4 in the Neuse River). Only one of those occurring in the Pamlico River estuary involved Atlantic menhaden with ulcerative lesions (NCDENR-DWQ, 2002).

**Liver-somatic index**

Liver-somatic indices may vary naturally with food availability, sexual maturation and life history (Goede and Barton, 1990). The variability in LSI observed in this study likely reflects both the health and nutritional state of Atlantic menhaden and the local
availability of specific dietary constituents. It is possible that Atlantic menhaden with lesions and low LSI, mainly those found in Broad Creek, may be food limited. Decrease in liver size has been observed in fish exposed to contaminants resulting in reduction in energy stores such as liver glycogen (Heath, 1995). Heath (1995) proposed the hypothesis that a decrease in LSI may be observed in fish limited by food or exposed for a short duration to pollutant stress, while chronic exposures to stress when food is not limited and feeding is normal would cause an increase in LSI. The higher LSI in Atlantic menhaden with ulcerative lesions caught in Blounts Bay (2001) and those caught in 2002 may reflect hyperplasia or hypertrophy of the hepatocytes. This may be an adaptive response to increase the capacity of the liver to detoxify contaminants (Heath, 1995).

**Spleno-somatic index**

The significantly higher SSI observed in Atlantic menhaden with ulcerative lesions may be due to hypertrophy or proliferation of leukocytes (Anderson, 1990). Splenomegaly has been reported in other diseased fish and is considered an indicator of disease and immune functional response in teleosts (Anderson et al., 1982; Goede and Barton, 1990). Increased SSI has been reported in juvenile rainbow trout, *Oncorhynchus mykiss* exposed to *Aeromonas salmonicida*, (Johansen et al., 1994) and other teleosts exposed to contaminants (Adams et al., 1992). Thus, SSI appears to be a good marker of splenic dysfunction and, by extension, a relatively good indicator of overall fish health.
Histopathology

Histopathology performed on the major internal organs confirmed that Atlantic menhaden in the Pamlico River estuary did not appear to have any major predisposing disease conditions that would lead to ulcerative lesions. Also, examination of sections of gill, liver, spleen, kidney, intestinal tract, and gonads did not distinguish between fish with and without lesions. Histopathology did not provide a sensitive enough tool to detect changes in the hematopoietic tissues that might signal immunocompromise, but inflammatory changes were reflected in the hemogram. This underscores the need for a suite of bioindicators at multiple levels of specificity or biological organization.

Only a few fish with gill lesions were collected and there were no apparent differences in the occurrence of gill pathology in fish with and fish without ulcerative lesions. The absence of gill changes in fish with ulcerative lesions suggest that their movement in the estuary did not carry them through any major environmental contaminants or other severe environmental changes during their residence in the Pamlico. A few fish had noticeable infiltration of secondary lamellae by small to moderate numbers of mixed inflammatory cells, characterized by lamellar thickening, atrophy/clubbing and, occasionally, fusion of two or more secondary lamellae. The presence of lamellar fusion due to hyperplasia of undifferentiated epithelial cells is considered a non-specific lesion associated with both infectious and non-infectious disease processes (Hinton et al., 1992).

Vacuolation of hepatocytes likely reflects nutritional stress, typical of many wild caught fish specimens, leading to fatty change in the liver. Chronic exposure to contaminants,
and increased storage products, such as glycogen or vitellogenin, may also affect the morphological appearance of hepatocytes (Hinton and Lauren, 1990). Hepatic congestion was only found in fish with lesions caught in 2001. Hepatocyte atrophy and cell swelling may reflect changes in oxygen tension, nutritional imbalances (fat/glycogen storage), or sub-lethal influences of toxins causing increased smooth endoplasmic reticulum due to enzyme induction, damage to mitochondria, or changes in protein production by rough endoplasmic reticulum (Heath, 1995).

**Hematology**

Many of the hematological parameters were significantly altered in Atlantic menhaden with ulcerative lesions. Lymphocytopenia, monocytosis, and neutrophilia have been observed in many other teleosts during stressful events and disease outbreaks (Ellsaesser and Clem, 1986; Pickering and Pottinger, 1987; Ellsaesser et al., 1985). Lymphocytopenia, monocytosis, and neutrophilia suggest a systemic, non-specific immune response, and have also been observed in our laboratory studies with Atlantic menhaden injected with the synthetic glucocorticoid, triamcinolone (Chapter 3).

Cortisone and ACTH caused lymphocytopenia and thrombocytopenia in rainbow trout *Oncorhynchus mykiss* (Blaxhall, 1972). Decreased thrombocyte and lymphocyte counts have also been observed in brown trout *Salmo trutta* and rainbow trout chronically stressed by crowding (Pickering and Pottinger, 1988). These changes in circulating levels of leukocytes are consistent with the suppression of the immune system and increased susceptibility to disease mediated by the hypothalamic-pituitary-interrenal axis (HPI).
The low hematocrit values observed in Atlantic menhaden with ulcerative skin lesions have also been reported in bacterial infected rainbow trout, *Oncorhynchus mykiss* (Barham *et al.*, 1980), in gilthead seabream, *Sparus aurata*, following simulated disturbance stress (Pages *et al.*, 1995), and rainbow trout exposed to copper effluent (Dethloff *et al.*, 2001). The decrease in hematocrit indicates a decrease in oxygen carrying capacity (Heath, 1995; Houston, 1990) and the loss of blood and inflammation from the open skin lesions in Atlantic menhaden may be partially responsible.

The low concentrations of total plasma protein, albumin, and globulin have also been detected in other diseased fish (Barham *et al.*, 1980; Hille, 1982) and since most of the calcium present in plasma is bound to albumin, the significantly lower calcium concentrations in fish with lesions are expected. Low total plasma protein has been shown to reflect infectious disease, kidney damage, and nutritional imbalance (Wedemeyer *et al.*, 1984). Gilthead seabream showed a reduction in plasma proteins after stress of pursuing animals with a net to simulate disturbance associated with aquaculture tasks (Pages *et al.*, 1995). The decrease in plasma proteins could result from an increase in protein catabolism (Mazeaud *et al.*, 1977) or possibly protein degradation associated with immunological responses (Pages *et al.*, 1995).

The lack of significant differences in muscle enzymes and other hematological parameters observed between fish with and without lesions may reflect our criterion used to define “fish with and without lesions.” Since the presence of gross ulcerative skin lesions was used to define fish with lesions, it is possible that fish without gross
ulcerative skin lesions may be at different stages of lesion development that are not yet obvious, grossly. This may be represented in some of the variability observed in several of the parameters measured. Also, variability in health parameters observed in fish with gross ulcerative skin lesions may reflect the severity of the lesions (e.g. early vs late stage lesions).

**TGF-β**

Splenic mononuclear cell TGF-β: β-actin mRNA ratios were significantly elevated in Atlantic menhaden with lesions relative to those without lesions. TGF-β is a cytokine that plays a central role in immunoregulation. Its immunomodulatory effects are primarily suppressive, although it has some pro-inflammatory effects. It down-regulates T and B cell proliferation, macrophage activation and macrophage respiratory burst (Ruscetti and Palladino, 1991). The elevated production of TGF-β observed in Atlantic menhaden with gross ulcerative lesions, taken in context with the other health parameters, is indicative of a suppression of the immune system. Increased production of TGF-β is important in the suppression of activated inflammatory cells, which is essential in the resolution of an inflammatory response (McCartney-Francis and Wahl, 1994). Therefore, it is likely that Atlantic menhaden with lesions do possess an active inflammatory response and are capable of regulating that response to prevent further tissue damage and promote healing.

**Water quality**

The timing of events is crucial to the disease outbreaks within the estuary. The observed temporal differences in salinity between the two years, with the occurrence of low
salinity in September 2001 versus November 2002 (Fig. 9), may partially explain some of
the differences observed in lesion prevalence between the two years. Usually, the
prevalence of Atlantic menhaden in the estuaries declines in the late fall as fish move
offshore and the temperature drops. In 2002 we caught very few fish by cast net in late
October, and sampling was terminated when fish could no longer be obtained. Therefore,
it is possible that the later occurrence of the low salinity events in 2002, when most of the
fish had already begun their migration out of the estuary, may have reduced the
prevalence of lesion events in the Pamlico River during that year.

Studies in the 1980s found low salinities to be associated with the ulcerative lesions in
Atlantic menhaden (Dykstra et al., 1986; Levine et al., 1990a) and low salinities favored
the growth of Aphanomyces (Dykstra et al., 1986). Also, abrupt salinity changes have
been reported to cause significant decreases in serum electrolytes and osmotic pressure in
Atlantic menhaden. Atlantic menhaden serum sodium and chloride concentrations,
obtained from pooled blood samples, decreased significantly after abrupt transfer from
high to low salinity (35 ppt to 3.5 ppt; Engel et al., 1987). Low salinities have also been
observed in the absence of ulcerative lesions (Dykstra et al., 1986), therefore, other
environmental factors are involved in the development of ulcerative lesions in menhaden.
The influence of many factors, both endogenous and exogenous, in the development of
ulcerative lesions in Atlantic menhaden has been reiterated by researchers since the early
1980s.
Atlantic menhaden examined during this study were collected by cast-net. Consequently, we are obtaining a sub-sample of the population at a single point in time, with an unknown exposure history. The movement of fish in the estuary carries them through different habitats and each location visited during their stay in the estuary poses specific risks. The absence of severe hepatic pathology and gill pathology, two indicators of exposure to some contaminants, provides some indication that exposure to these agents is minimal. However, to fully understand the factors that contribute to the incidence of ulcerative lesions in an estuary the exposure history of animals known not to have ulcerative lesions at the beginning of the observation period must be followed over time. Effective methods for conducting sentinel studies in cages and for marking and monitoring (e.g. telemetry) the movement of menhaden in the estuary need to be explored to help investigators more clearly define the factors associated with the development of ulcerative lesions and other health problems.

**SUMMARY**

Since the 1980s researchers have been investigating the link between ulcerative lesions in Atlantic menhaden and environmental factors. The mobility of fish, complexity of estuarine systems and the numerous factors, both endogenous and exogenous, which can affect the health of estuarine species, make understanding disease outbreaks in estuarine species challenging. The variability in health parameters observed in Atlantic menhaden from the different creeks with ulcerative lesions could be a consequence of the
environmental conditions present before or at the time of collection. Due to the limited water quality data obtained during the study period, no correlations can be made between the health parameters and water quality measurements. Notwithstanding, the various health indicators used in this study have provided plausible reasons for some of the differences in Atlantic menhaden health observed between the two years and at the different locations.

The approach of using multiple health indicators at three different levels of biological organization is aimed at better understanding the health status of Atlantic menhaden in the Pamlico River during the development of ulcerative lesions. Atlantic menhaden with ulcerative lesions had significantly higher spleno-somatic indices, liver-somatic indices, neutrophil and monocyte counts, splenic mononuclear cell TGF-β mRNA levels, and significantly lower lymphocyte counts, thrombocyte counts, hematocrit values, plasma proteins and calcium than those without. These health indicators have identified differences in the non-specific and specific response of the Atlantic menhaden immune system and overall health, thus increasing our knowledge of the changes in the health of Atlantic menhaden in the Pamlico River.

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Table 1. Morphometric, organosomatic, and immune function parameters for juvenile Atlantic menhaden sampled in 2001 and 2002. \( n \) = number of pooled samples.

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<td></td>
<td>Blow Bay (n = 5)</td>
<td>Broad Creek (n = 15)</td>
<td>Blow Bay (n = 5)</td>
<td>Upper Goose Creek (n = 10)</td>
</tr>
<tr>
<td>Fork length (mm)</td>
<td>81.0 ± 2.2</td>
<td>88.7 ± 3.0</td>
<td>83.0 ± 5.7</td>
<td>92.0 ± 8.4</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>7.2 ± 1.3</td>
<td>9.9 ± 1.1</td>
<td>7.8 ± 1.6</td>
<td>11.6 ± 3.2</td>
</tr>
<tr>
<td>Liver-somatic index</td>
<td>7.4 ± 1.9</td>
<td>4.6 ± 1.8</td>
<td>4.6 ± 0.9</td>
<td>4.2 ± 0.6</td>
</tr>
<tr>
<td>CONA (SI)</td>
<td>1.0 ± 0.2</td>
<td>1.0 ± 0.2</td>
<td>1.3 ± 0.5</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>LPS (SI)</td>
<td>0.6 ± 0.5</td>
<td>1.1 ± 0.4</td>
<td>0.7 ± 0.4</td>
<td>1.0 ± 0.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variable</th>
<th>2002 Lesioned</th>
<th>2002 Non-lesioned</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Broad Creek (n = 8)</td>
<td>Durham Creek (n = 5)</td>
</tr>
<tr>
<td>Fork length (mm)</td>
<td>88.0 ± 2.2</td>
<td>97.8 ± 3.6</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>9.7 ± 0.9</td>
<td>13.7 ± 1.5</td>
</tr>
<tr>
<td>Liver-somatic index</td>
<td>8.9 ± 1.3</td>
<td>9.7 ± 2.8</td>
</tr>
<tr>
<td>Spleno-somatic index</td>
<td>1.2 ± 0.5</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>CONA (SI)</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>LPS (SI)</td>
<td>1.0 ± 0.1</td>
<td>0.9 ± 0.0</td>
</tr>
</tbody>
</table>
Table 2. Hematology values for Atlantic menhaden sampled from all sample sites in the fall of 2001. \( n = \) number of pooled samples.

<table>
<thead>
<tr>
<th></th>
<th>Broad Creek</th>
<th>Blounts Bay</th>
<th>Upper Goose Creek</th>
<th>North Creek</th>
<th>Runyon Creek</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ±SD</td>
<td>n</td>
<td>Mean ±SD</td>
<td>n</td>
<td>Mean ±SD</td>
</tr>
<tr>
<td>Lesioned Neutrophils (%)</td>
<td>23.9 17.3 15</td>
<td>25.0 18.0 4</td>
<td>5.7 3.1 3</td>
<td>6.8 2.6 5</td>
<td></td>
</tr>
<tr>
<td>Non-lesioned Neutrophils (%)</td>
<td>7.2 9.1 5</td>
<td>4.9 1.5 5</td>
<td>42.2 10.4 5</td>
<td>3.8 3.6 5</td>
<td></td>
</tr>
<tr>
<td>Lesioned Lymphocytes (%)</td>
<td>69.0 5.0 5</td>
<td>42.2 12.9 5</td>
<td>49.4 12.4 10</td>
<td>43.6 19.3 5</td>
<td></td>
</tr>
<tr>
<td>Non-lesioned Lymphocytes (%)</td>
<td>7.2 9.1 5</td>
<td>4.9 1.5 5</td>
<td>42.2 10.4 5</td>
<td>3.8 3.6 5</td>
<td></td>
</tr>
<tr>
<td>Lesioned Monocytes (%)</td>
<td>14.8 10.0 14</td>
<td>20.3 7.6 4</td>
<td>3.3 2.9 4</td>
<td>3.3 2.1 8</td>
<td></td>
</tr>
<tr>
<td>Non-lesioned Monocytes (%)</td>
<td>1.5 0.6 4</td>
<td>3.3 2.1 8</td>
<td>4.9 1.5 5</td>
<td>1.9 0.0 2</td>
<td></td>
</tr>
<tr>
<td>Lesioned Thrombocytes (%)</td>
<td>46.2 5.2 5</td>
<td>48.2 11.8 5</td>
<td>44.3 12.9 10</td>
<td>51.0 15.7 5</td>
<td></td>
</tr>
<tr>
<td>Non-lesioned Thrombocytes (%)</td>
<td>2.0 4.2 5</td>
<td>3.3 2.1 8</td>
<td>4.9 1.5 5</td>
<td>1.9 0.0 2</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Hematology values for Atlantic menhaden sampled from all sample sites in the fall of 2002. \( n = \) number of pooled samples.

<table>
<thead>
<tr>
<th></th>
<th>Broad Creek</th>
<th>Durham Creek</th>
<th>Upper Goose Creek</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mean ±SD</td>
<td>n</td>
<td>Mean ±SD</td>
</tr>
<tr>
<td>Lesioned Neutrophils (%)</td>
<td>47.8 10.5 6</td>
<td>19.0 8.2 3</td>
<td>36.7 14.4 3</td>
</tr>
<tr>
<td>Non-lesioned Neutrophils (%)</td>
<td>44.8 7.6 9</td>
<td>45.0 3.6 3</td>
<td>36.7 14.4 3</td>
</tr>
<tr>
<td>Lesioned Neutrophils (%)</td>
<td>60.0 25.3 6</td>
<td>34.7 8.1 3</td>
<td>48.0 12.3 3</td>
</tr>
<tr>
<td>Non-lesioned Neutrophils (%)</td>
<td>76.0 19.1 6</td>
<td>87.1 26.8 9</td>
<td>68.7 11.4 3</td>
</tr>
<tr>
<td>Lesioned Lymphocytes (%)</td>
<td>4.7 2.3 6</td>
<td>3.6 2.4 3</td>
<td>4.0 0.6 3</td>
</tr>
<tr>
<td>Non-lesioned Lymphocytes (%)</td>
<td>3.9 1.1 6</td>
<td>4.0 5.7 8</td>
<td>2.7 1.4 3</td>
</tr>
<tr>
<td>Lesioned Lymphocytes (%)</td>
<td>8.5 5.4 6</td>
<td>9.7 4.5 3</td>
<td>8.7 2.1 3</td>
</tr>
<tr>
<td>Non-lesioned Lymphocytes (%)</td>
<td>5.3 1.6 6</td>
<td>4.8 6.8 8</td>
<td>4.0 2.0 3</td>
</tr>
<tr>
<td>Lesioned Lymphocytes (%)</td>
<td>19.0 6.4 6</td>
<td>10.3 1.1 3</td>
<td>12.6 4.7 3</td>
</tr>
<tr>
<td>Non-lesioned Lymphocytes (%)</td>
<td>29.1 16.9 6</td>
<td>25.9 10.1 9</td>
<td>21.5 6.7 3</td>
</tr>
<tr>
<td>Lesioned Lymphocytes (%)</td>
<td>34.8 15.9 6</td>
<td>31.0 8.9 3</td>
<td>25.7 4.5 3</td>
</tr>
<tr>
<td>Non-lesioned Lymphocytes (%)</td>
<td>36.2 18.4 6</td>
<td>30.3 8.9 9</td>
<td>32.0 10.6 3</td>
</tr>
<tr>
<td>Lesioned Monocytes (%)</td>
<td>4.6 5.0 5</td>
<td>8.0 4.2 3</td>
<td>4.7 5.9 2</td>
</tr>
<tr>
<td>Non-lesioned Monocytes (%)</td>
<td>1.6 1.1 4</td>
<td>1.8 1.2 7</td>
<td>3.4 2.5 3</td>
</tr>
<tr>
<td>Lesioned Monocytes (%)</td>
<td>7.8 8.3 5</td>
<td>22.0 6.2 3</td>
<td>13.5 17.7 2</td>
</tr>
<tr>
<td>Non-lesioned Monocytes (%)</td>
<td>2.3 1.5 4</td>
<td>2.3 1.3 7</td>
<td>4.7 2.9 3</td>
</tr>
<tr>
<td>Lesioned Thrombocytes (%)</td>
<td>30.5 21.3 6</td>
<td>8.6 1.8 3</td>
<td>26.7 13.5 3</td>
</tr>
<tr>
<td>Non-lesioned Thrombocytes (%)</td>
<td>40.9 11.5 6</td>
<td>54.7 19.7 9</td>
<td>39.1 11.8 3</td>
</tr>
<tr>
<td>Lesioned Thrombocytes (%)</td>
<td>47.2 14.7 6</td>
<td>25.0 2.8 3</td>
<td>52.7 16.4 3</td>
</tr>
<tr>
<td>Non-lesioned Thrombocytes (%)</td>
<td>55.5 14.9 6</td>
<td>62.0 8.9 9</td>
<td>56.3 9.5 3</td>
</tr>
<tr>
<td>Lesioned Total Plasma Protein (g dl^-1)</td>
<td>2.5 0.1 6</td>
<td>2.4 0.0 3</td>
<td>2.4 0.1 3</td>
</tr>
<tr>
<td>Non-lesioned Total Plasma Protein (g dl^-1)</td>
<td>2.9 0.3 6</td>
<td>2.9 0.4 9</td>
<td>2.7 0.2 3</td>
</tr>
</tbody>
</table>
Table 4. Hematology values for lesioned and non-lesioned Atlantic menhaden sampled in the fall of 2001 and 2002.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Unit</th>
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<th>2002</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lesioned Mean ±SD</td>
<td>n</td>
<td>Non-lesioned Mean ±SD</td>
<td>n</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>x 10^3/µl</td>
<td>5.75 ± 3.4</td>
<td>8</td>
<td>1.48 ± 0.54</td>
<td>6</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>%</td>
<td>20.46 ± 16.7</td>
<td>24</td>
<td>4.53 ± 5.3</td>
<td>17</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>x 10^3/µl</td>
<td>8.89 ± 6.56</td>
<td>8</td>
<td>34.4 ± 15.9</td>
<td>10</td>
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<tr>
<td>Lymphocytes</td>
<td>%</td>
<td>28.76 ± 16.08</td>
<td>25</td>
<td>50.72 ± 15.73</td>
<td>25</td>
</tr>
<tr>
<td>Monocytes</td>
<td>x 10^3/µl</td>
<td>3.07 ± 2.23</td>
<td>8</td>
<td>1.28 ± 0.87</td>
<td>7</td>
</tr>
<tr>
<td>Monocytes</td>
<td>%</td>
<td>13.35 ± 10.23</td>
<td>10</td>
<td>2.61 ± 2.06</td>
<td>18</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>x 10^3/µl</td>
<td>0.42 ± 0.05</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eosinophils</td>
<td>%</td>
<td>2.2 ± 1.32</td>
<td>10</td>
<td>1 ± 0</td>
<td>4</td>
</tr>
<tr>
<td>Thrombocytes</td>
<td>x 10^3/µl</td>
<td>30.97 ± 9.39</td>
<td>8</td>
<td>21.2 ± 11.4</td>
<td>10</td>
</tr>
<tr>
<td>Thrombocytes</td>
<td>%</td>
<td>32 ± 12.03</td>
<td>25</td>
<td>40.6 ± 17.49</td>
<td>25</td>
</tr>
<tr>
<td>PCV</td>
<td>%</td>
<td>24.75 ± 5.6</td>
<td>8</td>
<td>42.4 ± 3.75</td>
<td>10</td>
</tr>
<tr>
<td>WBC/Thrombocytes</td>
<td>x10^3/µl</td>
<td>30.97 ± 9.39</td>
<td>8</td>
<td>60.0 ± 22.3</td>
<td>10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Non-lesioned Mean ±SD</th>
<th>n</th>
<th>Lesioned Mean ±SD</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg dl⁻¹)</td>
<td>27.4 ± 13.5</td>
<td>33.3 ± 6.5</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Phosphorus (mg dl⁻¹)</td>
<td>11.5 ± 2.0</td>
<td>12.4 ± 2.0</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Calcium (mg dl⁻¹)</td>
<td>9.4 ± 2.2</td>
<td>12.0 ± 1.4</td>
<td>0.0002</td>
<td></td>
</tr>
<tr>
<td>TP (g dl⁻¹)</td>
<td>1.2 ± 0.7</td>
<td>2.1 ± 0.6</td>
<td>0.0004</td>
<td></td>
</tr>
<tr>
<td>Albumin (g dl⁻¹)</td>
<td>0.5 ± 0.3</td>
<td>0.8 ± 0.2</td>
<td>0.0010</td>
<td></td>
</tr>
<tr>
<td>Globulin (g dl⁻¹)</td>
<td>0.8 ± 0.5</td>
<td>1.4 ± 0.4</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Albumin/Globulin</td>
<td>0.6 ± 0.2</td>
<td>0.6 ± 0.3</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>AST (U L⁻¹)</td>
<td>346.7 ± 179.3</td>
<td>267.6 ± 67.6</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>CK (U L⁻¹)</td>
<td>5591.7 ± 1449.5</td>
<td>5977.2 ± 2295.3</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>LD (U L⁻¹)</td>
<td>2931.8 ± 1281.3</td>
<td>2433.6 ± 750.3</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Sodium (mmol⁻¹)</td>
<td>157.6 ± 5.8</td>
<td>160.3 ± 6.4</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Potassium (mmol⁻¹)</td>
<td>5.2 ± 2.3</td>
<td>6.2 ± 2.2</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Chloride (mmol⁻¹)</td>
<td>133.6 ± 4.8</td>
<td>137.4 ± 4.8</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Bicarbonate (mmol⁻¹)</td>
<td>7.3 ± 1.4</td>
<td>6.0 ± 1.8</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Anion Gap</td>
<td>21.9 ± 6.5</td>
<td>23.4 ± 4.0</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Sodium/Potassium</td>
<td>36.7 ± 19.6</td>
<td>29.2 ± 10.7</td>
<td>ns</td>
<td></td>
</tr>
</tbody>
</table>

Table 5. Plasma chemistry values for Atlantic menhaden caught in the fall of 2002. n = number of pooled samples.
Fig. 1. Map of the Pamlico River estuary creeks and ambient monitoring stations (●) sampled in the fall of 2001 and 2002.
Fig. 2. Temporal variability of juvenile Atlantic menhaden with ulcerative skin lesions in the Pamlico River estuary in (A) 2001 and (B) 2002. □ non-lesioned; ■, lesioned. Data courtesy of the Pamlico Rapid Response Team.
Fig. 3. Spatial distribution of Atlantic menhaden with ulcerative skin lesions in the Pamlico River estuary in 2001 and 2002. Data courtesy of the Pamlico Rapid Response Team.
Fig. 4. Liver-somatic indices (mean ± SE) for lesioned (■) and non-lesioned (□) Atlantic menhaden caught in the fall of 2001 and 2002 in the Pamlico River estuary. LSI were significantly higher for lesioned fish caught in 2002 than non-lesioned ($P = 0.0005$). Significant differences are indicated by an asterisk(*). $n = 30$, non-lesioned 2001; $n = 20$, lesioned 2001; $n = 31$, non-lesioned 2002; $n = 18$, lesioned 2002.
Fig. 5. Spleno-somatic indices (means ± SE) for lesioned (■) and non-lesioned (□) menhaden caught in the Pamlico River estuary during the fall of 2002. Spleno-somatic indices were significantly higher for lesioned fish (n = 41) than non-lesioned (n = 34; P = 0.0028).
Fig. 6. Atlantic menhaden with moderate to marked, diffuse, clear vacuolation of hepatocytes. This likely reflects nutritional stress as is seen in many wild caught fish specimens, leading to fatty change in the liver. Increased storage products, such as glycogen or vitellogenin, may also affect the morphological appearance of hepatocytes. H &E.
Fig. 7. Liver section of Atlantic menhaden with ulcerative skin lesions caught in the fall of 2001. Focal to multifocal areas of hepatic congestion (arrows) characterized by dilated sinusoids filled with erythrocytes and focal atrophy of hepatic cords were found in some lesioned fish. Surrounding hepatocytes are swollen or vacuolated. H &E.
Fig. 8. TGF-β: β-actin ratios for lesioned (■) and non-lesioned (□) Atlantic menhaden caught in the Pamlico River estuary during the fall of 2001. Values (means ± SE) were significantly higher for lesioned fish than non-lesioned (P = 0.0113). n = 11 for both groups.
Fig 9. Mean surface (1m depth) water quality measurements (± SD) collected from the ambient monitoring stations in Fig. 1.
Triamcinolone Induces Hyperglycemia, Leukogram Abnormalities, and Suppression of Transforming Growth Factor-β in Atlantic Menhaden, *Brevoortia tyrannus*

**ABSTRACT**

The immune-endocrine interaction was examined in captive Atlantic menhaden (*Brevoortia tyrannus*), an estuarine-dependent species plagued by ulcerative skin lesions in the estuaries along the eastern United States. Atlantic menhaden were acclimated in a closed system for two weeks prior to initiation of the study. The synthetic glucocorticoid, triamcinolone acetonide (10 mg kg\(^{-1}\) body weight), known to suppress the immune-system in some teleost fishes, was administered by intracoelomic injection. Its effects on Atlantic menhaden liver-somatic index, spleno-somatic index, hematology, plasma chemistry, lymphocyte mitogenesis, and splenic mononuclear cell TGF-β mRNA transcription were measured and compared to untreated fish at 48 and 96 hr post-treatment. Triamcinolone-treated Atlantic menhaden showed suppression of TGF-β mRNA production, neutrophilia, monocytosis, lymphopenia, and an increase in blood glucose levels. Knowledge of the interactions of the immune and endocrine systems provided by this study will improve our understanding of the immunodefense mechanisms of Atlantic menhaden and help us interpret some of the changes observed during the development of ulcerative lesions in wild caught menhaden and other aquatic species.
Atlantic menhaden (*Brevoortia tyrannus*), an estuarine-dependent species, have been plagued by outbreaks of ulcerative skin lesions in estuaries along the eastern United States (Noga and Dykstra, 1986; Noga *et al.*, 1988, Levine *et al.*, 1990b, Blazer *et al.*, 1999). The annual occurrence of ulcerative lesions in menhaden along with large scale fish kills have underscored the need to investigate the complex roles adverse environmental changes, pathogens, and host defenses play in the development of these health problems (Noga, 2000; Law, 2001). Additional attention has focused on the potential association of these health problems with blooms of *Pfiesteria* spp. in North Carolina and the Chesapeake Bay region (Burkholder *et al.*, 1992; Lewitus *et al.*, 1995; Noga *et al.*, 1996). A thorough understanding of the teleost immune system and its modulation by endocrine factors is essential in understanding disease resistance since both the endocrine and immune systems play an important role in maintaining homeostasis in vertebrates.

Glucocorticoids are important regulators of many essential physiological systems in vertebrates. Cardiovascular, metabolic, reproductive, and immune processes are all impacted by glucocorticoid activity (Wendelaar Bonga, 1997). In vertebrates, the major "stress hormones" are catecholamines and corticosteroids. In teleosts, the anterior kidney functions in both corticosteroid production (interrenal gland, located within the anterior kidney) and generating leukocytes for immunologic functions (Weyts *et al.*, 1999). Glucocorticoids are known primarily for their immunosuppressive and anti-inflammatory
effects. Cortisol, the major glucocorticoid produced by the teleost interrenal gland, has demonstrated some immunosuppressive effects in fish, including depressed lymphocyte mitogenesis (Espelid et al., 1996), circulating lymphocytes (McLeay, 1973; Pickering, 1984; Narnaware and Baker, 1996), and antibody production (Ellis, 1981). Triamcinolone acetonide, a long-acting synthetic glucocorticoid (Plumb, 1999), has been shown to suppress the immune system experimentally in some teleosts. Triamcinolone at 200 mg/kg body weight suppressed the resistance to ichthyophthiriasis in carp, *Cyprinus carpio* (Houghton and Matthews, 1986) and at a dose of 100 mg/kg body weight depressed the levels of circulating antibodies in striped bass, *Morone saxatilis*, exposed to infectious pancreatic necrosis virus (IPNV; Wechsler et al., 1986). In contrast, triamcinolone effects may also be in part immunostimulatory. Triamcinolone increased anterior kidney macrophage bactericidal activity, increased the splenic mononuclear cell pokeweed mitogen (PWM) stimulation index, and decreased transforming growth factor beta (TGF-β) mRNA levels in hybrid striped bass (*Morone saxatilis* x *M. chrysops*) at a concentration of 10 mg kg⁻¹ body weight (Harms et al., 2000a).

Cytokines are proteins and glycoproteins that are secreted *de novo* in response to immune and inflammatory stimuli (Ashwell et al., 2000). In mammals, glucocorticoids inhibit the production of many cytokines (e.g. IL-1, IL-2, IL-4, TNF-α and IFN-γ) by interfering with gene expression while increasing the production of others such as TGF-β (Wilckens and De Rijk, 1997). The cytokine TGF-β plays a central role in embryonic development, tumorigenesis, wound healing, fibrosis, and immunoregulation (Derynck, 1994). It inhibits production of many TH1 cytokines such as IFN-γ, TNF-α, TNF-β and IL-2
(Derynck, 1994) and it down-regulates thymocyte proliferation, T and B cell proliferation, cytotoxic T cell generation, and neutrophil adhesion to endothelium (Ruscetti and Palladino, 1991). It also suppresses cytokine production in T cells and mononuclear phagocytes (Roberts and Sporn, 1996), and inhibits macrophage activation and macrophage respiratory burst activity (Ruscetti and Palladino, 1991). In this study, a partial TGF-β sequence was isolated and cloned from Atlantic menhaden and a real-time quantitative RT-PCR assay was optimized to measure mRNA production of this important cytokine in Atlantic menhaden.

Immune-endocrine interactions are complex and factors such as the genetics and maturational state of a species, as well as its environmental conditions can affect a species’ health. This study was conducted to evaluate the effects of triamcinolone treatment on the Atlantic menhaden immune system (TGF-β mRNA transcription and lymphocyte mitogenesis), hematology and plasma chemistry, and general health indices (histopathology, liver-somatic and spleno-somatic indices).

**MATERIALS AND METHODS**

**Animals and Experimental Design**

Atlantic menhaden ranging from 46.8-69.7 g in weight and 150-175 mm fork length were collected by cast net in the White Oak River, NC, an estuarine system where ulcerative skin lesions have not been observed. A total of 40 fish, divided into 4 groups, were used for this study and the study was repeated once. All captured fish were free of visible skin
lesions and considered healthy. Fish were transported to the North Carolina State University College of Veterinary Medicine (NCSU-CVM) in Raleigh where they were acclimated for 2 weeks in two 250-L circular tanks (closed-system) at 12 g/L salinity. All procedures were conducted under the approval of the NCSU Institutional Animal Care and Use Committee. Fish were fed a commercial fish food (salmon starter crumble; Zeigler Bros, Inc., PA) once daily. Tanks were cleaned daily and water quality parameters (temperature, pH, ammonia and DO) were measured daily (nitrite and nitrate were measured weekly). Fish were kept at a photoperiod of 14h light:10h dark. After acclimation, five fish from each tank were randomly assigned to either a treatment or a control group, \( n = 10 \) per group. Fish in the treatment group were anesthetized by tricaine methanesulfonate (MS-222, Argent Chemical Laboratories) at 150 mg l\(^{-1}\), weighed, and injected intracoelomically with triamcinolone acetonide (10 mg kg\(^{-1}\) body weight; Kenalog-10, Apothecon, Princeton, New Jersey; 10 mg l\(^{-1}\) suspension in saline) following the protocol by Harms \textit{et al.}, (2000a). Fish were returned to their original tank after recovery. Control fish did not receive triamcinolone.

Five fish from each group (control and treated) were sacrificed 48 and 96 hour later. Both control and treated fish were anesthetized using MS-222 at 150 mg l\(^{-1}\) and bled from the caudal vein into heparinized syringes (1 ml syringe, 22 gauge needle). Blood samples were transferred to vacutainers and placed on ice. To reduce the effects of hormonal changes such as increased cortisol levels, care was taken to collect the blood immediately after handling the fish. As much blood as possible was removed to reduce peripheral blood content of the spleen (Harms \textit{et al.}, 2000a). Blood samples were collected for the
following assays: lymphocyte mitogenesis, hematology and plasma chemistry. Fish were then euthanized with an overdose of MS-222 (250 mg l\(^{-1}\)). Fork length, body weight and liver weight were measured. Samples of gill, heart, liver, intestine, gonads, and kidney were collected and fixed in 10% neutral buffered formalin for histopathology. Spleens were excised, weighed and stored in sterile cell culture medium, complete RPMI (RPMI-1640 plus 10% heat-inactivated fetal bovine serum, 100 U ml\(^{-1}\) penicillin, 100 µg ml\(^{-1}\) streptomycin, and 2 mM EDTA) for TGF-β mRNA assay. Spleen samples placed in complete RPMI were stored at 4°C and processed within 24 h for isolation of mononuclear cells to be used in a real-time RT-PCR assay developed for Atlantic menhaden TGF-β mRNA.

**Organosomatic Indices**

Liver-somatic index (LSI) and spleno-somatic index (SSI) were calculated as the organ weight (in mg) divided by the body weight (in mg; Goede and Barton, 1990).

**Histopathology**

Histopathology was performed on six fish from each group (\(n = 24\) fish total). Tissue samples were fixed in 10% neutral buffered formalin, routinely processed, embedded in paraffin, sectioned at 5 µm and stained with hematoxylin and eosin (H&E). All samples were examined by light microscopy by a single pathologist and microscopic changes
were assigned grades as follows: Grade 0 = no remarkable microscopic abnormalities; Grade 1 = very mild changes; Grade 2 = mild changes; Grade 3 = moderate changes; Grade 4 = moderately severe changes; and Grade 5 = severe, at the extreme range of pathology for the given lesion type (Hurty et al., 2002).

**Hematology**

Blood smears for differential leukocyte counts and whole blood samples were analyzed by the Clinical Pathology Laboratory at the NCSU College of Veterinary Medicine. Smears were stained with Wright-Giemsa stain (Volu-Sol, Inc., Salt Lake City, UT) and 100 leukocytes on each slide were identified. To determine white blood cells plus thrombocyte (WBC/thrombocyte) counts, cells were stained and diluted in Natt and Herrick’s solution and counted on a hemacytometer. The packed cell volume (PCV) or hematocrit values were determined using microhematocrit tubes, and reading the packed cell percentages. Plasma total solids were measured with a refractometer. The following substances were measured with a Hitachi 912 chemical analyzer (Roche Diagnostics, Indianapolis, IN): glucose, creatinine, phosphorus, calcium, total protein, albumin, aspartate aminotransferase (AST), creatinine kinase (CK), lactate dehydrogenase (LD), sodium (Na\(^+\)), potassium (K\(^+\)), chloride (Cl\(^-\)), and bicarbonate (HCO\(_3^-\)). The analyzer calculated albumin/globulin ratio, sodium/potassium ratio, and anion gap (AGAP). Anion gap was determined as follows: \([\text{Na}^+ + \text{K}^+ - (\text{Cl}^- + \text{HCO}_3^-)]\).
Lymphocyte Mitogenesis Assay

Isolation of Peripheral Blood Leukocytes (PBL)
The MTT (3, (4,5-dimethylthiazol-2-yl) 2,5-dipheyl-tetrazolium bromide) (M2128, Sigma, St. Louis, MO) assay used in this study was based on a modification of several techniques (Mosmann, 1983; Hansen et al., 1989; Daly et al., 1995). Blood samples were diluted 1:1 in PBS and centrifuged at 45 x g for 10 min in a swing-out rotor. The upper leukocyte layer was removed from the top of the red blood cell layer with a 1 ml pipette, diluted 1:1 in PBS and carefully layered over 2 ml of a 55% Percoll gradient (specific gravity 1.070 g ml$^{-1}$) in 0.15 M NaCl. Samples were centrifuged at 400 x g for 30 min at 22°C, the leukocyte rich interphase was collected and washed twice in 10 ml complete RPMI by centrifuging for 5 min at 300 x g. Viable cell counts were performed with cells suspended in 0.2% trypan blue (Gibco Laboratories, Grand Island, NY). Cell suspensions were diluted in complete RPMI to a concentration of 1.0 x 10$^6$ cells ml$^{-1}$ (5.0x10$^4$ cells/50µl).

Colorimetric MTT (tetrazolium) assay
The MTT was dissolved in sterile PBS at 5 mg/ml, sterilized by filtration and stored in a dark bottle at 4°C. Concanavalin A (Con A; C-0412, Sigma) and lipopolysaccharide (LPS; L-5262 from Vibrio cholerae, Sigma) were resuspended in PBS (1 mg/ml stock) and stored at –20°C. Both mitogens were diluted in complete RPMI to a final concentration of 10 µg/ml (LPS) and 2.5 µg/ml (Con A) before use. The mitogen doses and incubation times were optimized for Atlantic menhaden in preliminary experiments.
All mitogenesis assays were performed in quadruplicate. Cell suspensions (1.0 x 10^6 cells ml\(^{-1}\)) were loaded into 96 well round-bottomed tissue culture plates at 50 µl/well. Immediately after leukocyte plating, 50 µl of complete RPMI with or without mitogen (control) was added to each well and samples were incubated in a humidified container at 27°C for 5 days. After the incubation period, 10 µl stock MTT (5 mg/ml PBS) was added to each well and the samples were incubated at 27°C for 4 h for MTT cleavage. After incubation, formazan precipitate was dissolved by adding 120 µl of a solubilization buffer (pH = 4.7) consisting of 20% sodium diodecyl sulfate (SDS; Sigma, St. Louis, MO) in 50% \(N, N\)-dimethylformamide (DMF; Fisher Scientific, Suwannee, GA) per well and plates were incubated overnight. Plates were read at OD\(_{570}\) with reference wavelength of OD\(_{630}\) using a microplate reader. Stimulation indices were calculated using the formula:

\[
SI = \text{mean OD of stimulated culture/mean OD of non-stimulated control culture}
\]

**TGF-β real-time RT-PCR**

**Mononuclear cells**

Splenic mononuclear cell TGF-β transcription was determined by real-time RT-PCR procedures developed for Atlantic menhaden. Spleen samples in complete RPMI were minced finely, resuspended in complete RPMI and centrifuged on two-step Percoll gradients (35% and 55% stock solution in 0.15 M saline; specific gravity 1.046 and 1.070
Splenic mononuclear cells were harvested from the 35/55% interface, viable cell counts were performed with cells suspended in 0.2% trypan blue. Cell pellets were lysed in Tri Reagent (Tri Reagent, Molecular Research Ctr, Cincinnati, OH) at 1ml per 5-10 x 10⁶ cells and stored at –80°C.

**RNA Isolation and Reverse Transcription**

Isolation of RNA and reverse transcription were performed following the procedures of Harms *et al.*, (2000b). Total RNA was isolated by the guanidine thiocyanate method (Tri Reagent) following kit instructions. The RNA pellet was washed with 75% ethanol and resuspended in sterile diethyl pyrocarbonate (DEPC)-treated water. Splenic mononuclear cell RNA was resuspended to a concentration of 5 x 10⁴ cell equivalents µl⁻¹. Messenger RNA was reverse transcribed using Superscript II RT (Gibco BRL, Gaithersburg, Maryland) to cDNA with oligo dT₁₅ priming of 3 x 10⁶ cells equivalent. Samples of cDNA were stored at –20°C. Negative RT controls were run in parallel.

**Partial gene sequences for TGF-β and β-actin**

The PCR reaction for TGF-β was performed using primers and reaction conditions developed for hybrid striped bass (Harms *et al.*, 2000b). The PCR product was loaded onto a 1% ethidium bromide-stained agarose gel and a 1 kbp DNA molecular weight marker (Promega Corp., Madison, WI) was used to confirm the expected molecular weight of TGF-β fragment (225 bp). The reaction produced two bands approximately 200 bp and 800 bp. Both bands were purified using Qiaquick Gel Extraction Kit (Qiagen,
Valencia, CA) and sequenced at the UNC-CH Automated DNA Sequencing Facility on a Model 377 DNA Sequencer (Perkin Elmer, Applied Biosystems Division) using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (Perkin Elmer, Applied Biosystems Division). The sequences were verified using the BLAST program available at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). The large fragment was identified as a 5-lipoxygenase mRNA and the small fragment as a partial TGF-β mRNA (Fig.1). The housekeeping gene, β-actin, was used to normalize for sample-to-sample variation in RNA isolation, reverse transcription, and amplification. A partial β-actin sequence (Fig. 1) from a conserved region was obtained using primers derived from sequence alignments of β-actin from human, sheep, and four teleost species (striped bass, carp, fugu, grass carp; Harms et al., 2000b). Sequencing and verification of the β-actin amplicon were performed using the same method described above for TGF-β.

**Specific primers for real-time PCR**

The primers used for hybrid striped bass TGF-β were not specific for Atlantic menhaden TGF-β, therefore, primers specific for Atlantic menhaden TGF-β were designed from the sequenced 200 bp fragment. Primers for TGF-β and β-actin were designed using the software Primer 3, developed by Rosen and Skaletsky (2000) available on-line at http://www-genome.wi.mit.edu and Bio-Rad primer design instructions. Primer sets were checked for primer dimers using Oligo Analysis and Plotting tools (Qiagen). The
primers for Atlantic menhaden TGF-β and β-actin were purchased from Invitrogen Life Technologies (Carlsbad, CA). Primer pairs that showed homology for the gene of interest only using the BLAST program were considered specific (Table 1). Primers for TGF-β and β-actin yielded amplification products of 189 and 169 bp, respectively.

**Cloning of TGF-β and β-actin amplicons**

Products used for cloning were obtained from RT-PCR. PCR reactions contained: 25 µl iQ Supermix (consisting of 100mM KCl, 40 mM Tris-HCl, pH 8.4, 1.6 mM dNTPs, iTaq DNA polymerase, 50 units/ml, 6 mM MgCl₂ and stabilizers), 1.5µl of each primer (300nM), 2.5 µl cDNA, and sterile water to make a final volume of 50 µl. The PCR cycling conditions on the iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) were as follows: denaturation for 3 min at 95°C, followed by 40 cycles of 30 sec at 95°C and 30 sec at 55°C.

The PCR products were cloned into pGEM®-T easy vector (Promega Corp.). Recombinant plasmids were transformed in *Escherichia coli* JM109 competent cells (Promega Corp.) and grown on LB-medium according to kit instructions. Wizard® Plus SV Minipreps DNA Purification System (Promega Corp.) was used to isolate the plasmid DNA. The size and identity of the insert were confirmed by DNA sequencing (as above).

The molecular concentration of plasmid DNA with the insert was quantitated using the VersaFluor Fluorometer System and Fluorescent DNA Quantitation Kit (Bio-Rad Laboratories). The calculated molecular concentrations were diluted from 10⁸-10¹
molecules/uL. The standard curve dilutions for TGF-β and β-actin were aliquoted and stored at −20°C.

Both TGF-β and β-actin primers were further optimized for real-time PCR by amplifying different concentrations (300-500 nM) of each primer set with known amounts of each target. PCR and gel electrophoresis analysis were used to verify specificity of primer pairs for each target gene sequence. Primer concentrations that gave the greatest increase in fluorescence above baseline and produced a single band visualized on a 2% ethidium bromide-stained agarose gel were considered optimal for real-time RT-PCR.

**Optimized real-time PCR using SYBR Green 1**

PCR reactions contained 12.5 µl iQ SYBR Green Supermix (consisting of 100mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4mM of each dNTP (dATP, dCTP, dGTP, and dTTP), iTaq DNA polymerase, 50 units/ml, 6mM MgCl₂, SYBR Green 1, 20 nM fluorescein, and stabilizers), optimized concentrations of each primer set (Table 1), 1.25 µl cDNA, and sterile water to make a final volume of 25 µl. Each 96 well plate contained duplicates of unknown samples, standards for both TGF-β and β-actin, and controls. A no-template control (NTC) was included to detect fluorescence contamination in the sample, heat block and/or primer dimer formation from using SYBR green, and a no-template RT control was used to determine contamination during the RT step.

PCR cycling conditions were optimized for the iCycler and amplification of reverse transcribed cDNA samples and standards was performed in parallel. The cycling conditions were as follows: denaturation for 3 min at 95°C, followed by 40 cycles of 30
sec at 95°C and 30 sec at 60°C. Fluorescent data were specified for collection during the 60°C. To ensure that the fluorescent signal is from the desired PCR product, a melt curve analysis of each product was performed. Melt curves were obtained following a denaturation period of 5 sec at 95°C, 1 min annealing at 60°C, and 40 cycles of 1.0°C increments beginning at 60°C. PCR and melting procedures were detected in real-time with the iCycler instrument. PCR efficiencies were above 90% and correlation coefficient above 0.996. Results are reported as the ratio of TGF-β/β-actin.

**Statistical Analyses**

Values from control and triamcinolone-treated groups were compared using analysis of variance (ANOVA). The $P$ values for blood parameters were adjusted using a modified sequential Bonferroni test (Rice, 1988). This method was based on the total number of groups ($k = 5$) represented by the blood parameters measured. The groups were determined by their functional similarities and included: complete blood count (CBC; group 1); glucose, phosphorus and calcium (group 2); plasma proteins (group 3); muscle enzymes (group 4); and electrolytes (group 5). The total number of groups ($k$) was multiplied by the significance level ($P$ value) to give a more conservative alpha ($\alpha$). The Statistical Analysis System (SAS; SAS Institute Inc, Cary, NC) was used for all analyses. Significant ANOVAs ($P < 0.05$) were followed by Tukey’s multiple comparison tests (Neter et al., 1996). A visual assessment of residual plots was used to determine homogeneity of variance. To reduce variance heterogeneity, neutrophils, AST, LD,
chloride, Na⁺/K⁺ and TGF-β were log transformed, and monocyte counts and phosphorus were square root transformed (Neter et al., 1996).

RESULTS

Organosomatic Indices

Liver-somatic and spleno-somatic indices were not significantly different between triamcinolone-treated and control Atlantic menhaden (Table 2).

Histopathology

No remarkable differences in lesion prevalence between triamcinolone-treated and control fish were found in sections of heart, gill, intestine, liver, kidney, or gonad. In the gills of 7 fish, both treated and controls, there was mild to moderate secondary lamellar fusion, a common but relatively nonspecific change. In the intestinal tract, four fish from the control groups (48 and 96 hr) had mild to moderate trematode and nematode infestation. Approximately 50% of the kidney sections analyzed contained pigmented macrophage aggregates (PMAs), protozoa and granulomas. Spores of *Ichthyophonus* sp. found in kidney sections were surrounded by layers of macrophages admixed with scattered lymphocytes and necrotic cellular debris, usually within a distinctive, variable thick collar of amorphous eosinophilic material. In the liver of three treated and three control fish, there was mild to moderate, diffuse hepatocellular vacuolation. Similar to
the kidney sections, pigmented macrophages were found in more than 50% of the liver samples. Gonadal tissue was examined in order to identify sex and evaluate the presence of lesions. There were 18 males and 21 females (no gonad was found for one sample) and no remarkable microscopic changes observed in the gonads (Table 2).

**Hematology**

Triamcinolone-treated Atlantic menhaden blood neutrophil absolute counts increased at 48 and 96 hr compared to controls. Although the neutrophil counts were higher in triamcinolone-treated fish, differences were not significant \( P > 0.05 \); Fig. 2a). Monocyte counts increased significantly at 48 hr in triamcinolone-treated fish compared to controls at 48 and 96 hr \( P = 0.0077 \) and 0.0119 respectively; Fig.2b); but values returned to those of controls at 96 hr \( P > 0.05 \); Fig. 2b). Monocyte counts were also significantly higher in treated fish at 96 hr than 48 hr controls \( P = 0.0289 \); Fig. 2b). Lymphocyte counts were opposite that of neutrophils and monocytes. Lymphocyte counts decreased at 48 hr in triamcinolone-treated fish compared to controls and declined significantly further at 96 hr. Lymphocyte counts were significantly lower for treated fish at 96 hr than controls \( P = 0.0244 \); Fig. 2c). No significant differences were observed between controls and treated fish with respect to values for hematocrit (PCV), WBC/thrombocyte counts, and thrombocytes (Table 3).
Plasma chemistry

Glucose concentrations were significantly higher in triamcinolone-treated fish at 96 hr post-treatment than at 48 hr post-treatment ($P = 0.0273$) and higher than controls at either 48 or 96 hr ($P = 0.0036$ and 0.0063 respectively; Fig. 3). No significant differences were observed between controls and treated fish with respect to the other plasma chemistry values (Table 3).

Lymphocyte mitogenesis

Stimulation indices (ConA and LPS) were not significantly different between control and triamcinolone-treated Atlantic menhaden (Table 2).

TGF-β

Production of TGF-β mRNA by splenic mononuclear cells was significantly lower in triamcinolone-treated fish at 48 hr post-treatment than at 96 hr post-treatment ($P = 0.0167$) and lower than controls at either 48 or 96 hr ($P = 0.0128$ and 0.0104 respectively; Fig. 4). There were no significant differences in mean TGF-β mRNA transcription between control and triamcinolone treated Atlantic menhaden at 96 hr ($P > 0.05$; Fig. 4).
DISCUSSION

Since little is known about the response of the immune system of estuarine fish to the myriad stimuli in aquatic environments, controlled, laboratory-based studies are needed to establish baseline responses of estuarine species. In the present study, we used the well-known synthetic glucocorticoid, triamcinolone, as a model compound to generate baseline immune function data. We used a 3-tiered approach to measure the immune response in Atlantic menhaden: histopathology and the liver-somatic and spleno-somatic indices (Tier 1) were used to assess general health status and any major changes in immune-related tissues. In mammals, this would have included lymph nodes and bone marrow; in fish, the hematopoietic tissue is located in the anterior kidney. These analyses revealed only a small number of lesions commonly associated with wild-caught fish, and no significant differences between triamcinolone-treated fish and controls. Tier 1 analyses demonstrated that the fish used in the study were relatively healthy and that the changes noted in Tiers 2 and 3 could be attributed to the triamcinolone treatment. Of note, the degree of hepatocyte vacuolation (fatty change) in the liver was striking in some control and treated fish. This could be due to a less-than-optimal laboratory diet and/or to nutritional problems in the wild. We suspect that nutritional imbalances could be a major factor when large schools of menhaden are packed into small feeder creeks and other confined areas (Law, 2001).

Hematology and plasma biochemistry served as Tier 2 parameters, which are more specific for the immune system than Tier 1 tests but not as specific as Tier 3. Atlantic
menhaden injected with triamcinolone showed increased neutrophil and monocyte counts (neutrophilia and monocytosis) and decreased lymphocyte numbers (lymphopenia). The response of menhaden blood leukocytes to triamcinolone is a classic non-specific response of the immune system mediated by the endocrine system and observed by many investigators. Consistent with the immunosuppressive actions of glucocorticoids, triamcinolone has been shown to suppress the production of antibodies in rainbow trout, *Oncorhynchus mykiss* (Anderson *et al.*, 1982), increase susceptibility to the disease ichthyophthiriasis in carp *Cyprinus carpio* L. (Houghton and Matthews, 1986), and depress the levels of circulating antibodies in striped bass *Morone saxatilis* with infectious pancreatic necrosis virus (Wechsler *et al.*, 1986).

Contrary to these findings, glucocorticoids have been reported in some cases to have immunostimulatory effects. Cortisol caused an increase in leukocyte migration in rainbow trout (Iger *et al.*, 1995), increased circulating phagocytes in dab *Limanda limanda* (Pulsford *et al.*, 1994) and increased numbers of circulating neutrophils and monocytes in channel catfish, *Ictalurus punctatus* (Ellsaesser and Clem, 1987). Hybrid striped bass treated with triamcinolone showed increased anterior kidney macrophage bactericidal activity and splenic mononuclear cell PWM mitogen stimulation (Harms *et al.*, 2000a). The increased mobilization of phagocytic cells from hematopoietic tissue is thought to be an adaptative response of the cells to maintain basic functions (Weeks *et al.*, 1992). The observed decline in peripheral blood lymphocytes may have resulted from induced apoptosis and/or chemotaxis of lymphocytes and their sequestration into immune tissue such as the thymus and anterior kidney. The redistribution of lymphocytes to target
areas may enhance immunity (Wilckens and De Rijk, 1997). However, glucocorticoid suppression of lymphocytes as well as the reduction of phagocytic cells (neutrophils and monocytes) at inflammatory sites have been reported in other teleost and non-teleost species (Sapolsky et al., 2000; Schreck, 1996).

Blood glucose concentrations were significantly elevated in triamcinolone-treated Atlantic menhaden compared to untreated fish. Hyperglycemia was also observed in hybrid striped bass injected with triamcinolone (Harms et al., 2000a). The metabolic stress response of fish injected with triamcinolone is related to energy mobilization. Mobilization of glucose, inhibition of subsequent energy storage and hepatic gluconeogenesis are a result of elevated glucocorticoids and catecholamines (Sapolsky et al., 2000; Mayer et al., 1992a). Triamcinolone did not cause significant changes in any of the other plasma chemistry values. It is possible that a longer time course or different dose of triamcinolone may have revealed other differences between treated and non-treated fish plasma chemistry.

Tier 3 comprised measurements of lymphocyte mitogenesis and splenic TGF-β, the most specific immune function tests we used. No differences were found in peripheral blood lymphocyte mitogenesis between control and treated fish, which is similar to findings of Harms et al., (2000a). In their study, there was no significant difference between triamcinolone-treated and control fish in splenic lymphocyte proliferation using ConA although there was a significant difference with PWM. Mononuclear cell TGF-β mRNA transcription decreased significantly in triamcinolone treated Atlantic menhaden at 48 hr
but levels recovered at 96 hr to those of controls. This was similar to results found by Harms et al., (2000a) in which triamcinolone-treated hybrid striped bass showed a decline in anterior kidney mononuclear cell TGF-β mRNA production with the same concentration of triamcinolone (10 mg kg$^{-1}$). Cells producing TGF-β were probably down-regulated by triamcinolone and/or the effects of triamcinolone on the hypothalamic-pituitary-interrenal axis (Wilckens and De Rijk, 1997). Therefore, triamcinolone may cause TGF-β-producing cells to down-regulate their receptor affinity for TGF-β. For example, circulating monocytes recruited and activated by TGF-β down-regulate their TGF-β receptors, thereby preventing further activation of TGF-β. The low levels of TGF-β are important in establishing a chemotactic gradient for leukocyte recruitment into the target tissue (McCartney-Francis and Wahl, 1994).

The results of this study, when compared to previous studies, show that the actions of glucocorticoids can be either suppressive or stimulatory, depending on the species, organ source of cells (anterior kidney, spleen and peripheral blood) and the glucocorticoid dose. For example, the study by Harms et al., (2000a), in which the same dose of triamcinolone was administered, showed both similarities and differences relative to this study. Both studies showed suppression of TGF-β transcription (spleen and anterior kidney) and hyperglycemia. In contrast, plasma electrolytes were not affected by the triamcinolone treatment in our studies, while plasma electrolytes declined significantly in the previous study. The lack of change in electrolytes in our study may reflect the difference in salinity that the species were kept in; Atlantic menhaden were kept at 12 g/L salinity while hybrid striped bass were held in fresh water. Therefore, the variability between the
two studies is probably due to the differences in the organs examined as well as intrinsic and extrinsic factors of the respective species, and reflects the complexity of immune-endocrine interactions.

The concerted actions of glucocorticoids and immune function parameters are important for maintaining homeostasis. The immunomodulatory actions of triamcinolone observed in this study are consistent with those of other studies, and may be influenced by the animal’s physiology (e.g. sex, maturational status, genetics) and environmental factors. Therefore, examining multiple components of an organism’s physiology will improve our understanding of the diversity and complexity of immune-endocrine interactions in maintaining homeostasis. Furthermore, understanding these interactions will help us interpret some of the changes observed during the development of ulcerative lesions in wild caught Atlantic menhaden and other aquatic species, and perhaps help to clarify the different roles of environment, host, and pathogen during disease outbreaks.

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Table 1. Optimal TGF-β and β-actin primers and concentrations used for real-time PCR amplification

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>Size (bp)</th>
<th>Concentration (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2A Forward</td>
<td>5’ GGCTACTTTGCCAACTACTGC 3’</td>
<td>21</td>
<td>300</td>
</tr>
<tr>
<td>2B Reverse</td>
<td>5’ CTGCTCCACCTTTGTTGC 3’</td>
<td>19</td>
<td>300</td>
</tr>
<tr>
<td>3A Forward</td>
<td>5’GTTGCACTCAAGCTGTGC 3’</td>
<td>18</td>
<td>400</td>
</tr>
<tr>
<td>3B Reverse</td>
<td>5’TGAAGTGATGCTGCAAGTCA 3’</td>
<td>22</td>
<td>500</td>
</tr>
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Table 2. Morphometric and lymphocyte mitogenesis values for triamcinolone-treated and control Atlantic menhaden (F= females; M = males).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control (n=10)</th>
<th>Treated (n = 10)</th>
<th>Control (n = 10)</th>
<th>Treated (n = 10)</th>
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<tr>
<td></td>
<td>(F = 8; M = 2)</td>
<td>(F = 4; M = 6)</td>
<td>(F = 6; M = 4)</td>
<td>(F = 3; M =6)</td>
</tr>
<tr>
<td>Length (mm)</td>
<td>160.50 ± 7.25</td>
<td>163.89 ± 4.17</td>
<td>160.50 ± 7.25</td>
<td>160.00 ± 3.33</td>
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<tr>
<td>Body weight (g)</td>
<td>55.27 ± 7.33</td>
<td>57.14 ± 7.19</td>
<td>55.39 ± 4.23</td>
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<tr>
<td>Liver weight (g)</td>
<td>0.36 ± 0.11</td>
<td>0.35 ± 0.08</td>
<td>0.28 ± 0.03</td>
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</tr>
<tr>
<td>Spleen weight (g)</td>
<td>0.08 ± 0.04</td>
<td>0.06 ± 0.02</td>
<td>0.06 ± 0.02</td>
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<tr>
<td>Liver-somatic index</td>
<td>6.48 ± 1.75</td>
<td>6.18 ± 0.88</td>
<td>5.03 ± 0.71</td>
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<tr>
<td>Spleno-somatic index</td>
<td>1.43 ± 0.54</td>
<td>1.05 ± 0.34</td>
<td>1.17 ± 0.26</td>
<td>1.14 ± 0.48</td>
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<td>ConA (SI)</td>
<td>0.99 ± 0.04</td>
<td>0.98 ± 0.03</td>
<td>1.02 ± 0.05</td>
<td>0.94 ± 0.09</td>
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<tr>
<td>LPS (SI)</td>
<td>0.97 ± 0.06</td>
<td>0.95 ± 0.05</td>
<td>1.04 ± 0.07</td>
<td>1.00 ± 0.08</td>
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Table 3. Hematology and plasma chemistry values for control and triamcinolone-treated Atlantic menhaden.

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<td>96 Hour</td>
<td>48 Hour</td>
<td>96 Hour</td>
</tr>
<tr>
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<td>1.87 (7.3)</td>
<td>1.03 (6.7)</td>
<td>6.56 (17.0)</td>
</tr>
<tr>
<td>Monocytes</td>
<td>x10^3 µl⁻¹ (%)</td>
<td>0.50 (1.3)</td>
<td>0.47 (1.0)</td>
<td>2.96 (7.5)</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>µl⁻¹ (%)</td>
<td>95.0 (0.2)</td>
<td>190.0 (0.4)</td>
<td>135.0 (0.2)</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>x10^3 µl⁻¹ (%)</td>
<td>14.89 (48.5)</td>
<td>6.01 (8.9)</td>
<td>10.91 (31.3)</td>
</tr>
<tr>
<td>Thrombocytes</td>
<td>x10^3 µl⁻¹ (%)</td>
<td>13.53 (42.3)</td>
<td>7.29 (7.9)</td>
<td>13.43 (40.5)</td>
</tr>
<tr>
<td>WBC/Thrombo</td>
<td>x10^3 µl⁻¹ (%)</td>
<td>31.00</td>
<td>12.90</td>
<td>34.33</td>
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<tr>
<td>PCV</td>
<td>%</td>
<td>26.00</td>
<td>7.60</td>
<td>28.50</td>
</tr>
<tr>
<td>Glucose</td>
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<tr>
<td>Phosphorus</td>
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<td>0.9</td>
<td>6.4</td>
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<tr>
<td>Calcium</td>
<td>mg dL⁻¹</td>
<td>8.90</td>
<td>1.22</td>
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<td>g dL⁻¹</td>
<td>1.78</td>
<td>0.62</td>
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<td>0.767</td>
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<tr>
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<td>g dL⁻¹</td>
<td>1.15</td>
<td>0.38</td>
<td>1.72</td>
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<tr>
<td>Albumin/Globulin</td>
<td></td>
<td>0.54</td>
<td>0.12</td>
<td>0.49</td>
</tr>
<tr>
<td>AST</td>
<td>U L⁻¹</td>
<td>112.17</td>
<td>18.19</td>
<td>168.5</td>
</tr>
<tr>
<td>CK</td>
<td>U L⁻¹</td>
<td>7817.20</td>
<td>1022.92</td>
<td>2792.80</td>
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<td>U L⁻¹</td>
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<td>208.06</td>
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<td>mmol L⁻¹</td>
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<td>Bicarbonate</td>
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<td>Sodium/Potassium</td>
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<td>12.81</td>
<td>47.48</td>
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A. TGF-β

GTCCCAAGGGCTACTTTGCCAACTACTG

TGGAGCACCAGGACAAACTCTCAGATTCTAGCTGTATAAGCATCA

CAATCCCGGAGCATCAGGCCGCCCAGCCCTGTGGGTCCTCAGTCTTTGGAAC

CGCTGCCCATCATCTACTACGTTGGCCGGCAGCAACACACAGGTGGAGCAGCTGA

B. β-actin

CCAGATCATGTTTGAGACCTCCACCCCTGCCCATGTACGTGCCATCC

AGGCTGTGCTGCCTCTGATGTTCTGCTGCTGACTGTACCGTATCGTGATG

GACTCCCGGATGGGTCACCTACACTGTGCCCATCTACGGGTTACGC

CCTGCCCATGCCCATCCTCGTAGCTGACCTGACTGACCCATCTACGGGTTACGC

ACTACCTCATGAAGATCCTCACCGAGCGAG

Fig. 1. Partial nucleotide sequences of Atlantic Menhaden TGF-β (A) and β-actin (B). The numbers on the right indicate the nucleotide position. Primer sequences are marked by > (forward) and < (reverse).
Fig. 2. Effect of triamcinolone treatment on blood leukocytes of control (□) and treated (■) Atlantic menhaden; (A) neutrophil; (B) monocyte; (C) lymphocyte counts. Values are means ± SE (n = 6). Means that are significantly different (P < 0.05), have different letters.
Fig. 3. Effect of triamcinolone on plasma glucose levels (means ± SE) of Atlantic menhaden. Statistically significant differences ($P < 0.05$) between control and treated groups are denoted by different letters. Sample sizes are the same for both control (□) and treated (■) groups ($n = 6$).
Fig. 4. Effect of triamcinolone on TGF-β mRNA production by Atlantic menhaden splenic mononuclear cells. Absolute values (TGF-β: β-actin; means ± SE) were significantly lower for treated (■) fish at 48 hr than 96 hr and controls (□) at 48 and 96 hr (P < 0.05). Statistically significant differences (P < 0.05) between control and treated groups are denoted by different letters. Samples sizes are the same for all treatment groups (n = 9) except for 96 hr treated (n = 8).
Hypoxia-induced Immune Response in Atlantic menhaden, *Brevoortia tyrannus*

**ABSTRACT**

Mass mortalities of Atlantic menhaden (*Brevoortia tyrannus*) attributed to low dissolved oxygen (DO) are relatively common in North Carolina estuaries. However, little is known about the sublethal health effects of these low DO events. The effects of acute and subacute exposures to low DO were evaluated in Atlantic menhaden under controlled laboratory conditions. Atlantic menhaden were acclimated for 2 weeks in 2000-L circular tanks (closed-aquaria) prior to initiation of the study. Hematology, plasma chemistry, and spleno-somatic indices were measured at five different oxygen saturations (5, 10, 15, 20, and 84%) in the acute study. Splenic transforming growth factor-β (TGF-β) mRNA, lymphocyte mitogenesis, and blood parameters were measured at 20% and 84% oxygen saturation in the subacute exposure study. In both experiments, glucose and electrolytes were the blood parameters most affected by hypoxic conditions. Blood glucose concentrations were elevated in both studies. Fish exposed to 5% oxygen saturation in the acute study displayed “relative” blood acidosis while those exposed to 20% oxygen saturation in the subacute exposure study displayed “relative” blood alkalosis. These effects may have consequences in estuarine systems where fish are exposed to multiple stressors, which may cumulatively affect their ability to endure adverse environmental conditions and resist disease.
INTRODUCTION

Estuaries are dynamic, highly productive and complex ecosystems that serve as nursery areas for many species important to both commercial and recreational fisheries. Like most estuarine systems, the estuaries of North Carolina are predominantly nitrogen (N) limited (Paerl, 1997). Both natural and anthropogenic (point and non-point) sources of N have resulted in increased primary production, loss of seagrass communities, food web alterations, occurrence of nuisance algal blooms, and extended periods of bottom hypoxia and anoxia (Paerl, 1997). Low dissolved oxygen or hypoxia is considered the most common environmental stressor that fish encounter (Walters and Plumb, 1980; Van Ginneken et al., 1998) and its occurrence seems to be increasing in estuaries along the eastern United States (Paerl and Ramus, 1998).

In North Carolina’s estuaries, hypoxic events occur mainly during the warmer months, and are relatively common in both the Pamlico and Neuse estuaries (NCDENR-DWQ, 2001; NCDENR-DWQ, 2002). While the lethal effects of hypoxia are obvious, less severe health effects are more obscure and complicated by other endogenous and exogenous factors. Effects of hypoxia are usually metabolic (Adams, 1990). Hypoxia may result in alteration of cellular components such as enzymes, cell membranes, or impairment of vital functions such as respiration, circulation, immune response, osmoregulation, and hormonal regulation. The higher energy cost of maintaining homeostasis may result in reduced growth, impaired reproduction, vulnerability to other environmental factors, and increased susceptibility to disease (Ellis, 1981; Adams, 1990).
Atlantic menhaden (*Brevoortia tyrannus*), an estuarine-dependent species important to the commercial fishing industry, have been subjected to periods of hypoxia and anoxia driven by eutrophication. At times, episodic mortality events are observed when oxygen depletion is induced by the movement of large densely packed schools into small tributaries (Smith, 1999; Law, 2001; NCDENR-DWQ, 2001). Fish that survive these events may become susceptible to predators and infectious diseases such as epizootic ulcerative syndrome (EUS; Law, 2001). In this study, two aspects of low dissolved oxygen stress were evaluated in Atlantic menhaden under controlled laboratory conditions. First, the effects of acute exposure (1 h) to abrupt decreases in dissolved oxygen concentrations on Atlantic menhaden spleno-somatic indices and blood parameters were measured; and second, the effects of subacute exposure (72 h) to low dissolved oxygen concentrations on Atlantic menhaden immune function and blood parameters were determined. Immune function parameters measured were TGF-β mRNA and lymphocyte mitogenesis. The alterations in immune and health parameters observed in the laboratory will help elucidate variations in Atlantic menhaden health indices that occur during rapid DO changes and periods of constant non-lethal low DO exposure in the estuaries.
MATERIALS AND METHODS

Collection and holding of fish

Atlantic menhaden weighing 49.8-100.6 g and measuring 155-195 mm fork length (FL) were collected by cast net in the White Oak River, NC. Fish were transported to the North Carolina State University College of Veterinary Medicine (NCSU-CVM) in Raleigh where they were acclimated for 2 weeks in 2000-L circular tanks (closed-aquaria). All procedures were conducted under the approval of the NCSU Institutional Animal Care and Use Committee. Fish were fed a commercial fish food (salmon starter crumble; Zeigler Bros, Inc., PA) once daily. Salinity was maintained at 12 g/L (± 0.5) and temperature ranged from 22-24°C. Tanks were cleaned daily and water quality parameters (temperature, pH, ammonia and DO) were measured daily (nitrite and nitrate were measured weekly). Fish were kept at a photoperiod of 14h light:10h dark. Fish were transferred to experimental tanks 72 h prior to experiments and feeding was terminated 24 h prior to the start of each experiment. The dissolved oxygen concentration in each experimental tank was regulated using Neptune Systems Aquacontroller Pro unit (www.neptunesystems.com) described in detail previously (Lehman et al., in press) and concentrations were verified using a portable hand-held YSI 85 oxygen meter.
Acute hypoxia exposure study

Atlantic menhaden were exposed to five different DO concentrations in 250-L circular tanks. Treatments were 5% (0.39 mg/L), 10% (0.79 mg/L), 15% (1.19 mg/L), 20% (1.59 mg/L), and 84% (6.7 mg/L) oxygen saturation. Three fish were exposed to 84% oxygen saturation (control) and five fish to each of the other four concentrations (n = 23 fish total). The initial drop in DO levels was rapid (< 2 h) and fish were exposed to each DO concentration for a maximum of 1 h.

Blood and Tissue Sampling: Fish were removed from the exposure tank, anesthetized with tricaine methanesulfonate (MS-222, Argent Chemical Laboratories) at 150 mg/l, and bled from the caudal vein. Immediate analyses of some hematological parameters were determined using the i-STAT portable clinical analyzer (i-STAT PCA; i-STAT Corp., East Windsor, NJ) with the i-STAT E7+ cartridge. Non-heparinized blood samples (< 100 µl) were analyzed within a minute of collection and results were obtained within 2 min. Blood samples for routine hematological analysis and lymphocyte mitogenesis were collected with heparinized syringes, transferred to vacutainers, and placed on ice. To reduce the effects of hormonal changes such as increased cortisol levels, care was taken to collect the blood immediately after handling the fish. Blood samples were also collected for hematocrit, white blood cell plus thrombocyte counts (WBC/thrombocyte counts), differential leukocyte counts, and plasma chemistries. Fish were then euthanized with an overdose of MS-222 (250 mg/l) and fork length (FL), body weight, and spleen
weight were measured. Tissue samples of gill, heart, liver, intestine, gonads, and kidney were collected for histopathology.

**Histopathology:** Histopathology was performed on all fish \((n = 23)\). Tissue samples were fixed in 10% neutral buffered formalin, routinely processed, embedded in paraffin, sectioned at 5 \(\mu\)m, and stained with hematoxylin and eosin (H&E). All samples were examined by light microscopy by a single pathologist and microscopic changes were assigned grades as follows: Grade 0 = no remarkable microscopic abnormalities; Grade 1 = very mild changes; Grade 2 = mild changes; Grade 3 = moderate changes; Grade 4 = moderately severe changes; and Grade 5 = severe, at the extreme range of pathology for the given lesion type (Hurty et al., 2002).

**Hematology:** Blood smears for differential leukocyte counts and whole blood samples were analyzed by the Clinical Pathology Laboratory at the College of Veterinary Medicine (CVM). Blood smears were stained with Wright-Giemsa stain (Volu-Sol, Inc., Salt Lake City, UT) and 100 leukocytes on each slide were identified. To determine WBC/thrombocyte counts, cells were stained and diluted in Natt and Herrick’s solution and counted on a hemacytometer. Plasma total solids were measured with a refractometer (Leica Microsystems, Germany). The following substances were measured with a Hitachi 912 chemical analyzer (Roche Diagnostics, Indianapolis, IN): glucose, creatinine, calcium, phosphorus, total protein, albumin, aspartate aminotransferase (AST), creatinine kinase (CK), lactate dehydrogenase (LD), chloride \((Cl^-)\). The analyzer calculated albumin/globulin ratio. Packed cell volume (PCV) or hematocrit, ionized
calcium (iCa\(^+\)), sodium (Na\(^+\)), potassium (K\(^+\)), PCO\(_2\), and pH were measured using the i-STAT PCA and the analyzer calculated bicarbonate (HCO\(_3^-\)). The sodium/potassium ratio was determined using measurements from the i-STAT PCA. Values for PCO\(_2\) and pH from the i-STAT PCA were not temperature corrected. Ashwood et al., (1983) recommends using pH and PCO2 at 37°C when assessing acid-base status and performing temperature corrections for measurements below 2°C and above 37°C.

**Subacute hypoxia exposure study**

Atlantic menhaden were randomly assigned to 850-L control and treatment tanks (n = 40 fish per tank). The treatment group was exposed to 20% oxygen saturation, determined in the acute study, because this concentration of DO was stressful but not lethal within 1 hr. Fish in the treatment tank were exposed to 20% oxygen saturation for 72 h after which oxygen was increased in the tank to 84% oxygen saturation for an additional 84 h. The decrease and increase in DO took approximately 1h and fish were monitored every 2-8 h. The control tank was kept at 84% oxygen saturation for the duration of the experiment (156 h). Five fish each from both control and treatment groups were collected at 48, 72, 84, and 156 h (n = 40). The experiment was repeated once (n = 80 fish total; 40 treatment and 40 control fish).

**Blood and Tissue Sampling:** The procedures in this study were the same as the acute hypoxia study, with the addition of the following two assays: lymphocyte mitogenesis
assay and TGF-β mRNA assay. Blood samples were collected for the lymphocyte mitogenesis assay and spleen samples for the TGF-β mRNA assay. Spleens were excised and stored in sterile cell culture medium, complete RPMI (RPMI-1640 plus 10% heat-inactivated fetal bovine serum, 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, and 2 mM EDTA) for TGF-β mRNA assay. Spleen samples placed in complete RPMI were stored at 4°C and processed within 24 h for isolation of mononuclear cells to be used in a real-time RT-PCR assay developed for Atlantic menhaden TGF-β mRNA. The study was repeated once (n = 80 total).

**Histopathology**

Six fish (n = 3 fish per treatment) from each time period (48, 72, 84, and 156 h) were processed for histopathology (n = 24 total). Tissues were prepared and processed for histopathology assessment as noted above.

**Hematology**

A total of 24 samples (n = 3 fish per treatment) were processed by routine hematology, and analyzed by the iSTAT PCA described in the acute hypoxia study.

**Lymphocyte Mitogenesis Assay**

The MTT assay used in this study was based on a modification of techniques described previously (Mosmann, 1983; Hansen *et al.*, 1989; and Daly *et al.*, 1995; Johnson *et al.*, 2004). Blood samples were diluted 1:1 in PBS and centrifuged at 45 x g for 10 min in a swing-out rotor. The leukocyte layer was removed from the top of the red blood cell
layer with a 1 ml pipette, diluted 1:1 in PBS and carefully layered over 2 ml of a 55% Percoll gradient (specific gravity 1.070 g ml$^{-1}$) in 0.15 M NaCl. Samples were centrifuged at 400 x g for 30 min at 22°C, the leukocyte rich interphase was collected and washed twice in 10 ml complete RPMI by centrifuging for 5 min at 300 x g. Viable cell counts were performed with cells suspended in 0.2% trypan blue (Gibco Laboratories, Grand Island, NY). Cell suspensions were diluted in complete RPMI to a concentration of 1.0 x 10$^6$ cells ml$^{-1}$ (5.0x10$^4$ cells/50µl).

The MTT (3, (4,5-dimethylthiazol-2-yl) 2,5-dipheyl-tetrazolium bromide) (M2128, Sigma, St. Louis, MO) was dissolved in sterile PBS at 5 mg/ml, sterilized by filtration and stored in a dark bottle at 4°C. Concanavalin A (Con A; C-0412, Sigma) and lipopolysaccharide (LPS; L-5262 from *Vibrio cholerae*, Sigma) were resuspended in PBS (1 mg/ml stock) and stored at –20°C. Both mitogens were diluted in complete RPMI to a final concentration of 10 µg/ml (LPS) and 2.5 µg/ml (Con A) before use. The mitogen doses and incubation times were optimized for Atlantic menhaden in preliminary experiments. Stimulation indices ranged from 1.09-1.7 for Con A and 1.15-1.4 for LPS. These stimulation indices were similar to those obtained by Espelid *et al.*, 2003).

All mitogenesis assays were performed in quadruplicate. Cell suspensions (1.0 x 10$^6$ cells ml$^{-1}$) were loaded into 96 well round-bottomed tissue culture plates at 50 µl/well. Immediately after leukocyte plating, 50 µl of complete RPMI with or without mitogen (control) was added to each well and samples were incubated in a humidified container at 27°C for 5 days. After the incubation period, 10 µl stock MTT (5 mg/ml PBS) was added
to each well and the samples were incubated at 27°C for 4 h for MTT cleavage. After incubation, formazan precipitate was dissolved by adding 120 µl SDS/DMF solution (20% SDS/50% DMF, pH = 4.7) per well and plates were incubated overnight. Plates were read at OD₅₇₀ with reference wavelength of OD₆₃₀ using a microplate reader. Stimulation indices were calculated using the formula:

\[ \text{SI} = \frac{\text{mean OD of stimulated culture}}{\text{mean OD of non-stimulated control culture}} \]

**TGF-β real-time RT-PCR**

Splenic mononuclear cell TGF-β transcription was determined by real-time RT-PCR procedures developed for Atlantic menhaden (chapter 3). Spleen samples in complete RPMI were minced finely, resuspended in complete RPMI and centrifuged on two-step Percoll gradients (35% and 55% stock solution in 0.15 M saline; specific gravity 1.046 and 1.070 g ml⁻¹). Splenic mononuclear cells were harvested from the 35/55% interface, viable cell counts were performed with cells suspended in 0.2% trypan blue. Cell pellets were lysed in Tri Reagent (Tri Reagent, Molecular Research Ctr, Cincinnati, OH) at 1ml per 5-10 x 10⁶ cells and stored at –80°C.

The RNA isolation and reverse transcription were performed following published procedures (Harms et al., 2000). Total RNA was isolated by the guanidine thiocyanate method (Tri Reagent) following kit instructions. The RNA pellet was washed with 75% ethanol and resuspended in sterile diethyl pyrocarbonate (DEPC)-treated water. Splenic mononuclear cell RNA was resuspended to a concentration of 5 x 10⁴ cell equivalents/µl.
Messenger RNA was reverse transcribed using Superscript II RT (Gibco BRL, Gaithersburg, Maryland) to cDNA with oligo dT₁₅ priming of 3 x 10⁶ cells equivalent. Samples of cDNA were stored at –20°C. Negative RT controls were run in parallel.

Real-time PCR was performed using primers developed specifically for Atlantic menhaden and procedures described previously (chapter 3). Amplification of reverse transcribed cDNA samples and standards was performed in parallel. The PCR efficiencies were above 90% and correlation coefficient above 0.996. Results are reported as the ratio of TGF-β/β-actin.

**Statistical Analyses**

Comparisons between control (84% oxygen saturation) and hypoxic groups in the acute experiment were analyzed using Dunnett’s t tests (Zar, 1996). Analysis of variance (ANOVA) was used to independently compare oxygen saturation at specific time points in the subacute experiment. The Statistical Analysis System (SAS; SAS Institute Inc, Cary, NC) was used for all analyses. To satisfy homogeneity of variance, neutrophil percentages, phosphorus, AST, LD, total protein, albumin, albumin/globulin ratio, and calcium were log transformed, and monocyte percentages and CK were square root transformed in both experiments study (Neter et al., 1996). In the acute study, glucose, chloride and K were log transformed, and WBC/thrombocyte counts were square root transformed. Globulin and PCO₂ were log transformed in the subacute experiment. Tukey’s multiple comparison tests was used to determine significance among the
different time points in the subacute study (Neter et al., 1996). Differences were considered significant at $P < 0.05$ in both experiments.

**RESULTS**

**Acute hypoxia exposure study**

**Fish survival and behavior:** During the acute hypoxia experiment mortalities occurred only in the 5% oxygen saturation group. Four fish died within 15 min and 1 at 50 min after 5% oxygen saturation was reached. Hyperventilation and short bursts of activity at the tank surface followed by a decrease in activity and loss of equilibrium were observed in most Atlantic menhaden at this oxygen concentration. Fish with pronounced hyperventilation and escape reactions noted by short bursts of activity at the surface died very shortly afterwards. There were no significant differences in length and weight of Atlantic menhaden among treatment groups (Table 1).

**Histopathology**

No remarkable differences in the presence of histopathologic lesions presence were detected in sections of heart, gill, liver, intestine, kidney, or gonad of fish exposed to different oxygen saturation levels. Hepatocellular vacuolation, pigmented macrophage aggregates (PMAs) in the liver and kidney, and aggregates of protozoal spores consistent
with *Kudoa* sp. in the heart were observed in all 5 oxygen saturation groups. More than 80% of kidney sections contained PMAs.

**Spleno-somatic index**

Spleno-somatic indices were not significantly different between the control and hypoxic groups although indices were highest for the control group and lowest for the 10% oxygen saturation group (Table 1).

**Hematology and plasma chemistry**

Marked differences in blood parameters were observed after acute exposure to different oxygen saturation levels (Table 1). Neutrophil counts were significantly higher at 10 and 15% oxygen saturation than control (84% oxygen saturation; \( P < 0.05 \); Fig. 1a). Monocyte counts, glucose and calcium concentrations were highest at 5% but values were not significantly different from control (Fig. 1b, 1c, and 1d respectively). Phosphorus concentrations were significantly higher at 10% and 15% oxygen saturation than control (\( P < 0.05 \); Fig. 1e). Fish from both the 5% oxygen saturation and control groups showed the lowest phosphorus concentrations (Table 1; Fig. 1e). The muscle enzymes AST, CK, and LD were lowest at 5% oxygen saturation, but differences were not significant (Table 1).

Sodium concentrations increased as the oxygen saturation declined, with the highest concentrations measured at 5% oxygen saturation (Fig. 1f; Table 1). Sodium
concentrations were significantly higher at 5% saturation than control ($P < 0.05$).

Potassium concentrations were significantly higher at 5 and 10% oxygen saturation than control ($P < 0.05$; Fig. 1g). The ratio of Na/K was significantly lower at 5 and 10% oxygen saturation than control (Table 1; $P < 0.05$). Bicarbonate, and pH (Fig. 1h), were lowest at 5% saturation but levels were not significantly different from control (Table 1).

**Subacute hypoxia exposure study**

**Fish survival and behavior**

In the first subacute hypoxia trial, 16 fish died (~36%) in the exposure tank. The highest mortalities were observed at 48 and 57 hr (4 and 7 fish respectively). Only one death was observed during the post-exposure period and no mortalities were observed in the control tank. There were no mortalities in the second trial. There were differences observed between the two trials in Atlantic menhaden behavior. During the first experiment, menhaden in the hypoxia-exposed tank responded much slower to stimuli such as capture and handling than the controls. In the second experiment there were no differences observed between controls and hypoxia-exposed fish. Both groups were very active during capture and initial MS-222 induction. Fish in this study were similar in length and weight (Table 2a).

**Histopathology**

No remarkable differences in lesion prevalence were detected in sections of heart, gill, liver, intestine, kidney, or gonad of hypoxia exposed and control fish with the exception of liver vacuolation. Mild to moderate diffuse hepatocellular vacuolation was more
prevalent in hypoxia-exposed fish than controls (n = 9 and 4 respectively). Of the 48 fish collected for histopathology, approximately 50% (10 hypoxia exposed and 13 controls) had mild to moderate pigmented macrophage aggregates (PMAs) in the liver. PMAs were found in approximately 80% of the kidney sections (19 hypoxia exposed and 20 controls). Gonads were not found for one fish in the 84 h control group (Table 2b).

**Hematology and plasma chemistry**

Most hematological parameters were not altered in Atlantic menhaden during the subacute hypoxia exposure (Table 2b). Absolute leukocyte counts were not significantly different between hypoxia-exposed and control fish at the different time points, although some parameters were elevated (Table 2b). Monocyte counts were higher in hypoxia-exposed Atlantic menhaden than controls at 48 h but counts were not significant (Table 2b). Relative to controls, hypoxia-exposed fish had lower lymphocyte counts at all time points but values were not significantly different (Table 2b). Glucose concentrations were significantly higher in hypoxia-exposed fish than controls at 72 h \( P = 0.026 \), but concentrations returned to that of controls during the subsequent recovery period (Fig. 2a). Phosphorus concentrations were significantly higher for hypoxia-exposed fish at 48h than 72 and 84 h \( P = 0.021 \) and \( 0.0007 \) respectively, but concentrations were significantly lower for hypoxia-exposed fish than controls at 84 h \( P = 0.018 \); Fig. 2b). Concentrations returned to those observed in the control group by the end of the experiment (156 h).
Sodium and potassium concentrations were lower in hypoxia-exposed fish than controls at all time points but the concentrations were not significantly different (Table 2b). Bicarbonate concentrations were significantly higher for hypoxia-exposed fish at 48 h than all other time points \((P < 0.05)\) except for hypoxia-exposed fish sacrificed at 72 h (Fig. 2c; Table 2b). Relative to controls, blood pH was significantly higher for hypoxia-exposed fish at 48 h \((P = 0.049;\) Fig. 2d). Blood pH was also significantly higher for hypoxia-exposed fish at 48 h \((P = 0.029)\) and 72 h \((P = 0.034)\) than controls at 156 h (Table 2b).

**Lymphocyte mitogenesis**

ConA stimulation indices (SI) were lower at 72 and 84 h for hypoxia treated Atlantic menhaden than controls but differences were not significant (Table 2a). Stimulation indices of LPS were similar for both control and hypoxia treated Atlantic menhaden sacrificed at each time point (Table 2a).

**TGF-β**

Splenic mononuclear cell TGF-β mRNA production was lower in hypoxia treated fish than controls at 72 h but differences were not significant (Table 2a). Levels of TGF-β mRNA at each of the other time points were similar for both controls and hypoxia treated fish (Table 2a).
DISCUSSION

Acute hypoxia

The lowest oxygen concentration (5% or 0.39 mg/L) used in this experiment was similar to that reported as the lethal concentration of oxygen (LC$_{50}$) for Atlantic menhaden. Thornton (1975) and Burton et al. (1980) reported a LC$_{50}$ of 0.4mg/L and Shimps (2003) reported a 12hr LC$_{50}$ of approximately 0.9mg/L at 25°C. Most menhaden tried to avoid the hypoxic conditions, and this was evidenced by quick bursts of activity at the surface of the tank. Avoidance behavior in response to low dissolved oxygen was observed previously in Atlantic menhaden under laboratory conditions (1mg/L; Wannamaker and Rice, 2000). Loss of equilibrium prior to death was also observed in channel catfish, and was attributed to depression of the central nervous system due to acidosis (Caillouet, 1968).

Spleno-somatic index

Altered spleno-somatic index can be an indicator of splenic dysfunction and an overall indicator of fish health. The gradual decline in SSI, although not significant, may have been caused by contraction of the spleen and erythrocyte recruitment into the blood (Van Ginneken et al., 1998). Splenic contraction to increase oxygen carrying capacity of the blood has been observed in trout exposed to acute hypoxia (Wells and Weber, 1990).
decrease in SSI was also observed in juvenile rainbow trout, *Oncorhynchus mykiss*, exposed for 24 h to tetrachloroguaiacol (Johansen et al., 1994). The short survival time of fish at 5% oxygen saturation may have prevented this change.

**Hematology and plasma chemistry**

The period of exposure in this study was limited to 1 h and less for fish in the 5% oxygen saturation group. It is possible that the fish in the 5% oxygen saturation group did not survive long enough for an increase in neutrophilic response to be observed. Atlantic menhaden treated with triamcinolone in our laboratory showed both neutrophilia and monocytosis (Johnson et al., 2004). Both monocytosis and neutrophilia are non-specific responses observed in many other animals, including teleosts, during stressful events and disease (Ellsaesser and Clem, 1986; Pickering and Pottinger, 1987). The elevation of blood glucose in the 5% oxygen saturation group, although not significant, has been reported in other teleosts during hypoxia. Hyperglycemia in response to hypoxia was reported in carp during 90 min deep hypoxia treatment (Van Raajj et al., 1996) and channel catfish after 24 h hypoxia treatment (Scott and Rogers, 1981). Glucose mobilization results from elevated glucocorticoids and catecholamines and is also considered a non-specific stress response (Thomas, 1990) occurring within minutes of the onset of the stress (Heath, 1995).

Blood acidosis in the 5% oxygen saturation group was probably due to the depletion of glycogen and a build up of lactic acid (Heath, 1995). This is a very common occurrence in fish exposed to severe hypoxia (Heath, 1995). Diffusion of lactic acid from white
muscle and adrenergic stimulation of Na\(^+/\)H\(^+\) exchange in erythrocytes have been reported to cause blood acidosis, and an increase in Na\(^+\) is a means to balance the lactate ions (Heath, 1995; Wendelaar Bonga, 1997). As reported in channel catfish Caillouet, 1965, this acidosis likely contributed to the loss of equilibrium observed in the present study. Blood acidosis as indicated by the decrease in pH may be the proximate cause of death in Atlantic menhaden at 5% oxygen saturation.

Phosphorus concentrations were significantly higher at 10 and 15% oxygen saturation but levels dropped at 5% oxygen saturation. The observed hyperventilation and anaerobic metabolism are energetically costly and these may be responsible for the depletion of phosphorus seen at 5% (Heath, 1995). Catecholamines have been reported to cause a decrease in intracellular organic phosphates and an elevation of oxygen-hemoglobin affinity in fish (Perry and Thomas, 1991). It has also been reported that erythrocyte ATP concentration decreases near complete anoxia (Greaney and Powers, 1978), and that cortisol injection caused a decline in ATP concentration in red snapper, *Pagrus auratus* (Bollard *et al.*, 1993). Phosphorus is important in many aspects of metabolism; increased plasma phosphorus concentrations at 10 and 15% oxygen saturation levels may reflect critical cellular demand for the ATP needed for cellular function during hypoxia.

**Subacute hypoxia study**

Interestingly, the behavior of the fish in the two replicates in response to hypoxia was very different. This may reflect an aggregate difference in physiologic state between the two groups, perhaps associated with their nutritional status or some other factor,
especially since the two groups were collected at different times in the field.

Nonetheless, this seemed to underscore one of the drawbacks of using wild-caught fish in the laboratory setting.

**Histopathology**

Hepatocellular macrovesicular vacuolation, consistent with fatty change in the liver, may by due to cellular injury, nutritional imbalance or normal lipid storage (Adams et al., 2003; Law, 2001; Heath, 1995). Cellular injury due to hypoxia may have caused the hepatic lipidosis in the first trial, since only hypoxia-exposed fish (n=4) had moderate liver vacuolation. In the second trial, equal numbers of fish in the control and treatment groups had hepatocellular vacuolation and most of these were observed at 84 and 156 h. These changes may have been due to the nutritional state of the fish (Hinton and Lauren, 1990). Although these fish appeared healthy before the study, with wild caught specimens it is difficult to know if adequate food sources were available or were properly utilized before capture. It is quite common to see hepatocellular vacuolation in teleosts in field studies. Hepatocellular vacuolation, attributed to nutritional stress, was found in spot (*Leiostomus xanthurus*) and southern flounder (*Paralichthys lethostigma*) in a recent evaluation of the Pamlico and Core Sounds, NC (Adams et al., 2003).

**Hematology and plasma chemistry**

Similar to the acute study, hyperglycemia was also observed in the subacute study. In our laboratory studies, Atlantic menhaden injected with the synthetic glucocorticoid triamcinolone also showed an increase in glucose concentration (Johnson et al., In press).
This mobilization of energy can result from elevated glucocorticoids and catecholamines (Sapolsky et al., 2000) and is considered a non-specific stress response (Thomas, 1990).

Hypoxia causes a decrease in the hemoglobin oxygen saturation and hyperventilation in fish. A “relative blood alkalosis” and not acidosis was observed in Atlantic menhaden during the subacute hypoxia study. This may be due to the difference in exposure periods. Nikinmaa and Salama (1998) reported that plasma alkalinization is interrupted by an initial phase of plasma acidification (1-2 min after the onset of hypoxia) in many teleosts during acute hypoxia. The acidification of plasma represents the activation of the adrenergic Na\(^+\)/H\(^+\) exchange, protons are extruded from the red blood cells in exchange for Na\(^+\). The increase in pH and HCO\(_3\)\(^-\) at 48 and 72 h and the subsequent decline observed during the recovery period suggest that Atlantic menhaden will probably re-establish acid-base equilibrium. But the relatively short post-exposure observation period precluded making these observations. The increase in pH and HCO\(_3\)\(^-\) allowed Atlantic menhaden to increase their hemoglobin oxygen affinity. The increase in hemoglobin oxygen affinity due to the adrenergic activation of the red blood cell Na\(^+\)/H\(^+\) exchanger improved oxygen loading during hypoxia in carp, *Cyprinus carpio* and other teleosts (Nakinmaa et al., 1987, Nikinmaa and Salama, 1998; Thomas and Perry, 1992).

Our initial hypothesis was that these levels of hypoxia would lead to abnormalities in menhaden immune function and would help explain the recent rise in the proportion of Atlantic menhaden with lesions observed in outbreaks. However, there were no significant differences between treated animals and controls in either lymphocyte
mitogenesis, a marker for the adaptive immune system, or TGF-β mRNA, a multifunction, negative regulator of immune function. It is possible that longer periods of hypoxia are required before changes in these bioindicators are seen. Alternatively, a different component of the immune process than was measured, such as phagocytic capacity or mucosal immunity, may be affected by hypoxia.

Data from other work in our laboratory suggest that Atlantic menhaden have a remarkable ability to deal with oxidative stress, perhaps due to their high oil content and high concentrations of tocopherols which may serve as a type of “sink” for reactive oxygen species (Lehmann et al., In press). Menhaden exposed to hypoxic conditions did not develop muscle necrosis nor did they show significant increases in several markers of oxidative damage. This is in contrast to the deep muscle necrosis followed by secondary infections reported in channel catfish exposed to hypoxia reported by Plumb et al., 1976. Hypoxia may be a necessary but not sufficient factor in the development of lesions reported as EUS in many estuarine species such as menhaden and flounder.

Conclusions

In both experiments, glucose and electrolytes were the blood parameters most affected by hypoxic conditions. These health parameters did offer information about the differences between the lethal (5%) and the sublethal (20%) oxygen saturation levels. Fish exposed to acute lethal oxygen saturation showed signs of “relative blood acidosis” while those exposed to sublethal subacute hypoxia showed signs of “relative blood alkalosis”.
Atlantic menhaden spawn offshore, and North Carolina’s estuaries serve as nurseries for juveniles (Ahrenholz, 1991). As summer temperatures rise, massive oxygen depletion-related mortality of menhaden sometimes occurs in the Tar-Pamlico and Neuse estuaries. Schools of Atlantic menhaden may try to avoid areas of low oxygen, but mortality due to extremely low DO may be inevitable since blood acidosis occurs very rapidly. As Atlantic menhaden schools move into creeks to feed or escape from predators, the collective biomass of these schools may also deplete the oxygen supply available within small tributaries and result in mortality (NCDENR-DWQ, 2001; Smith, 1999). Mortality rates may be affected by other environmental conditions as well as the health status of the fish experiencing the low DO event. Low dissolved oxygen related fish kills in estuaries may be due to blood acidosis, and as noted previously, the schooling behavior of Atlantic menhaden (Smith, 1999; Law, 2001; NCDENR-DWQ, 2001).

Little difference was observed in the majority of the health indicators that we used to compare the response of fish exposed to hypoxic conditions with controls. It is possible that more marked differences in these health parameters would have been observed if the study was extended for a longer period of time. “Relative blood alkalosis” was experienced at 48, and 72 h after low DO exposure. During the recovery phase of the experiment most parameters that were significantly affected by the low DO returned to levels observed in the control group. Blood pH recovery was much slower and although levels declined during the recovery phase, levels did not return to that of controls. Therefore acid-base balance may require a longer time to recover after extended periods
of low DO exposure. This may have consequences in estuarine systems where fish are exposed to multiple factors with potential deleterious health effects, and exposure to these stressors may cumulatively affect their ability to withstand additional stressful events and resist disease. The ability to effectively examine the potential consequences of exposure to these factors in the field, however, is severely impaired by the movement of fish in the estuary, which limits documentation of the exposure history of individual fish or schools. Sentinel cohort studies with penned fish may improve efforts to interpret the effects of changes in the estuarine environment on estuarine species.

ACKNOWLEDGEMENTS

We thank Captain Jeffrey Cronk of Fish’N4Life Charters for help with specimen collection, and Dr. Cavell Brownie for statistical help. Funding for this study was provided, in part, by the North Carolina Department of Environment and Natural Resources (Project No. EW 200020) and the North Carolina Department of Health and Human Services/Centers for Disease Control funds (Project No. 01081-01).

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Table 1. Morphometric and blood parameters measured for Atlantic menhaden after acute exposure to different oxygen saturation (%).  F = female; M = male.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Unit</th>
<th>5% (M=4; F=1)</th>
<th>10% (M=4; F=1)</th>
<th>15% (M=3; F=2)</th>
<th>20% (M=2; F=3)</th>
<th>84% (control) (M=1; F=2)</th>
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<tbody>
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<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Body Weight g</td>
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<td>82.7</td>
<td>17.8</td>
<td>65.4</td>
<td>15.3</td>
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<td>0.4</td>
<td>1.3</td>
<td>0.3</td>
</tr>
<tr>
<td>PCV %</td>
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<td>8.7</td>
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<td>10.6</td>
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<tr>
<td>WBC/Thrombocytes x10³ µl⁻¹</td>
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<td>34.5</td>
<td>58.8</td>
<td>23.9</td>
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<td>1.7</td>
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<td>4.2</td>
<td>8.6</td>
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<td>Eosinophils x10³ µl⁻¹</td>
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<td>Lymphocytes x10³ µl⁻¹</td>
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<td>15.9</td>
<td>12.0</td>
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<td>2.8</td>
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<td>17.0</td>
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<td>Glucose mg dL⁻¹</td>
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<td>Phosphorus mg dL⁻¹</td>
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<td>iCalcium nmol/L</td>
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<td>1.5</td>
<td>0.0</td>
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<tr>
<td>Total Protein g dL⁻¹</td>
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<td>1.3</td>
<td>0.6</td>
<td>1.7</td>
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<tr>
<td>Albumin g dL⁻¹</td>
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<td>0.6</td>
<td>0.2</td>
<td>0.5</td>
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<tr>
<td>Globulin g dL⁻¹</td>
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<td>0.7</td>
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<tr>
<td>Albumin/Globulin</td>
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<td>AST U L⁻¹</td>
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<td>51.9</td>
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<td>CK U L⁻¹</td>
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<td>LD U L⁻¹</td>
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<td>Sodium nmol L⁻¹</td>
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<td>158.5</td>
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<td>Potassium nmol L⁻¹</td>
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<td>Sodium/Potassium</td>
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<td>Chloride mmol L⁻¹</td>
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<td>151.6</td>
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<td>Bicarbonate mmol L⁻¹</td>
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<td>pH</td>
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<td>TCO2 mM</td>
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Table 2. Morphometric and immune function parameters (A) and blood parameters (B) for Atlantic menhaden exposed to hypoxic (20% oxygen saturation) and normoxic (84% oxygen saturation) conditions. F = female; M = male.

### A

<table>
<thead>
<tr>
<th>Variables</th>
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<th></th>
<th>72 Hour</th>
<th></th>
<th>84 Hour</th>
<th></th>
<th>156 Hour</th>
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<td>Treatment (M=4; F=5)</td>
<td>Control (M=4; F=7)</td>
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<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
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<tr>
<td>Body Weight</td>
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<td>SD</td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
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<tr>
<td>TGF-β</td>
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<td></td>
<td></td>
<td>Control (M=4; F=6)</td>
<td>Treatment (M=5; F=5)</td>
<td>Control (M=3; F=7)</td>
<td>Treatment (M=4; F=7)</td>
<td>Control (M=6; F=4)</td>
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### B

<table>
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<tr>
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</thead>
<tbody>
<tr>
<td></td>
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<td>Control (M=3; F=2)</td>
<td>Treatment (M=3; F=3)</td>
<td>Control (M=2; F=4)</td>
<td>Treatment (M=1; F=5)</td>
<td>Control (M=3; F=1)</td>
<td>Treatment (M=2; F=4)</td>
<td>Control (M=2; F=4)</td>
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<tr>
<td>Neutrophils</td>
<td>x10^3 µL⁻¹</td>
<td>6.37</td>
<td>9.25</td>
<td>4.76</td>
<td>5.55</td>
<td>1.73</td>
<td>2.08</td>
<td>5.56</td>
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<td>Monocytes</td>
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<td>0.27</td>
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<td>5.45</td>
<td>2.80</td>
<td>3.16</td>
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<tr>
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<td>0.99</td>
<td>1.01</td>
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<td>0.49</td>
<td>0.57</td>
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<tr>
<td>Lymphocytes</td>
<td>x10^3 µL⁻¹</td>
<td>20.32</td>
<td>3.40</td>
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<td>16.50</td>
<td>25.26</td>
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<tr>
<td>Thrombocytes</td>
<td>x10^3 µL⁻¹</td>
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<td>4.40</td>
<td>19.36</td>
<td>16.10</td>
<td>28.16</td>
<td>15.11</td>
<td>18.76</td>
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<tr>
<td>WBC/Thrombocyte</td>
<td>x10^3 µL⁻¹</td>
<td>40.33</td>
<td>14.00</td>
<td>27.73</td>
<td>76.00</td>
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<td>Treatment (M=3; F=3)</td>
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<tr>
<td>Glucose mg dL⁻¹</td>
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<td>33.00</td>
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<td>1.14</td>
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<td>3.28</td>
<td>7.37</td>
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<td>Albumin/Globulin</td>
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Fig. 1. Comparison of blood parameters measured in Atlantic menhaden during acute hypoxia. Oxygen saturations of 5%, 10%, 15%, and 20% were compared to 84% (control). Asterisks (*) indicate significant difference between the hypoxia-exposed and the control group ($P < 0.05$). Mean values ± SE; $n = 5$ for all groups except 84% ($n = 3$).
Fig. 2. Blood parameter measurements for Atlantic menhaden exposed to oxygen concentrations of 20% (●) and 84% (▲) saturation for 48, 72, 84, and 156 hours. Treatment group was exposed to 20% oxygen saturation for 72 hours. During the recovery period, oxygen saturation was the same as the control group (84%). Time points for which significant differences between hypoxia-exposed and control menhaden were observed are denoted by asterisks (*). Mean values ± SE; n = 6.