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, 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 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

(Press and Evensen, 1999). 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 menhadens (*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 (YOY; 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 chevron 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

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

(Anderson, 1990; Schreck, 1996). 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. Behavorial 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 methanosulfonate (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 (HCO_3^-). The analyzer calculated albumin/globulin ratio, sodium/potassium ratio, and anion gap (AGAP). Anion gap was determined as follows: $[(Na^+ + K^+)-(Cl^- + HCO_3^-)]$.

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°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 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°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 (1.0×10^6 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 = mean OD of stimulated culture/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, Na^+, K^+,

Na^+/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 ($P = 00128$ 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 ($P = 0.0357$; 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; 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 ($P < 0.05$; 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 ($P < 0.02$; Table 3) whereas globulin concentrations were significantly higher in June than July and October ($P = 0.0049$ and 0.0327 respectively; Table 3).

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

Sodium concentrations were significantly lower in fish collected in September than all other months ($P < 0.05$; 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 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 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 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).

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 seasonal 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.

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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.

Collection Dates	Sites	Estuarine System	n
June 8	Whittaker Creek	Neuse	10
June 17	Swansboro	White Oak	12
June 19	Oriental	Neuse	10
June 25	Upper Broad Creek	Neuse	10
July 4	Oriental	Neuse	8
July 9	Upper Broad Creek	Neuse	10
July 16	Oriental	Neuse	10
July 27	Swansboro	White Oak	12
July 30	Blounts Bay	Pamlico	13
August 2	Trent River	Neuse	10
August 19	Bath Creek	Pamlico	10
August 25	Swansboro	White Oak	12
September 4	Durham Creek	Pamlico	10
September 6	Black Beacon & Johnson Pt	Neuse	10
September 13	Swansboro	White Oak	12
September 16	Johnson Pt	Neuse	10
September 18	Broad Creek	Pamlico	6
September 30	Broad Creek	Pamlico	6
October 2	Swansboro	White Oak	13
October 4	Goose Creek	Neuse	10
October 17	Upper Goose Creek	Pamlico	6
October 23	Durham Creek	Pamlico	5

Table 2. Morphometric, organosomatic, hematological, and immune function parameters for Atlantic menhaden from the White Oak, Neuse and Pamlico river estuaries in 2002.

| | June | | | | July | | | | | | August | | | |
| | White Oak | | Neuse | | White Oak | | Neuse | | Pamlico | | White Oak | | Neuse | |
	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD
Fork Length (mm)	148.9	4.9	109.4	30.8	158.3	6.9	92.3	4.8	149.8	21.0	162.3	4.8	89.5	2.8
Body Weight (g)	43.3	6.0	23.6	20.4	56.4	8.2	10.6	1.6	51.2	24.1	70.3	6.4	10.3	0.8
Liver-somatic index	4.1	0.9	5.2	0.5	6.4	1.0	5.0	2.7	11.2	4.0	6.8	0.8	7.4	2.8
Spleno-somatic index	0.5	0.2	1.3	0.8	0.6	0.4	0.6	0.2	1.0	0.5	0.9	0.1	0.5	0.3
PCV (%)	26.3	5.8	33.4	5.2	35.3	5.2	40.3	5.5	40.2	7.2	42.2	3.5	48.2	4.3
WBC/Thrombo (/μl)	209.7	190.6	138.2	169.5	132.3	69.4	81.0	26.1	87.0	28.5	117.7	38.1	129.7	40.1
Neutrophils (/μl)	22780	18362	3460	2163	7430	6062	6373	4953	11545	17223	13023	14346	4167	4915
Eosinophils (/μl)	6400	7354	996	560	2948	2037	903	327	1060	113	3765	3824	1733	372
Lymphocytes (/μl)	5094	20973	36895	51166	27207	8901	28331	18967	21840	10204	15903	6011	59770	23181
Monocytes (/μl)	7260	7077	2621	3877	5440	6872	1753	1432	2390	1659	4040	4284	3616	1615
Thrombocytes (/μl)	79390	77892	93757	121777	89317	57543	42304	17326	53100	19201	75377	14250	56213	13566
Total Plasma Protein (g/dl)	2.5	0.2	2.5	0.2	3.1	0.6	2.5	0.2	2.8	0.9	3.5	0.4	3.1	0.3
ConA (SI)	0.99	0.17	1.02	0.21	0.96	0.12	1.03	0.13	0.98	0.14	0.89	0.09	1.21	0.14
LPS (SI)	0.98	0.13	1.00	0.18	1.00	0.09	0.98	0.10	0.97	0.13	0.89	0.05	1.16	0.14

| | September | | | | | | October | | | | |
| | White Oak | | Neuse | | Pamlico | | White Oak | | Neuse | | |
	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mea
Fork Length (mm)	162.9	4.5	94.3	2.9	100.0	9.6	135.0	6.1	104.0	3.2	101
Body Weight (g)	69.7	5.4	11.4	1.0	13.6	3.1	38.5	4.1	16.9	1.0	16.
Liver-somatic index	6.2	0.6	6.3	1.7	7.0	1.8	5.0	0.6	5.7	0.9	6.6
Spleno-somatic index	0.8	0.2	0.8	0.3	0.8	0.4	0.6	0.1	0.5	0.1	1.3
PCV (%)	40.5	6.2	45.2	3.3	48.3	4.1	40.0	6.1	39.5	6.0	41.
WBC/Thrombo (/μl)	110.0	44.6	91.3	33.3	87.3	21.3	120.3	54.1	68.7	21.8	66.
Neutrophils (/μl)	6507	5279	8738	8528	3131	1290	9343	4925	3187	2687	486
Eosinophils (/μl)	3730	1287	2316	1657	890	156	3176	2602	520		134
Lymphocytes (/μl)	28937	15494	26700	15401	28657	13565	34753	14234	18953	13111	214
Monocytes (/μl)	2240	1075	3236	2095	1740	950	2145	1248	1260	453	251
Thrombocytes (/μl)	61377	26981	51247	29216	53248	17083	71603	38716	44760	9809	359
Total Plasma Protein (g/dl)	3.3	0.5	2.7	0.3	2.9	0.3	2.9	0.5	2.9	0.2	2.
ConA (SI)	0.99	0.07	1.05	0.07	1.01	0.07	1.05	0.07	1.00	0.05	1.0
LPS (SI)	0.99	0.08	1.03	0.09	1.00	0.06	1.03	0.08	0.97	0.06	0.9

Table 3. Monthly plasma chemistry values for Atlantic menhaden collected from the White Oak, Neuse and Pamlico River estuaries in 2002.

| | June | | | | July | | | | | | August | | | | | |
| | *White Oak* | | *Neuse* | | *White Oak* | | *Neuse* | | *Pamlico* | | *White Oak* | | *Neuse* | | *Pamlico* | |
Variable	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD
Glucose (mg/dl)	39.8	11.1	19.3	16.0	53.8	11.8	29.4	13.0	65.5	24.6	69.7	15.7	36.5	11.3	41.8	4.3
Phosphorus (mg/dl)	12.7	3.9	17.0	6.0	13.1	2.7	13.3	2.1	8.3	1.1	14.2	2.1	15.4	2.9	13.4	2.2
Calcium (g/dl)	11.1	3.4	13.2	1.7	13.9	2.2	12.7	2.6	11.8	1.4	15.0	1.7	13.0	0.8	14.4	0.7
Albumin (g/dl)	0.50	0.17	0.63	0.14	0.58	0.46	0.77	0.17	0.70	0.30	1.05	0.11	0.88	0.08	0.93	0.14
Globulin (g/dl)	1.37	0.47	1.68	0.83	1.60	0.42	1.18	0.23	1.32	0.38	1.85	0.22	1.60	0.19	1.33	0.19
Albumin/Globulin	0.4	0.2	0.4	0.4	0.6	0.1	0.7	0.2	0.5	0.1	0.6	0.1	0.6	0.1	0.7	0.1
AST (U/L)	282	52	358	101	379	94	315	116	136	49	435	194	403	98	206	51
CK (U/L)	428	267	1246	280	2836	1929	2849	2388	3099	2287	2031	2148	4951	2259	6621	2474
Sodium (mmol/l)	188.3	17.0	172.8	12.9	186.7	7.4	172.9	5.6	167.7	7.5	196.2	10.7	165.3	4.8	171.0	4.6
Chloride (mmol/l)	161.7	10.1	149.2	14.3	154.5	2.3	148.7	11.7	147.2	4.0	165.7	5.6	135.8	4.2	142.2	2.6
Na/K	34.0	28.3	57.3	45.0	77.8	22.3	68.2	18.5	60.6	32.1	113.9	20.7	42.5	30.7	37.3	15.8
LD (U/L)	1726	398	2114	561	2389	745	2110	1104	1078	619	3259	1159	3928	886	2291	558
Anion Gap	32.0	7.5	24.3	4.6	31.4	7.0	22.0	8.9	19.6	3.8	28.9	4.9	30.4	4.7	29.1	2.4
Potassium (mmol/l)	10.5	7.7	5.1	3.2	2.6	0.8	2.8	1.0	3.8	2.3	1.8	0.3	5.9	3.7	5.5	2.5
Bicarbonate (mmol/l)	5.3	1.0	4.8	0.9	4.0	0.0	5.2	1.4	4.8	1.2	4.2	0.4	5.0	1.1	5.2	0.8

| | September | | | | | | October | | | | | |
| | *White Oak* | | *Neuse* | | *Pamlico* | | *White Oak* | | *Neuse* | | *Pamlico* | |
Variable	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD
Glucose (mg/dl)	59.7	10.3	37.8	14.8	33.7	4.8	45.8	5.0	34.5	9.3	32.7	9.7
Phosphorus (mg/dl)	15.1	2.6	12.5	3.5	12.8	2.3	12.9	2.5	12.6	1.0	11.6	1.1
Calcium (g/dl)	15.2	0.9	12.8	1.4	12.1	1.2	13.9	0.7	13.0	1.2	11.6	1.9
Albumin (g/dl)	0.97	0.05	0.85	0.18	0.82	0.14	0.87	0.16	0.88	0.04	0.72	0.27
Globulin (g/dl)	1.62	0.31	1.47	0.17	1.34	0.36	1.57	0.26	1.18	0.43	1.56	0.34
Albumin/Globulin	0.6	0.1	0.6	0.1	0.7	0.3	0.6	0.1	0.9	0.4	0.5	0.1
AST (U/L)	252	66	373	99	279	65	225	66	196	54	246	73
CK (U/L)	866	658	10558	9013	6405	2237	2392	1356	5925	1574	5122	2363
Sodium (mmol/l)	199.8	4.0	157.8	6.3	163.4	4.3	179.8	4.4	169.8	3.3	154.2	5.7
Chloride (mmol/l)	168.8	2.5	134.3	5.6	139.8	3.4	152.0	3.6	139.5	1.8	132.7	3.4
Na/K	153.3	25.6	21.0	5.6	30.7	10.8	60.4	38.5	51.9	16.9	26.0	10.8
LD (U/L)	2084	790	3057	837	2610	640	2295	820	1488	542	2081	889
Anion Gap	26.5	2.3	23.3	2.5	24.2	3.5	26.3	4.3	28.5	2.2	21.9	5.0
Potassium (mmol/l)	1.3	0.2	7.9	1.8	5.9	1.8	4.4	2.8	3.8	2.0	6.9	2.9
Bicarbonate (mmol/l)	5.8	1.6	8.1	1.4	5.4	1.3	5.6	0.9	5.8	1.2	7.4	2.1
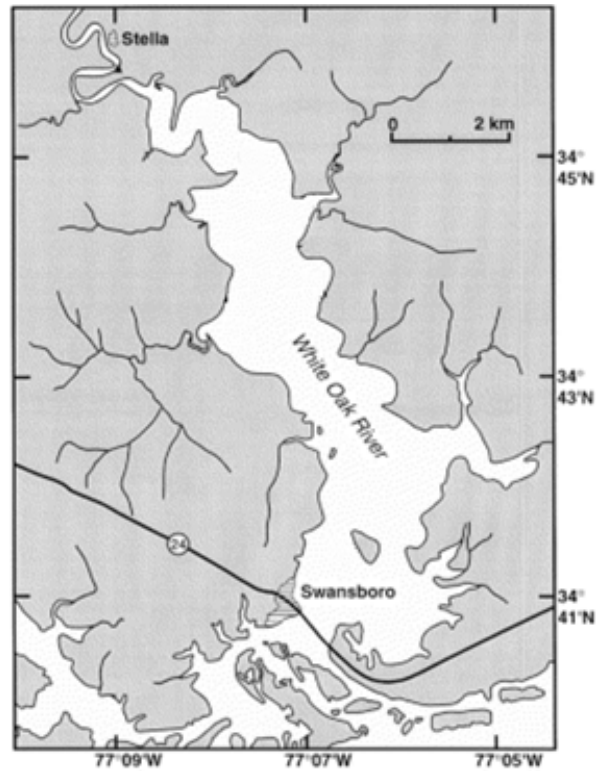
Fig. 1. Map of the White Oak River estuary. Atlantic menhaden were collected near the Highway 24 bridge in Swansboro from June to October in 2002.
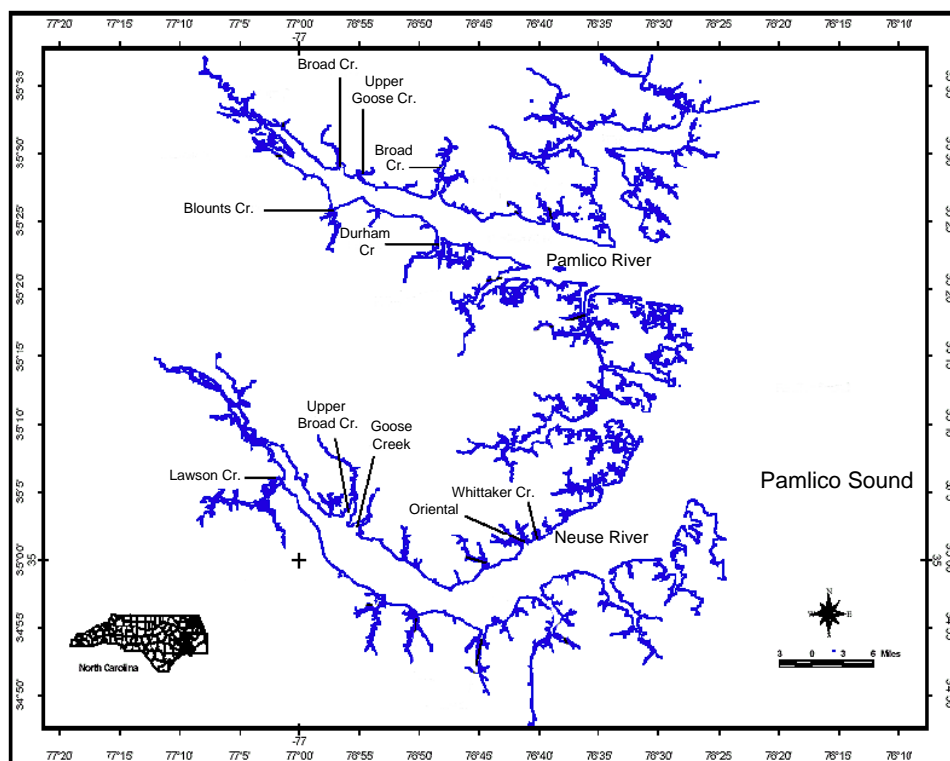
Fig. 2. Map of the Pamlico and Neuse River estuaries showing creeks sampled in 2002.
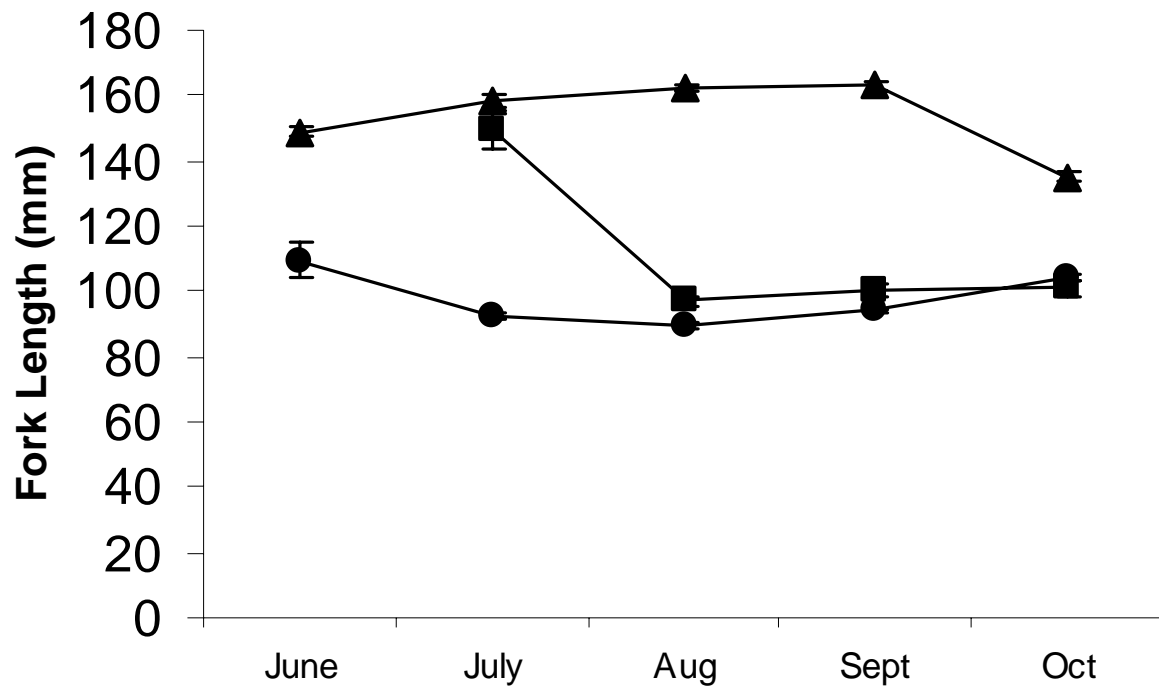
Fig. 3. Mean fork length (mm) for Atlantic menhaden collected by cast net in the White Oak (▲), Neuse (●), and Pamlico (■) River estuaries during 2002. Values are means ± standard error.

White Oak

Neuse

Pamlico

White Oak
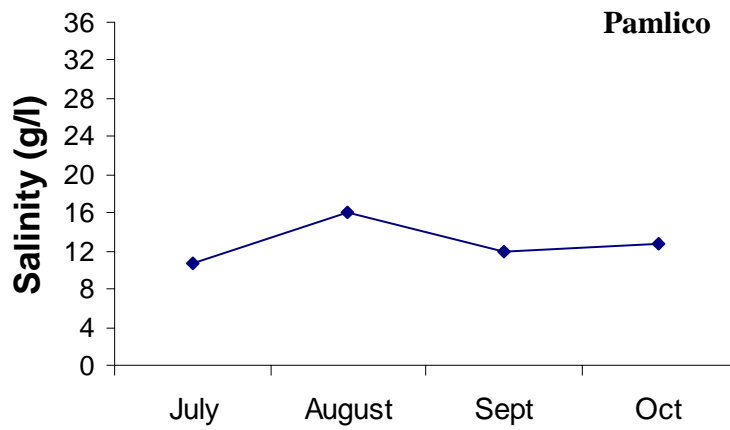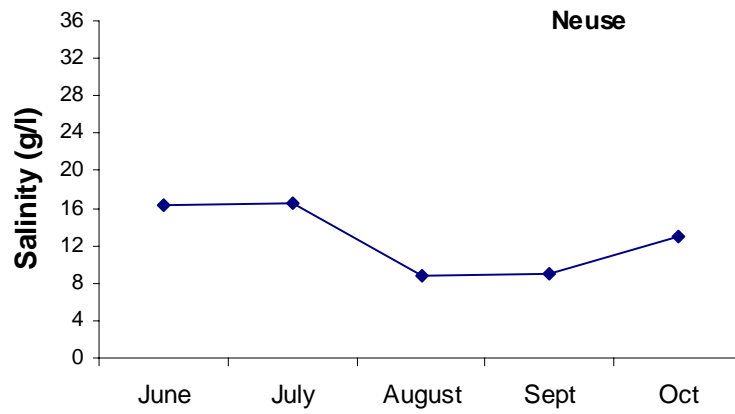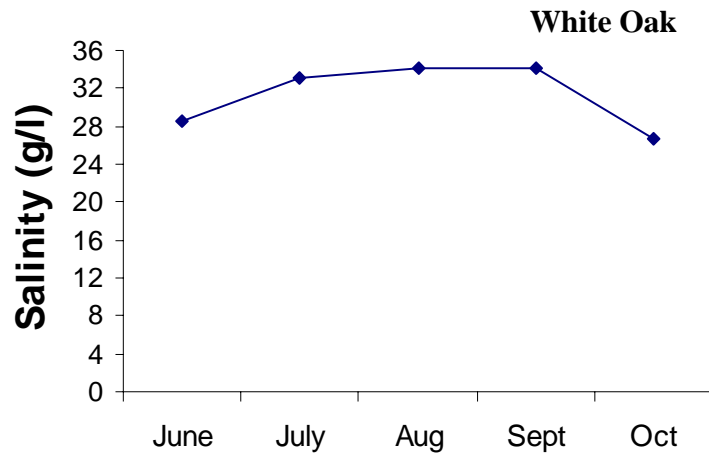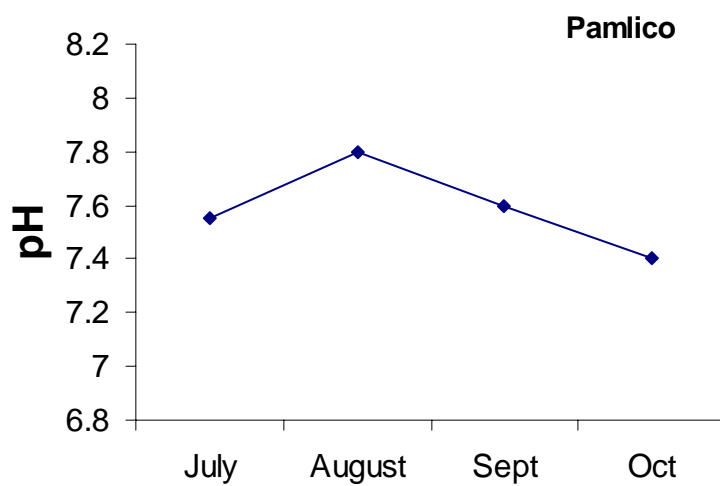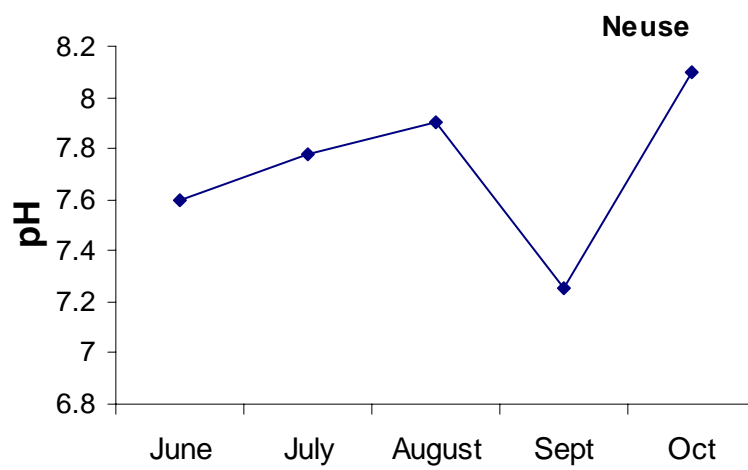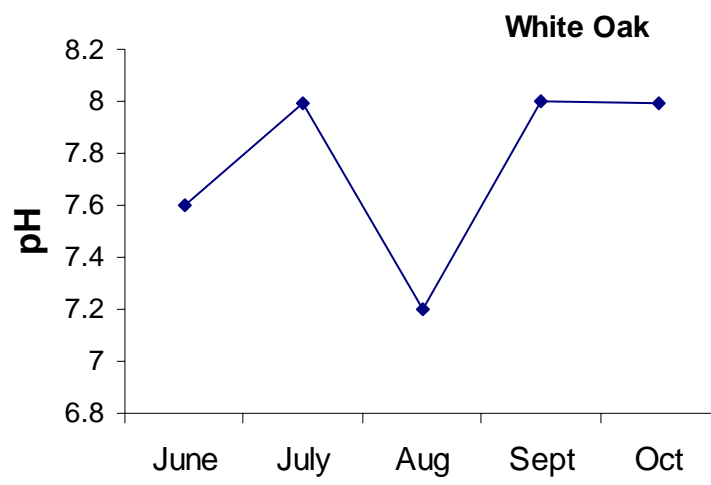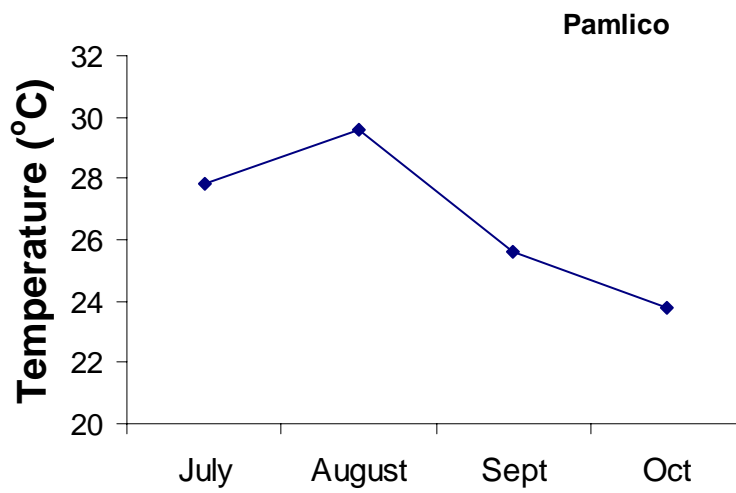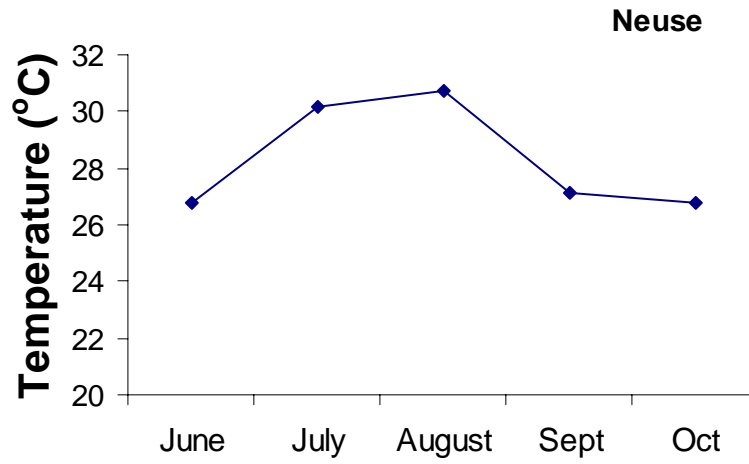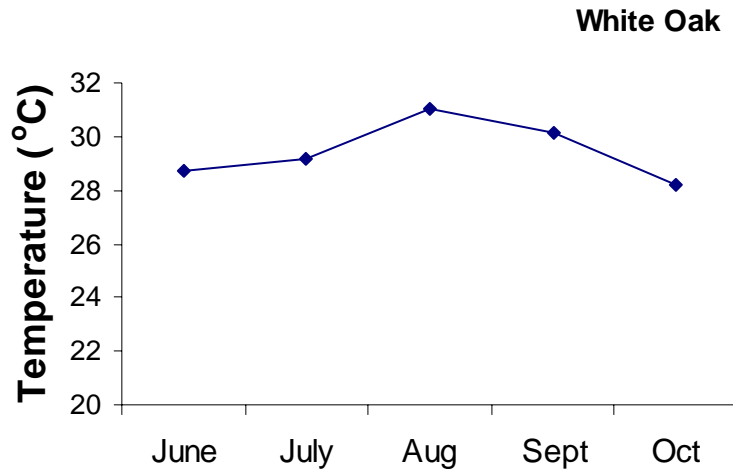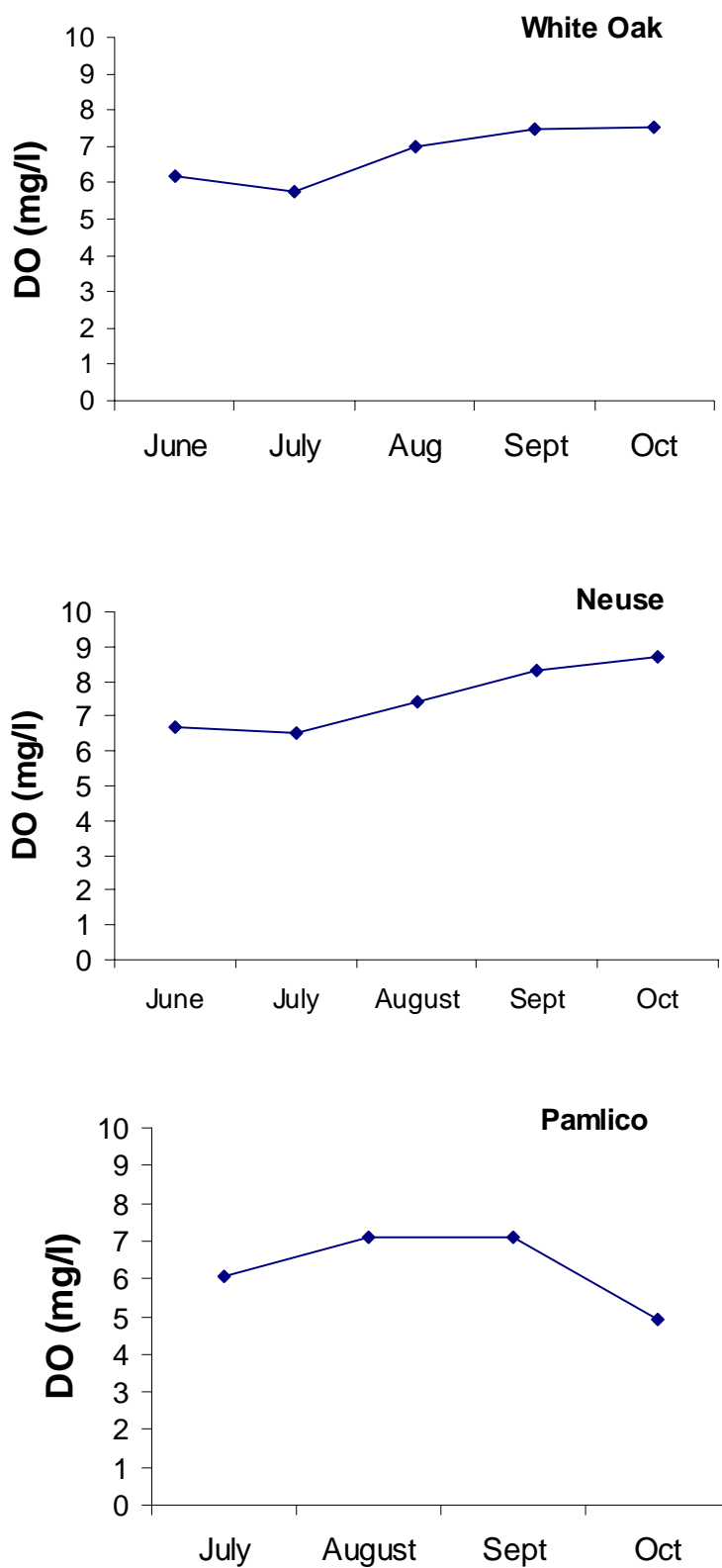
Neuse

Pamlico

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.
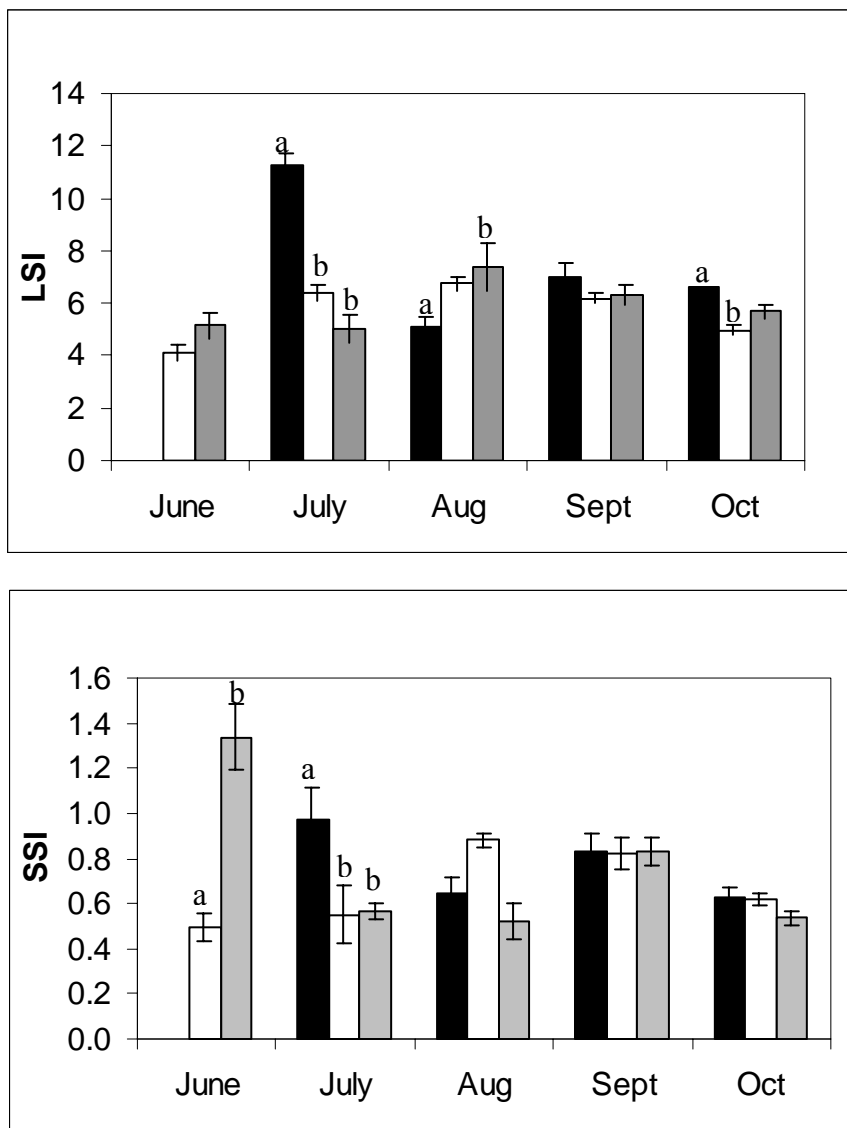
Fig. 5. Comparison of (a) liver-somatic and (b) spleno-somatic indices for Atlantic menhaden collected in the White Oak (□), Neuse (▨), and Pamlico (■) River estuaries. Significant differences among the river systems for each month are indicated by different letters. In July, both SSI and LSI were significantly 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.
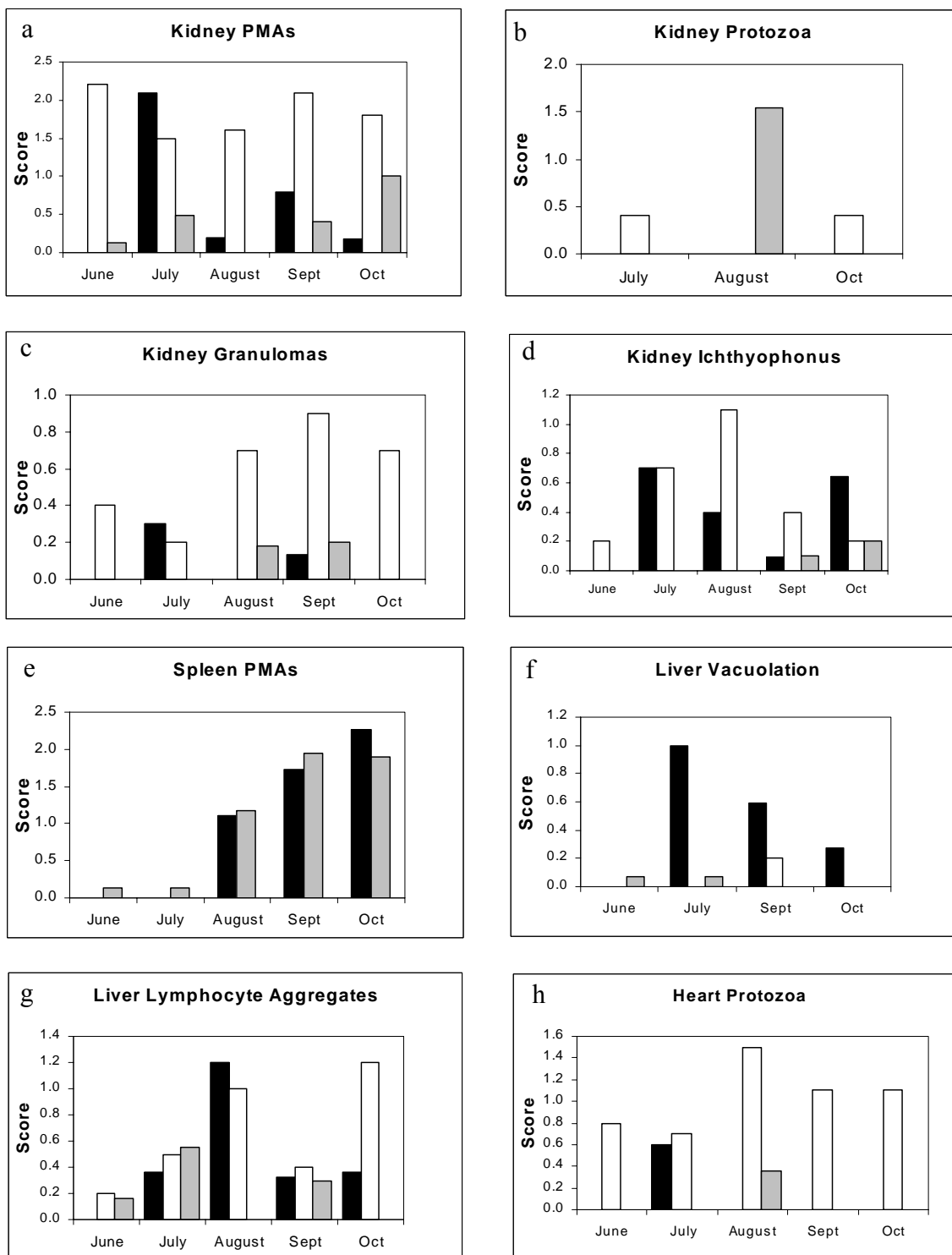
Fig. 6. Mean monthly lesion scores for Atlantic menhaden collected in the White Oak (□), Neuse (▨), and Pamlico (■) Rivers from June to October 2002. Sample sizes (*n*) are the same as in Table 1 except for the Pamlico River where n = 11 for July.
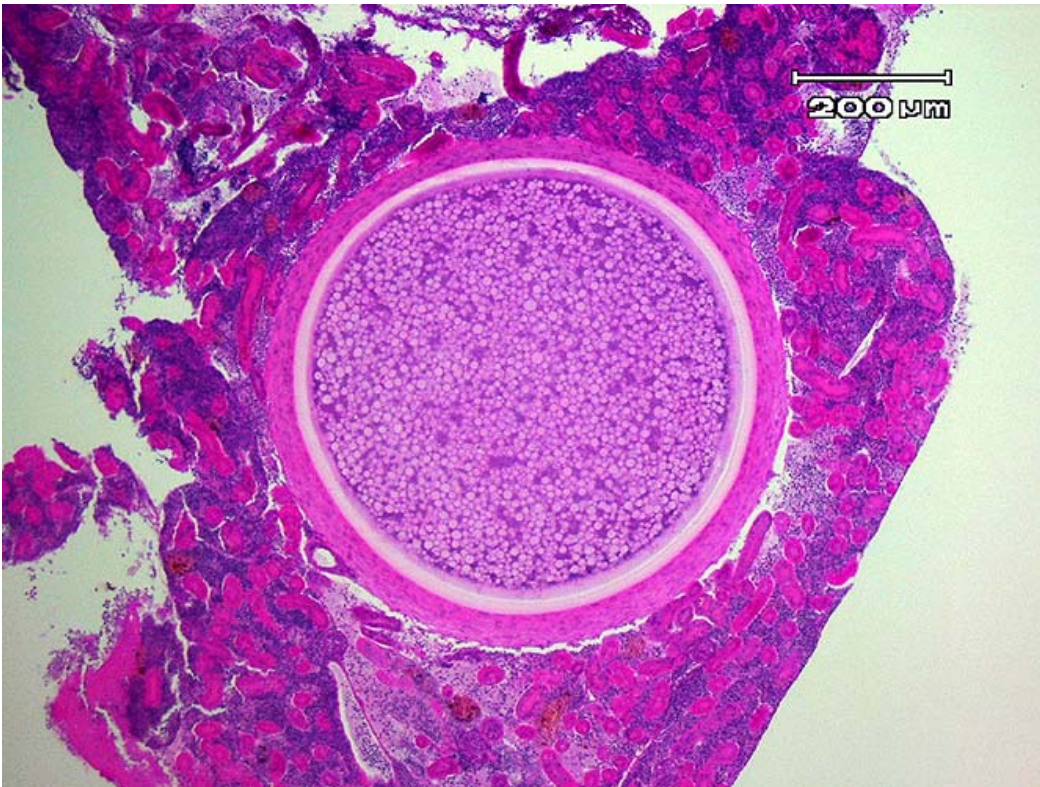
Fig. 7. Section of kidney showing a multinucleate spore, consistent with *Ichthyophonus* sp. A focal area of renal tubules and intervening hematopoietic tissue has been expanded and replaced by this large parasite granuloma. The multinucleate spore, consistent with *Ichthyophonus* sp., has a thick wall 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.
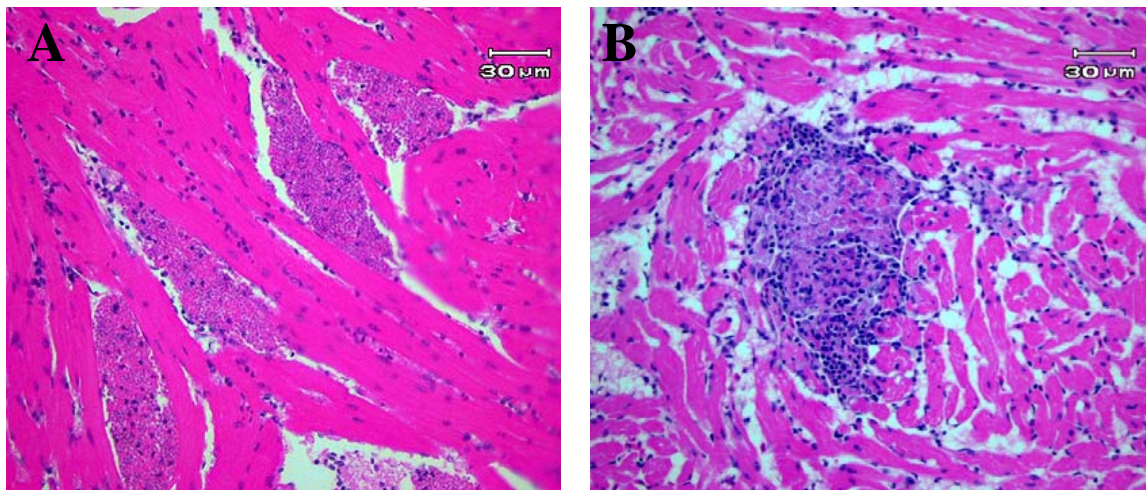
Fig. 8. Section of heart tissue showing myxosporean spores consistent with *Kudoa* sp. from Atlantic menhaden caught in the White Oak River. (A) The myocardial interstitium is expanded and replaced by aggregates of 1 to 2μm protozoal spores. These spores incited little to no surrounding host inflammatory 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.
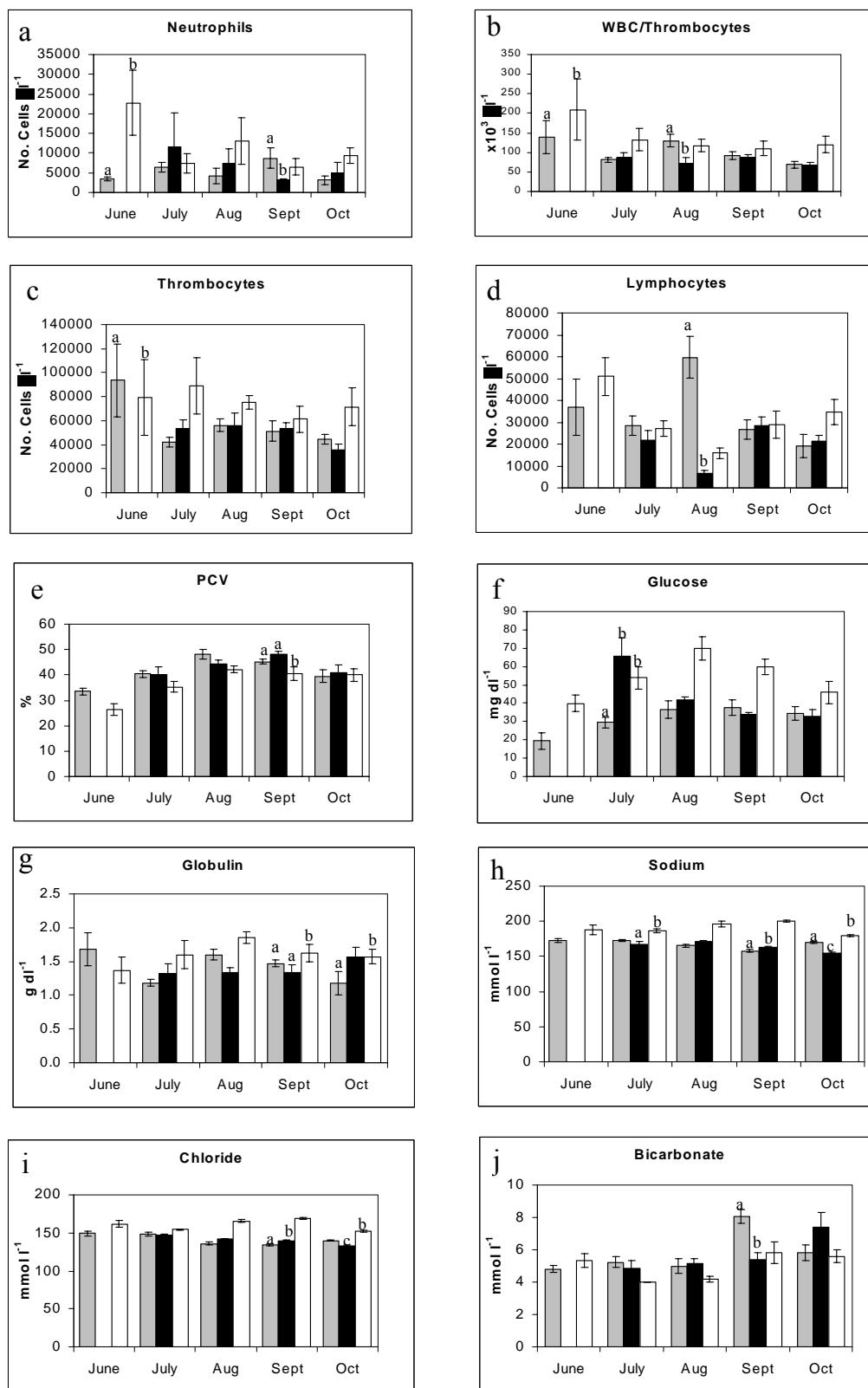
Fig. 9. Blood parameter measurements for Atlantic menhaden collected in the White Oak
(□), Neuse (▨), and Pamlico (■) River estuaries during 2002. Months for which
significant differences ($P < 0.05$) among the three river estuaries were observed are
denoted by different letters. Values are means ± standard error.
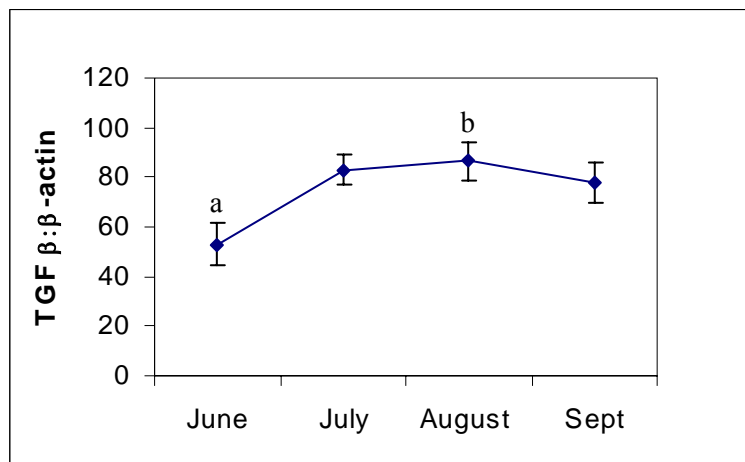
Fig. 10. Monthly variability in TGF-β:β-actin ratios for Atlantic menhaden caught in the White Oak River estuary. Means that are significantly different ($p < 0.05$) have different letters. Sample sizes are the same for each month ($n = 8$). Values are means $\pm$ standard error.

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 (HCO_3^-). The analyzer calculated albumin/globulin ratio, sodium/potassium ratio, and anion gap (AGAP). Anion gap was determined as follows: $(Na^+ + K^+)-(Cl^- + HCO_3^-)$.

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/50µl). 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-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 OD_{570} with reference wavelength of OD_{630} using a microplate reader.

Stimulation indices were calculated using the formula:

SI = mean OD of stimulated culture/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 10^6 cells and stored at $-80°C$.

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 x 10^4 cell equivalents/µl. 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 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-β/β-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 ± 4.42 and 73.08 ± 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 ± 0.6 and 3.15

± 0.72 respectively; $P = 0.0002$; 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 ± 0.91 and 1.81 ± 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

($P < 0.0001$; Table 4). 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.

2001													
	Lesioned				Non-lesioned								
	Blounts Bay		Broad Creek		Blounts Bay		Upper Goose Creek		North Creek		Runyon Creek		
	(n = 5)		(n = 15)		(n = 5)		(n = 5)		(n = 10)		(n = 10)		
Variable	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	
Fork length (mm)	81.0	2.2	88.7	3.0	83.0	5.7	92.0	8.4	104.0	6.1	83.5	3.4	
Body weight (g)	7.2	1.3	9.9	1.1	7.8	1.6	11.6	3.2	16.3	3.3	7.9	0.9	
Liver-somatic index	7.4	1.9	4.6	1.8	4.6	0.9	4.2	0.6	5.6	1.9	6.1	1.9	
CONA (SI)	1.0	0.2	1.0	0.2	1.3	0.5	1.2	0.4	0.9	0.2	0.9	0.2	
LPS (SI)	0.6	0.5	1.1	0.4	0.7	0.4	1.0	0.2	1.2	0.2	1.1	0.3	

2002												
	Lesioned						Non-lesioned					
	Broad Creek		Durham Creek		Upper Goose Creek		Broad Creek		Durham Creek		Upper Goose Creek	
	(n = 8)		(n = 5)		(n = 10)		(n = 12)		(n = 5)		(n = 6)	
Variable	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD
Fork length (mm)	88.0	2.2	97.8	3.6	99.3	1.5	93.6	4.7	105.7	12.2	100.0	2.6
Body weight (g)	9.7	0.9	13.7	1.5	13.9	0.6	11.5	1.9	17.1	10.2	14.7	1.0
Liver-somatic Index	8.9	1.3	9.7	2.8	7.7	1.9	7.4	2.1	6.9	1.2	5.4	1.3
Spleno-somatic index	1.2	0.5	0.8	0.2	0.8	0.2	0.7	0.3	0.9	0.4	0.6	0.2
CONA (SI)	1.0	0.1	1.0	0.0	1.0	0.1	1.0	0.1	1.0	0.1	1.0	0.1
LPS (SI)	1.0	0.1	0.9	0.0	0.9	0.1	1.0	0.1	1.0	0.1	0.9	0.1

Table 2. Hematology values for Atlantic menhaden sampled from all sample sites in the fall of 2001. n = number of pooled samples.

		2001 Creeks													
		Broad Creek			Blounts Bay			Upper Goose Creek			North Creek			Runyon Creek		
		Mean	±SD	n	Mean	±SD	n	Mean	±SD	n	Mean	±SD	n	Mean	±SD	n
Neutrophils (%)	Lesioned	23.9	17.3	15	25.0	18.0	4							6.6	2.6	5
	Non-lesioned				7.2	9.1	5	5.7	3.1	3	2.0	1.4	4	3.2	2.3	5
Lymphocytes (%)	Lesioned	20.5	9.5	15	40.2	21.9	5							42.2	10.4	5
	Non-lesioned				69.0	5.0	5	42.2	12.9	5	49.4	12.4	10	43.6	19.3	5
Monocytes (%)	Lesioned	14.8	10.0	14	20.3	7.6	4							3.8	3.6	5
	Non-lesioned				1.5	0.6	4	3.3	2.9	4	3.3	2.1	8	1.0	0.0	2
Thrombocytes (%)	Lesioned	35.2	20.9	15	12.6	8.1	5							41.8	16.9	5
	Non-lesioned				15.2	5.2	5	48.2	11.8	5	44.3	12.9	10	51.0	15.7	5

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

		2002 Creeks								
		Broad Creek			Durham Creek			Upper Goose Creek		
		Mean	±SD	n	Mean	±SD	n	Mean	±SD	n
PCV (%)	Lesioned	32.7	10.4	6	19.0	8.2	3	36.7	14.4	3
	Non-lesioned	47.8	5.2	6	44.8	7.6	9	45.0	3.6	3
WBC/Thrombocytes (x10^3/μl)	Lesioned	60.0	25.3	6	34.7	8.1	3	48.0	12.5	3
	Non-lesioned	76.0	19.1	6	87.1	25.8	9	68.7	11.4	3
Neutrophils (x10^3/μl)	Lesioned	4.7	2.3	6	3.6	2.4	3	4.0	0.6	3
	Non-lesioned	3.9	1.1	6	4.0	5.7	8	2.7	1.4	3
Neutrophils (%)	Lesioned	8.5	5.4	6	9.7	4.5	3	8.7	2.1	3
	Non-lesioned	5.3	1.6	6	4.8	6.2	8	4.0	2.0	3
Lymphocytes (x10^3/μl)	Lesioned	19.0	6.4	6	10.3	1.1	3	12.6	4.7	3
	Non-lesioned	29.1	16.9	6	25.9	10.1	9	21.5	6.7	3
Lymphocytes (%)	Lesioned	34.8	15.9	6	31.0	8.9	3	25.7	4.5	3
	Non-lesioned	36.2	18.4	6	30.3	8.9	9	32.0	10.6	3
Monocytes (x10^3/μl)	Lesioned	4.6	5.0	5	8.0	4.2	3	4.7	5.9	2
	Non-lesioned	1.6	1.1	4	1.8	1.2	7	3.4	2.5	3
Monocytes (%)	Lesioned	7.8	8.3	5	22.0	6.2	3	13.5	17.7	2
	Non-lesioned	2.3	1.5	4	2.3	1.3	7	4.7	2.9	3
Thrombocytes (x10^3/μl)	Lesioned	30.5	21.3	6	8.6	1.8	3	26.7	13.5	3
	Non-lesioned	40.9	11.5	6	54.7	19.7	9	39.1	11.8	3
Thrombocytes (%)	Lesioned	47.2	14.7	6	25.0	2.6	3	52.7	16.4	3
	Non-lesioned	55.5	14.9	6	62.0	8.9	9	56.3	9.5	3
Total Plasma Protein (g dl^{-1})	Lesioned	2.5	0.1	6	2.4	0.0	3	2.4	0.1	3
	Non-lesioned	2.9	0.3	6	2.9	0.4	9	2.7	0.2	3

Table 4. Hematology values for lesioned and non-lesioned Atlantic menhaden sampled in the fall of 2001 and 2002.

| | | 2001 | | | | | | 2002 | | | | | |
| | | Lesioned | | | Non-lesioned | | | Lesioned | | | Non-lesioned | | |
Variable	Unit	Mean	±SD	n	Mean	±SD	n	Mean	±SD	n	Mean	±SD	n
Neutrophils	x 10^3/μl	5.75	3.4	8	1.48	0.54	6	4.23	1.94	12	3.74	3.88	17
Neutrophils	%	20.46	16.7	24	4.53	5.3	17	8.83	4.26	12	4.82	4.3	17
Lymphocytes	x 10^3/μl	8.89	6.56	8	34.4	15.9	10	15.2	6.23	12	26.25	12.01	18
Lymphocytes	%	28.76	16.08	25	50.72	15.73	25	31.58	12.15	12	32.56	12.56	18
Monocytes	x 10^3/μl	3.07	2.23	8	1.28	0.87	7	5.63	4.62	10	2.07	1.54	14
Monocytes	%	13.35	10	23	2.61	2.06	18	13.2	10.79	10	2.79	1.88	14
Eosinophils	x 10^3/μl	0.42	0.05	3				3.49	1.98	3	1.16	0.87	5
Eosinophils	%	2.2	1.32	10	1	0	4	7.33	4.16	3	1.4	0.89	5
Thrombocytes	x 10^3/μl	30.97	9.39	8	21.2	11.4	10	24.08	18.15	12	47.5	17.1	18
Thrombocytes	%	32	20.43	25	40.6	17.49	25	43	16.49	12	58.89	11.1	18
PCV	%	24.75	5.6	8	42.4	3.75	10	30.25	12.15	12	45.83	6.2	18
WBC/Thrombocytes	x10^3 μl^{-1}	30.97	9.39	8	60.0	22.3	10	50.67	21.2	12	80.3	22.1	18

Table 5. Plasma chemistry values for Atlantic menhaden caught in the fall of 2002. *n* = number of pooled samples.

| | | Lesioned (n =12) | | Non-lesioned (n = 18) | | |
Variable	Unit	Mean	±SD	Mean	±SD	P
Glucose	mg dl^{-1}	27.4	13.5	33.3	6.5	ns
Phosphorus	mg dl^{-1}	11.5	2.0	12.4	2.0	ns
Calcium	mg dl^{-1}	9.4	2.2	12.0	1.4	0.0002
TP	g dl^{-1}	1.2	0.7	2.1	0.6	0.0004
Albumin	g dl^{-1}	0.5	0.3	0.8	0.2	0.0010
Globulin	g dl^{-1}	0.8	0.5	1.4	0.4	<0.0001
Albumin/Globulin		0.6	0.2	0.6	0.3	ns
AST	U L^{-1}	346.7	179.3	267.6	67.6	ns
CK	U L^{-1}	5591.7	1449.5	5977.2	2295.3	ns
LD	U L^{-1}	2931.8	1281.3	2433.6	750.3	ns
Sodium	mmol^{-1}	157.6	5.8	160.3	6.4	ns
Potassium	mmol^{-1}	5.2	2.3	6.2	2.2	ns
Chloride	mmol^{-1}	133.6	4.8	137.4	4.8	ns
Bicarbonate	mmol^{-1}	7.3	1.4	6.0	1.8	ns
Anion Gap		21.9	6.5	23.4	4.0	ns
Sodium/Potassium		36.7	19.6	29.2	10.7	ns
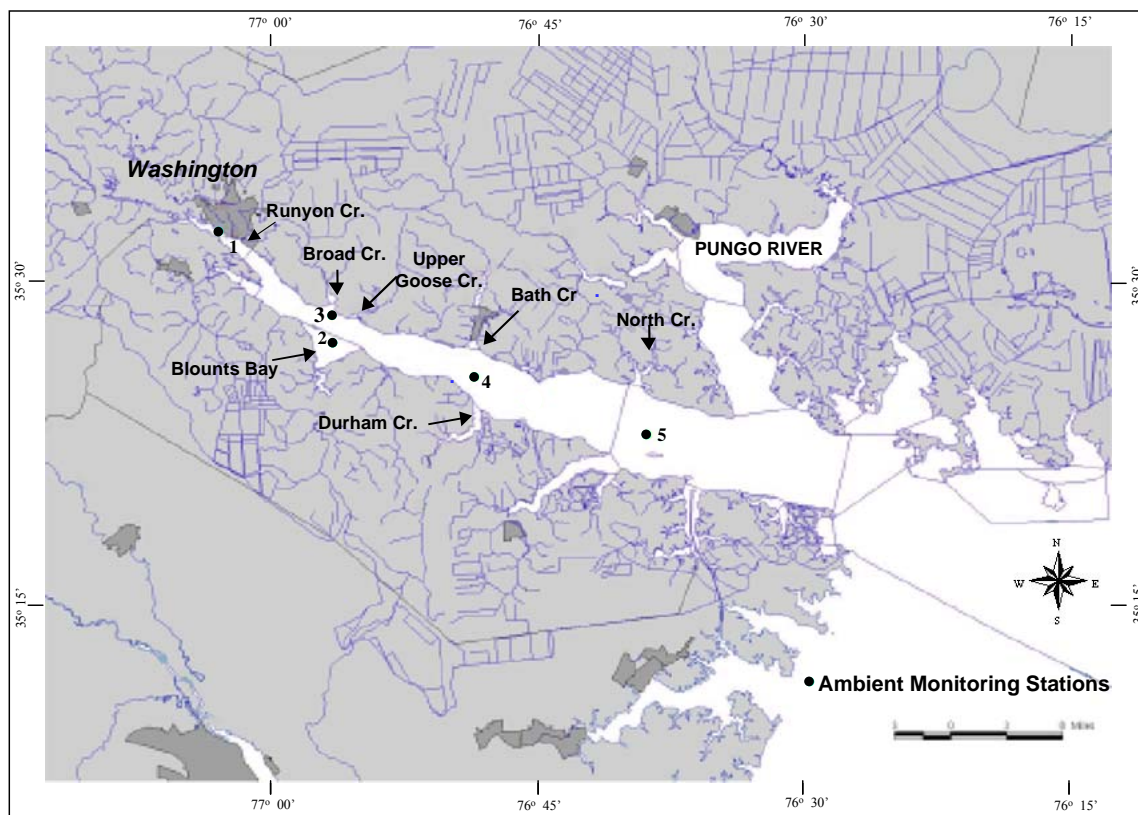
Fig. 1. Map of the Pamlico River estuary creeks and ambient monitoring stations (•) sampled in the fall of 2001 and 2002.
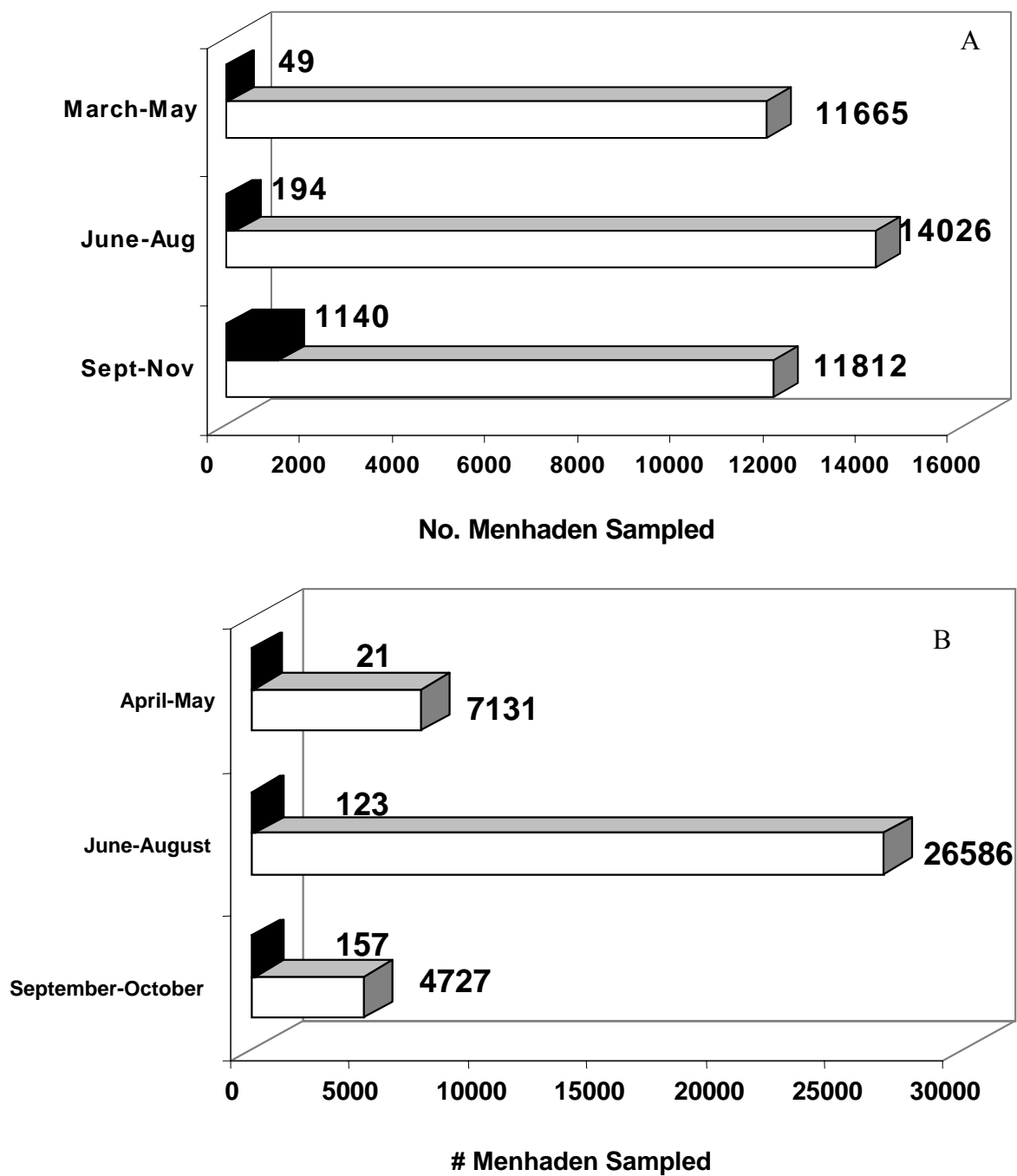
Fig. 2. Temporal variability of juvenile Atlantic menhaden with ulcerative skin lesions in the Pamlico River estuary in (A) 2001 and (B) 2002. □ non-lesioned; ■, lesioned. Data courtesy of the Pamlico Rapid Response Team.
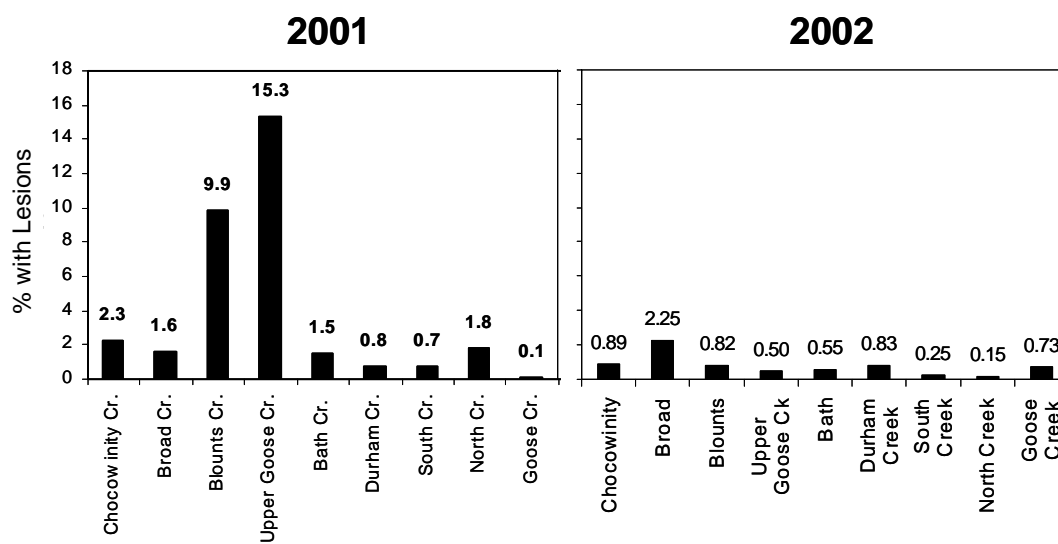
Fig. 3. Spatial distribution of Atlantic menhaden with ulcerative skin lesions in the Pamlico River estuary in 2001 and 2002. Data courtesy of the Pamlico Rapid Response Team.
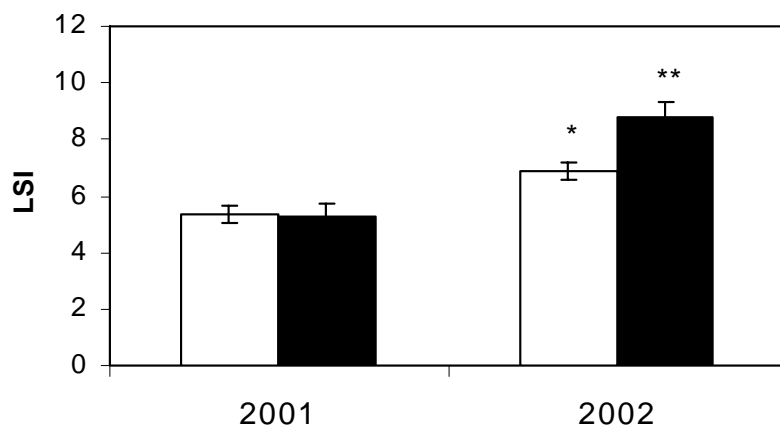
Fig. 4. Liver-somatic indices (mean ± SE) for lesioned (■) and non-lesioned (□) Atlantic menhaden caught in the fall of 2001 and 2002 in the Pamlico River estuary. LSI were significantly higher for lesioned fish caught in 2002 than non-lesioned ($P = 0.0005$). Significant differences are indicated by an asterisk(*). n =30; non-lesioned 2001; $n = 20$, lesioned 2001; $n = 31$, non-lesioned 2002; $n = 18$, lesioned 2002.
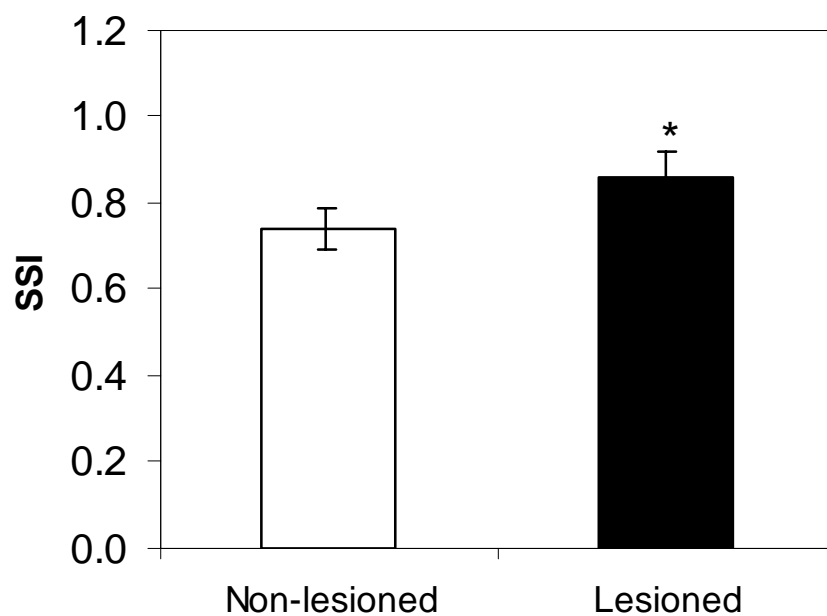
Fig. 5. Spleno-somatic indices (means ± SE) for lesioned (■) and non-lesioned (□) menhaden caught in the Pamlico River estuary during the fall of 2002. Spleno-somatic indices were significantly higher for lesioned fish ($n = 41$) than non-lesioned ($n = 34$; $P = 0.0028$).
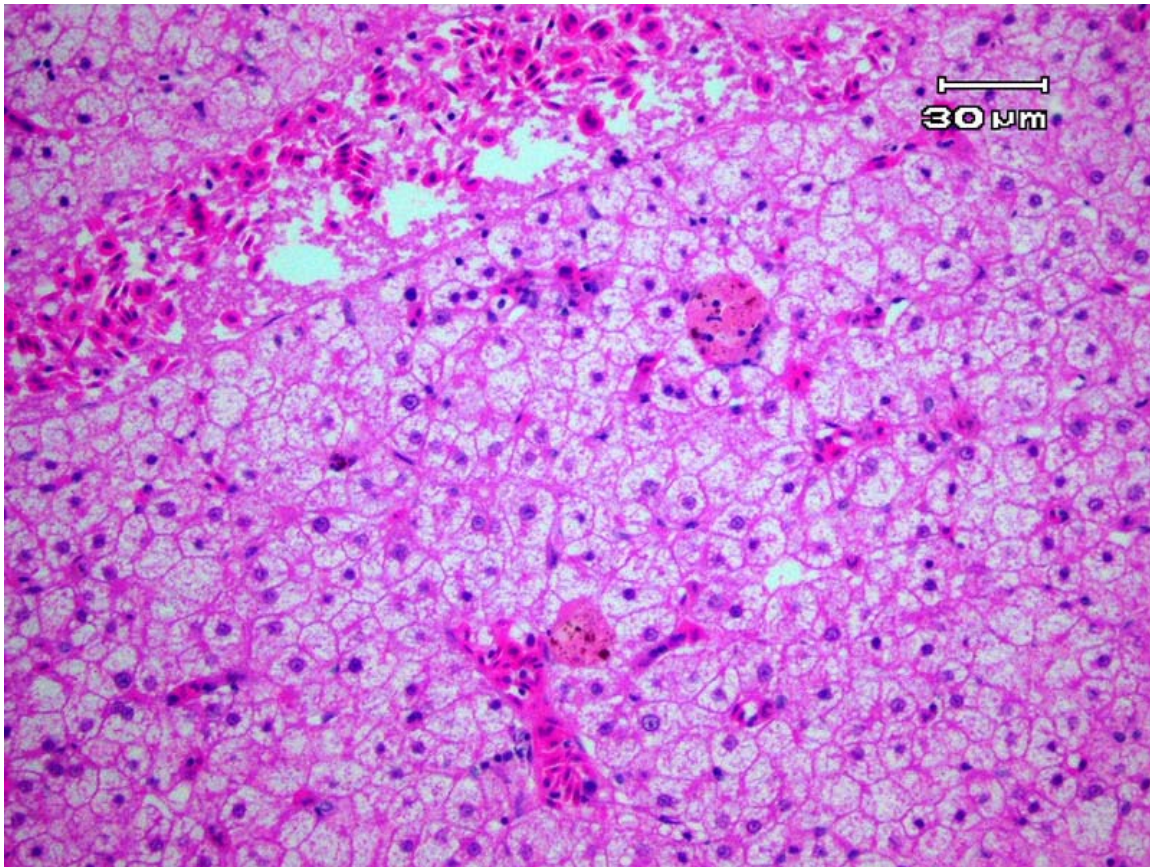
Fig. 6. Atlantic menhaden with moderate to marked, diffuse, clear vacuolation of hepatocytes. This likely reflects nutritional stress as is seen in many wild caught fish specimens, leading to fatty change in the liver. Increased storage products, such as glycogen or vitellogenin, may also affect the morphological appearance of hepatocytes. H &E.
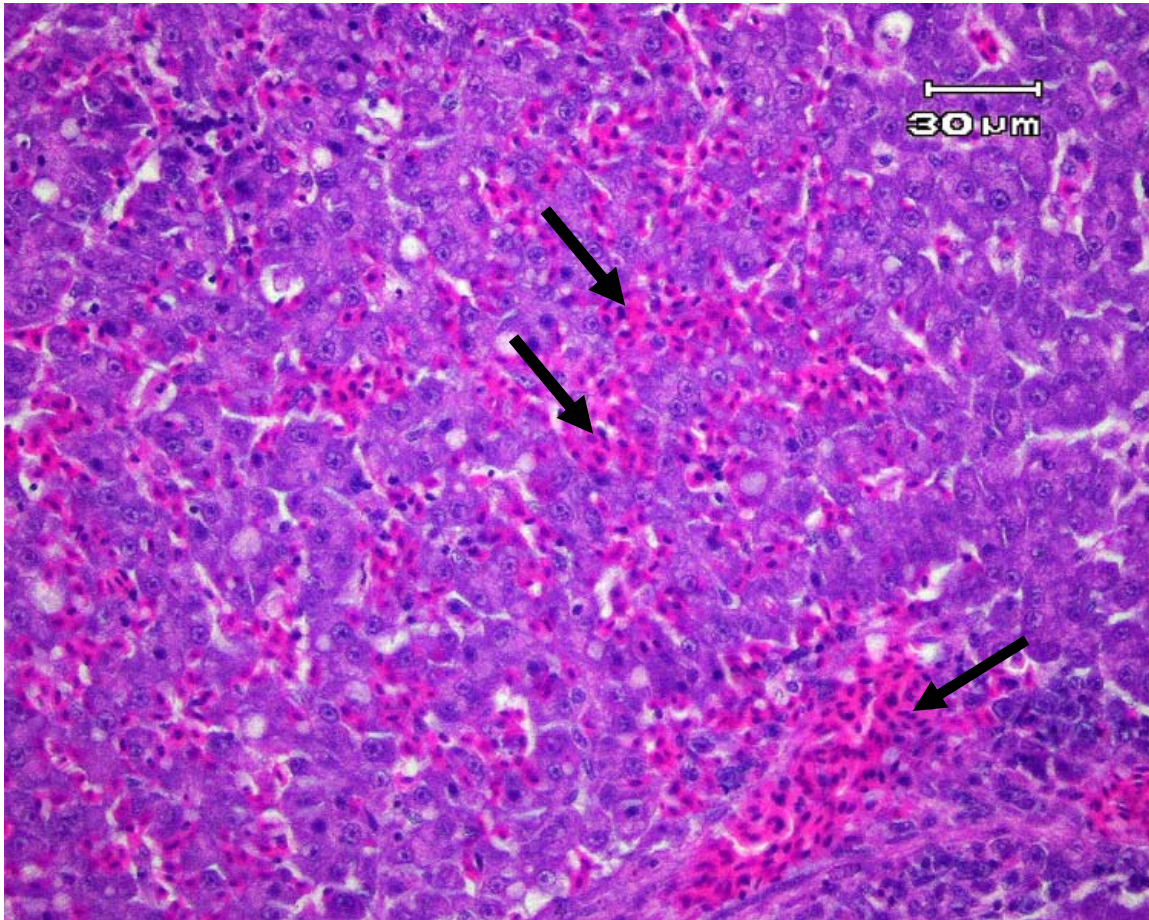
Fig. 7. Liver section of Atlantic menhaden with ulcerative skin lesions caught in the fall of 2001. Focal to multifocal areas of hepatic congestion (arrows) characterized by dilated sinusoids filled with erythrocytes and focal atrophy of hepatic cords were found in some lesioned fish. Surrounding hepatocytes are swollen or vacuolated. H &E.
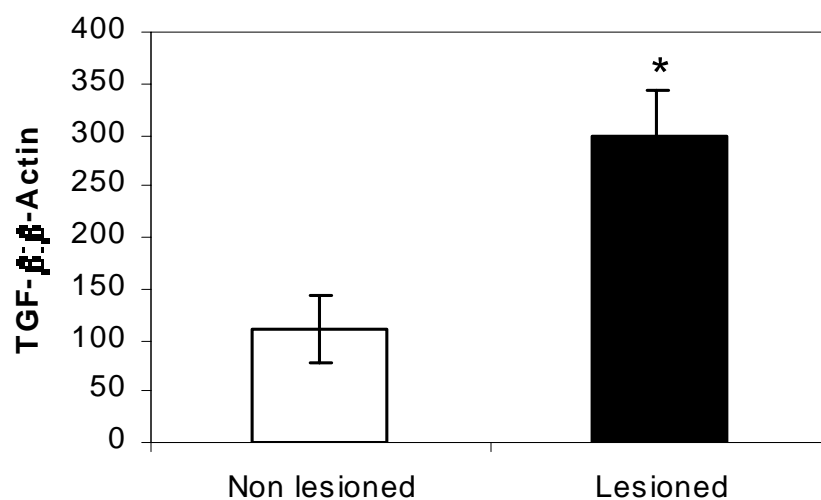
Fig. 8. TGF-β: β-actin ratios for lesioned (■) and non-lesioned (□) Atlantic menhaden caught in the Pamlico River estuary during the fall of 2001. Values (means ± SE) were significantly higher for lesioned fish than non-lesioned ($P = 0.0113$). $n = 11$ for both groups.
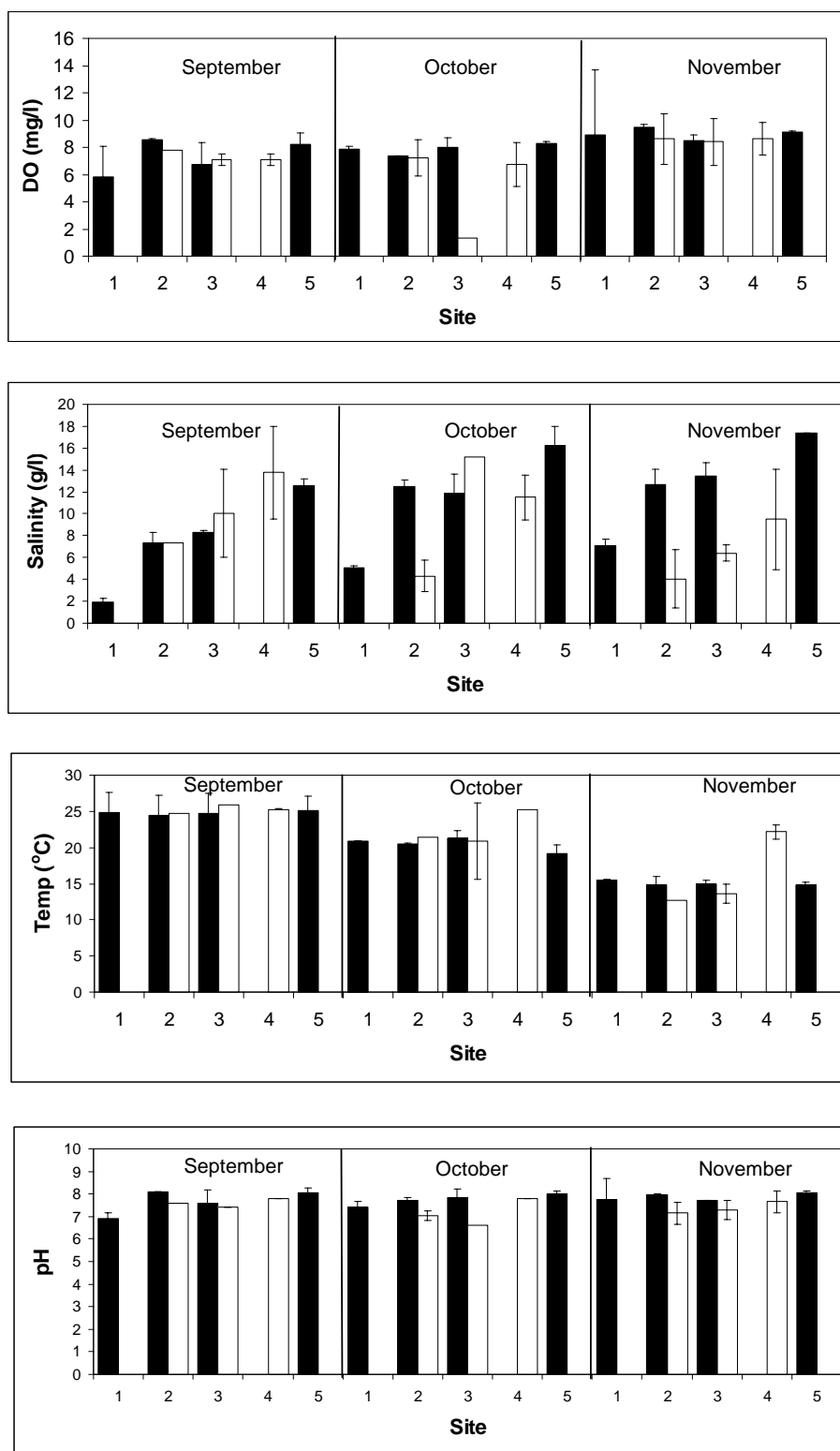
Fig 9. Mean surface (1m depth) water quality measurements (± SD) collected from the
ambient monitoring stations in Fig. 1.

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.

INTRODUCTION

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 TH$_1$ 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 *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 *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: $[(Na^+ + K^+)-(Cl^- + HCO_3^-)]$.

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}

(5.0×10^4 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 (1.0×10^6 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 = 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

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

10^6 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 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.

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 $MgCl_2$ 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 10^8-10^1

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 $MgCl_2$, 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

multipled by the significance level (*P* value) to give a more conservative alpha (α). 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 (96

hr; $P = 0.0244$; 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.

ACKNOWLEDGMENTS

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

	Oligonucleotide	Sequence	Size (bp)	Concentration (nM)
2A	Forward	5' GGCTACTTTGCCAACTACTGC 3'	21	300
2B	Reverse	5' CTGCTCCACCTTGTGTTGC 3'	19	300
3A	Forward	5'GTTGCCATCCAGGCTGTGC 3'	18	400
3B	Reverse	5' TGAGGTAGTCTGTCAGGTCACG 3'	22	500

Table 2. Morphometric and lymphocyte mitogenesis values for triamcinolone-treated and control Atlantic menhaden (F= females; M = males).

	48 Hour						96 Hour					
	Control (n=10)			*Treated (n = 10)*			*Control (n = 10)*			*Treated (n = 10)*		
	(F = 8; M = 2)			(F = 4; M = 6)			(F = 6; M = 4)			(F = 3; M =6)		
Variable	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE
Length (mm)	160.50	7.25	2.29	163.89	4.17	1.32	160.50	7.25	2.29	160.00	3.33	1.05
Body weight (g)	55.27	7.33	2.32	57.14	7.19	2.27	55.39	4.23	1.34	51.86	2.28	0.72
Liver weight (g)	0.36	0.11	0.03	0.35	0.08	0.02	0.28	0.03	0.01	0.28	0.04	0.01
Spleen weight (g)	0.08	0.04	0.01	0.06	0.02	0.01	0.06	0.02	0.00	0.06	0.02	0.01
Liver-somatic index	6.48	1.75	0.55	6.18	0.88	0.28	5.03	0.71	0.23	5.38	0.81	0.26
Spleno-somatic index	1.43	0.54	0.17	1.05	0.34	0.11	1.17	0.26	0.08	1.14	0.48	0.15
ConA (SI)	0.99	0.04	0.01	0.98	0.03	0.01	1.02	0.05	0.02	0.94	0.09	0.03
LPS (SI)	0.97	0.06	0.02	0.95	0.05	0.02	1.04	0.07	0.02	1.00	0.08	0.03

Table 3. Hematology and plasma chemistry values for control and triamcinolone-treated Atlantic menhaden.

		48 Hour				96 Hour			
		Control (n = 6)		Treated (n = 6)		Control (n = 6)		Treated (n = 6)	
Variable	Unit	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Neutrophils	x10^3 µl^{-1} (%)	1.87 (7.3)	1.03 (6.7)	6.56 (17.0)	6.14 (7.6)	3.56 (8.5)	2.78 (6.3)	9.32 (22.8)	11.92 (17.3)
Monocytes	x10^3 µl^{-1} (%)	0.50 (1.3)	0.47 (1.0)	2.96 (7.5)	1.66 (5.1)	0.78 (2.0)	0.76 (2.2)	1.67 (4.2)	0.1 (1.3)
Eosinophils	µl^{-1} (%)	95.0 (0.2)	190.0 (0.4)	135.0 (0.2)	270 (0.4)	135.0 (0.3)	270 (0.8)	193.3 (0.2)	334.9 (0.4)
Lymphocytes	x10^3 µl^{-1} (%)	14.89 (48.5)	6.01 (8.9)	10.91 (31.3)	9.96 (9.8)	23.15 (54.0)	10.88 (19.6)	4.87 (16.2)	2.501 (12.5)
Thrombocytes	x10^3 µl^{-1} (%)	13.53 (42.3)	7.29 (7.9)	13.43 (40.5)	7.98 (7.4)	16.77 (34.3)	12.86 (19.9)	22.26 (55.2)	14.92 (25.4)
WBC/Thrombo	x10^3 µl^{-1}	31.00	12.90	34.33	23.17	44.67	13.97	38.50	187.91
PCV	%	26.00	7.60	28.50	6.86	25.50	5.75	25.00	5.73
Glucose	mg dL^{-1}	45.17	9.00	67.17	8.42	52.83	6.74	87.67	9.89
Phosphorus	mg dL^{-1}	6.47	0.9	6.4	1.24	6.43	0.67	6.33	1.39
Calcium	mg dL^{-1}	8.90	1.22	9.88	1.49	10.75	1.29	10.30	1.49
Total protein	g dL^{-1}	1.78	0.62	2.48	0.57	2.52	0.57	2.42	0.69
Albumin	g dL^{-1}	0.63	0.25	0.767	0.38	0.85	0.21	0.833	0.29
Globulin	g dL^{-1}	1.15	0.38	1.72	0.53	1.67	0.37	1.58	0.41
Albumin/Globulin		0.54	0.12	0.49	0.22	0.51	0.04	0.52	0.08
AST	U L^{-1}	112.17	18.19	168.5	95.39	91.17	56.78	135.5	122.11
CK	U L^{-1}	7817.20	1022.92	2792.80	2423.08	2834.00	743.21	2622.83	2527.20
LD	U L^{-1}	1077.17	208.06	1396.5	8376.98	794.17	441.15	923.5	754.21
Sodium	mmol L^{-1}	159.17	4.96	164.83	4.36	162.67	1.86	164.83	3.06
Potassium	mmol L^{-1}	5.70	2.03	4.07	1.42	2.75	1.26	2.67	1.47
Chloride	mmol L^{-1}	148.67	3.78	150.5	5.99	146.67	2.94	148.33	2.8
Bicarbonate	mmol L^{-1}	5.50	0.58	5.75	0.96	6.00	0.71	5.80	0.84
Anion Gap		11.2	1.74	13.4	1.54	13.25	1.75	13.67	4.56
Sodium/Potassium		31.42	12.81	47.48	25.15	66.7	20.52	78.77	41.82

A. TGF-β

GTCCCAAG==GGCTACTTTGCCAACTACTGC==GTGGGCTCCTGCACCTACATC 50
         >>>>>>>>>>>>>>>>>>>>>>>>>>
TGGAGCACCGAGGACAAACACTCTCAGATTCTAGCTCTGTATAAGCATCA 100

CAATCCCGGAGCATCGGCCCAGCCCTGTTGCGTCCCTCACGTCTTGGAAC 150

CGCTGCCCATCATCTACTACGTTGGCCG==GCAACACAAGGTGGAGCAG==CTGA 201
                            <<<<<<<<<<<<<<<<<<<<<<<<<

B. β-actin

CCAGATCATGTTTGAGACCTTCAACACCCCTGCCATGTAC==GTTGCCATCC== 50
                                        >>>>>>>>>>>>>
==AGGCTGTGC==TGTCCCTGTATGCCTCTGGTCGTACCACTGGTATCGTGATG 100
>>>>>>>>>>>
GACTCCGGTGATGGTGTCACCCACACTGTGCCCATCTACGAGGGTTACGC 150

CCTGCCCCATGCCATCCTGCGTCTGGACTTGGCTGGC==CGTGACCTGACAG== 200
                                     <<<<<<<<<<<<<<<<<
==ACTACCTCA==TGAAGATCCTCACCGAGCGAG 230
<<<<<<<<<<<

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).
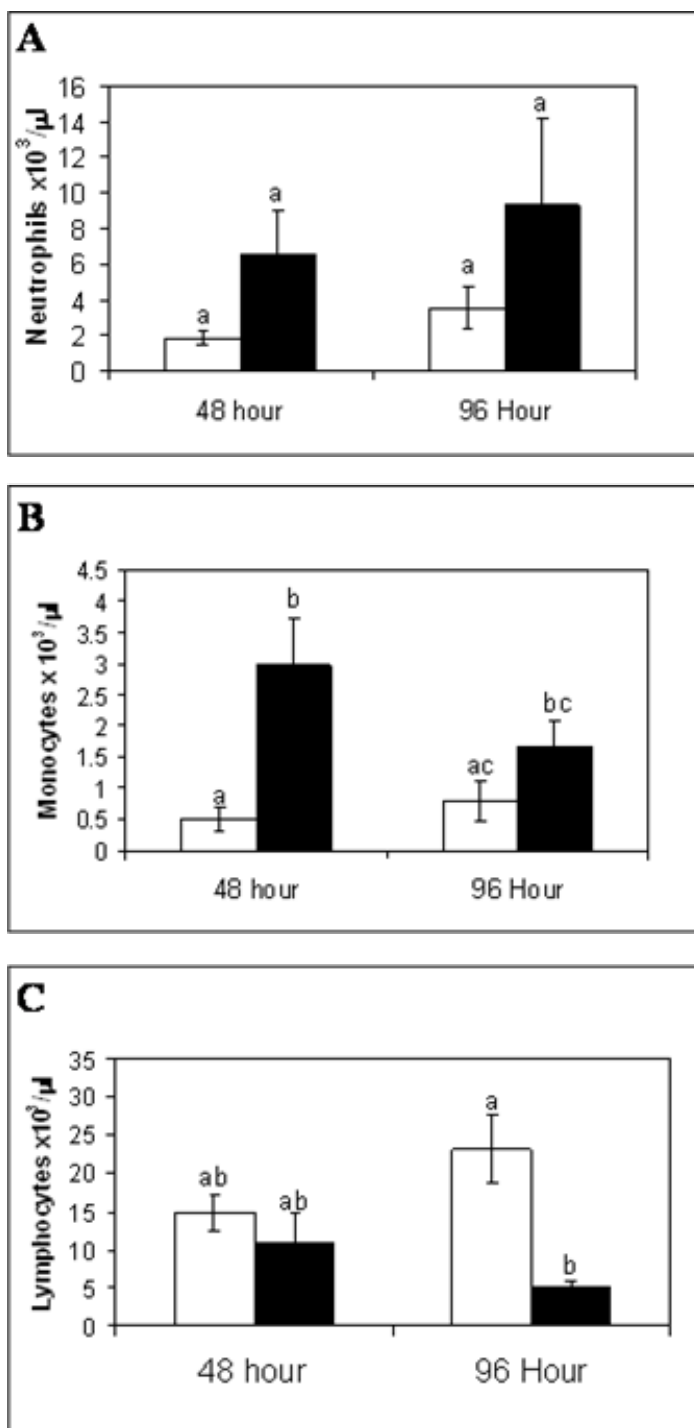
Fig. 2. Effect of triamcinolone treatment on blood leukocytes of control (□) and treated (■) Atlantic menhaden; (A) neutrophil; (B) monocyte; (C) lymphocyte counts. Values are means ± SE ($n = 6$). Means that are significantly different ($P < 0.05$), have different letters.
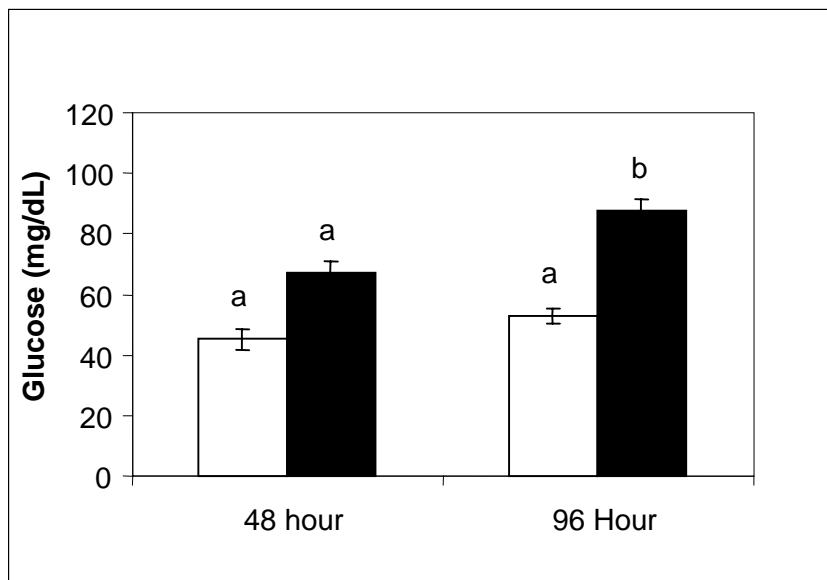
Fig. 3. Effect of triamcinolone on plasma glucose levels (means ± SE) of Atlantic menhaden. Statistically significant differences (*P* < 0.05) between control and treated groups are denoted by different letters. Sample sizes are the same for both control (□) and treated (■) groups (*n* = 6).
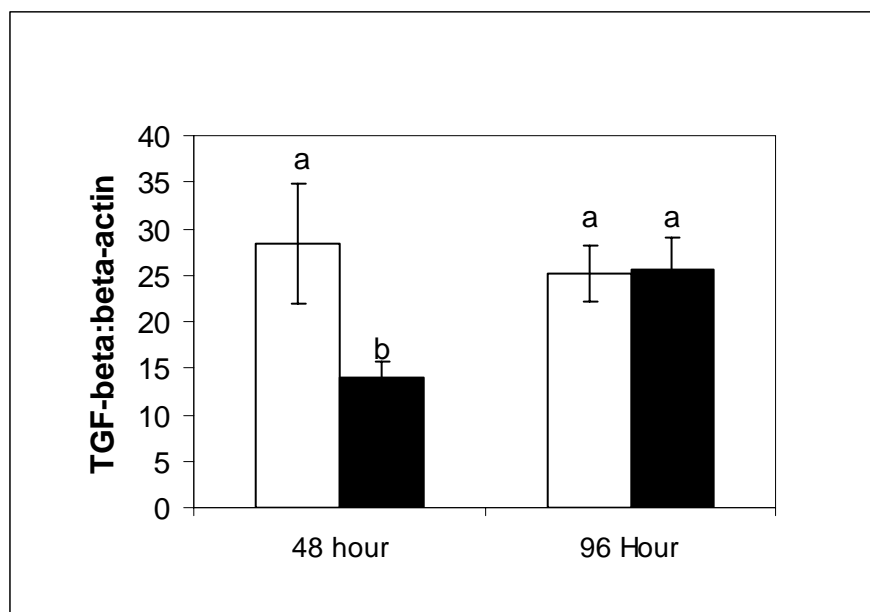
Fig. 4. Effect of triamcinolone on TGF-β mRNA production by Atlantic menhaden splenic mononuclear cells. Absolute values (TGF-β: β-actin; means ± SE) were significantly lower for treated (■) fish at 48 hr than 96hr and controls (□) at 48 and 96 hr ($P < 0.05$). Statistically significant differences ($P < 0.05$) 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 ($\pm$ 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 $^{EG}7^+$ 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 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 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 OD_{570} with reference wavelength of OD_{630} using a microplate reader. Stimulation indices were calculated using the formula:

SI = mean OD of stimulated culture/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 10^6 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 10^4 cell equivalents/μl.

Messenger RNA was reverse transcribed using Superscript II RT (Gibco BRL, Gaithersburg, Maryland) to cDNA with oligo dT_{15} priming of 3×10^6 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 (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 PCO_2 were log transfomed 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 25ºC. 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). A

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 (Nikinmaa *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.

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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.

		Oxygen Saturation									
		5% (M=4; F=1)		10% (M=4; F=1)		15% (M=3; F=2)		20% (M=2; F=3)		84% (control) (M=1; F=2)	
Variables	Unit	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Body Weight	g	71.0	13.9	82.7	17.8	65.4	15.3	73.0	18.1	67.8	15.2
Fork Length	mm	173.0	11.0	184.0	10.8	170.0	10.6	175.0	14.6	171.7	16.1
SSI		1.4	0.1	1.2	0.4	1.3	0.3	1.6	0.2	1.6	0.6
PCV	%	37.8	11.5	27.0	8.7	33.3	10.6	37.3	5.9	27.0	5.6
WBC/Thrombo	$\times 10^3\ \mu l^{-1}$	54.8	34.5	58.8	23.9	59.2	14.2	37.2	12.4	40.0	12.1
Neutrophils	$\times 10^3\ \mu l^{-1}$	2.5	1.7	7.5	4.2	8.6	8.0	2.4	1.4	0.7	0.9
Eosinophils	$\times 10^3\ \mu l^{-1}$	1.0	0.0	1.0		1.0	0.0				
Lymphocytes	$\times 10^3\ \mu l^{-1}$	17.4	11.5	15.9	12.0	20.7	4.3	16.7	8.0	14.1	8.8
Monocytes	$\times 10^3\ \mu l^{-1}$	3.9	2.7	2.0	2.8	2.6	2.2	1.4	0.4	0.5	0.1
Thrombocytes	$\times 10^3\ \mu l^{-1}$	29.3	18.0	33.0	17.0	24.2	11.5	16.9	6.6	24.8	2.7
Glucose	mg dL^{-1}	92.8	31.7	29.4	13.7	50.2	19.7	55.5	13.4	48.3	6.0
Phosphorus	mg dL^{-1}	9.2	1.9	15.9	3.3	11.0	1.9	8.7	2.1	6.7	0.8
iCalcium	nmol/L	1.7	0.1	1.5	0.0	1.6	0.0	1.5	0.0	1.6	0.0
Total Protein	g dL^{-1}	1.6	0.4	1.3	0.6	1.7	0.5	1.5	0.5	1.5	0.2
Albumin	g dL^{-1}	0.5	0.2	0.6	0.2	0.5	0.3	0.5	0.1	0.5	0.4
Globulin	g dL^{-1}	1.0	0.2	0.7	0.5	1.2	0.3	1.0	0.4	1.0	0.2
Albumin/Globulin		0.5	0.1	1.1	0.9	0.4	0.0	0.5	0.0	0.6	0.5
AST	U L^{-1}	28.0	12.7	124.4	51.9	193.6	80.7	73.0	28.3	101.0	72.0
CK	U L^{-1}	1102.4	467.0	4289.0	1976.4	9631.4	4945.9	4866.0	1238.9	4199.0	2468.9
LD	U L^{-1}	269.4	66.5	1130.4	509.9	1689.8	515.8	1036.0	415.8	1009.3	934.2
Sodium	nmol L^{-1}	165.3	2.6	163.0	3.6	158.5	1.3	157.0	2.7	158.3	2.3
Potassium	nmol L^{-1}	6.9	1.0	6.7	1.2	4.9	0.6	4.4	0.1	4.4	0.2
Sodium/Potassium		24.3	3.0	24.9	4.9	32.5	3.8	36.0	0.7	35.8	2.1
Chloride	mmol L^{-1}	149.2	2.6	151.6	4.5	143.2	3.1	146.0	0.0	150.0	4.6
Bicarbonate	mmol L^{-1}	2.3	1.0	3.3	0.6	3.0	0.0	3.3	0.6	3.7	0.6
pH		6.7	0.1	6.9	0.1	6.8	0.1	6.9	0.1	6.8	0.0
PCO2	mm Hg	18.7	4.2	17.6	3.8	18.5	4.5	20.5	5.8	23.3	0.7
TCO2	mM	2.8	1.0	4.0	0.0	3.5	0.6	4.3	0.6	4.0	0.0

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

		48 Hour				72 Hour				84 Hour				156 Hour			
		Control (M=4; F=6)		Treated (M=5; F=5)		Control (M=3; F=7)		Treated (M=4; F=6)		Control (M=7; F=2)		Treated (M=3; F=7)		Control (M=6; F=4)		Treated (M=4; F=6)	
Variables	Unit	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Fork Length	mm	164.5	8.0	164.0	8.4	165.0	5.8	169.5	6.9	164.5	5.5	165.5	7.3	161.0	5.7	163.5	7.1
Body Weight	g	54.9	6.4	57.1	9.4	60.6	7.1	62.8	7.8	56.3	6.8	57.0	8.1	54.6	4.8	53.6	5.7
ConA	SI	1.0	0.1	1.1	0.1	1.1	0.1	1.0	0.0	1.0	0.1	1.0	0.1	1.0	0.0	1.0	0.1
LPS	SI	1.0	0.1	1.1	0.1	1.0	0.1	1.0	0.1	1.0	0.1	1.0	0.1	1.0	0.1	1.0	0.0
TGF-β		12.6	6.1	16.8	14.6	11.9	6.1	8.8	4.5	15.4	7.8	18.8	19.0	9.9	6.8	12.0	8.7

B

		48 Hour				72 Hour				84 Hour				156 Hour			
		Control (M=3; F=3)		Treated (M=3; F=3)		Control (M=2; F=4)		Treated (M=1; F=5)		Control (M=4; F=1)		Treated (M=1; F=5)		Control (M=3; F=3)		Treated (M=2; F=4)	
Variables	Unit	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Neutrophils	$x10^3\ \mu l^{-1}$	6.37	9.25	4.76	5.55	1.73	2.08	5.56	4.54	2.17	1.92	7.78	7.41	6.64	3.09	3.87	4.59
Monocytes	$x10^3\ \mu l^{-1}$	0.70	0.27	4.03	5.45	2.80	3.16	1.16	0.84	2.39	1.60	1.43	0.95	5.00	2.84	1.48	0.87
Eosinophils	$x10^3\ \mu l^{-1}$	1.00	0.99	1.01	0.30	0.49	0.57	0.30	0.43	0.46	0.43	0.22	0.25	0.86	0.71	0.17	0.30
Lymphocytes	$x10^3\ \mu l^{-1}$	20.32	3.40	20.14	1.23	42.35	16.50	25.26	8.88	29.41	14.13	24.18	13.76	28.72	9.65	26.05	17.61
Thrombocytes	$x10^3\ \mu l^{-1}$	12.16	4.40	19.36	16.10	28.16	12.20	27.82	15.11	18.76	9.64	20.89	13.17	12.89	8.41	9.29	2.83
WBC/Thrombocyte	$x10^3\ \mu l^{-1}$	40.33	14.00	49.20	27.73	76.00	29.90	60.00	24.98	53.67	18.70	55.17	23.20	55.00	16.20	41.00	19.50
PCV	%	28.33	3.56	37.50	8.69	33.50	3.94	33.00	4.98	26.83	7.57	30.50	8.50	35.67	4.27	29.33	4.37
Glucose	mg dL^{-1}	40.67	7.87	39.00	19.33	33.00	13.55	52.30	8.94	42.83	7.55	48.80	10.78	40.50	7.48	41.30	3.14
Phosphorus	mg dL^{-1}	7.60	1.14	9.70	3.28	7.37	2.17	6.47	1.22	8.17	0.92	6.18	0.54	8.07	0.94	7.35	0.67
iCalcium	mg dl^{-1}	1.62	0.05	1.97	1.25	1.55	0.03	1.47	0.07	1.62	0.04	1.57	0.05	1.65	0.04	1.59	0.04
Total Protein	g dL^{-1}	1.77	0.53	1.98	0.48	1.53	0.64	1.82	0.55	2.12	0.50	1.88	0.60	2.02	0.35	1.67	0.63
Albumin	g dL^{-1}	0.70	0.18	0.77	0.10	0.68	0.16	0.68	0.25	0.78	0.17	0.82	0.29	0.72	0.12	1.00	0.81
Globulin	g dL^{-1}	1.22	0.47	1.22	0.43	0.85	0.60	1.14	0.46	1.33	0.45	1.07	0.60	1.30	0.25	1.08	0.34
Albumin/Globulin		0.75	0.37	0.68	0.18	0.96	0.95	0.73	0.60	0.65	0.29	0.85	0.52	0.56	0.07	0.79	0.45
AST	U L^{-1}	107.0	82.7	166.5	151.1	77.2	93.5	76.0	59.1	119.3	114.5	111.8	41.9	250.0	135.9	193.8	118.6
CK	U L^{-1}	1684.3	1500.0	1699.0	1612.6	2554.8	2448.5	1735.8	1571.9	2398.2	1736.3	4336.3	3084.8	4771.7	990.7	5318.0	2182.3
LD	U L^{-1}	992.7	730.2	1468.8	1407.5	868.0	701.6	688.7	416.0	916.8	1149.2	818.7	409.1	2131.5	1022.6	1633.7	847.0
Sodium	nmol L^{-1}	155.30	1.51	154.17	2.32	155.00	2.10	154.00	4.52	158.17	2.79	157.67	2.50	158.00	0.89	157.50	0.55
Potassium	nmol L^{-1}	4.78	0.64	4.27	0.38	4.85	0.69	4.25	0.38	4.80	0.67	4.32	0.97	4.77	1.14	4.33	0.23
Sodium/Potassium		32.96	4.42	36.38	3.38	32.47	4.32	36.45	3.04	33.44	2.24	38.05	8.23	32.95	7.53	36.44	2.10
Chloride	mmol L^{-1}	149.17	4.54	147.67	7.06	147.70	3.08	146.33	2.50	148.17	4.02	150.00	4.69	150.80	2.99	149.67	1.97
Bicarbonate	mmol L^{-1}	5.00	0.89	8.17	1.33	5.67	1.37	7.17	0.98	4.50	0.55	5.00	1.10	4.33	0.82	4.00	1.55
pH		6.82	0.07	7.00	0.07	6.86	0.06	6.99	0.18	6.87	0.05	6.95	0.08	6.83	0.10	6.95	0.07
PCO$_2$	mm Hg	29.50	4.97	33.58	9.07	31.30	3.99	32.12	11.48	24.58	2.62	21.50	3.98	25.83	1.79	19.07	9.69
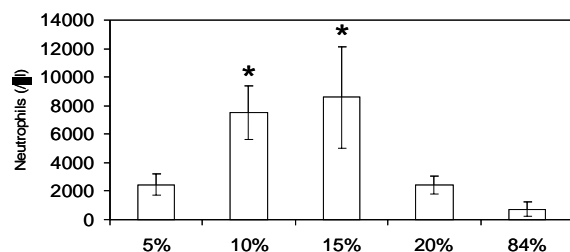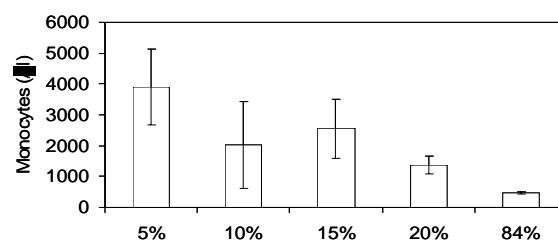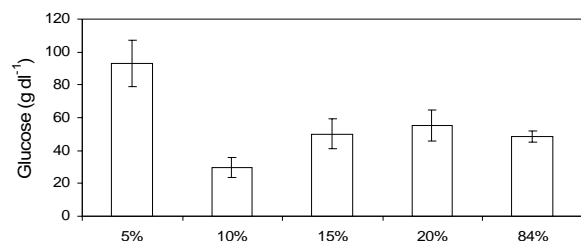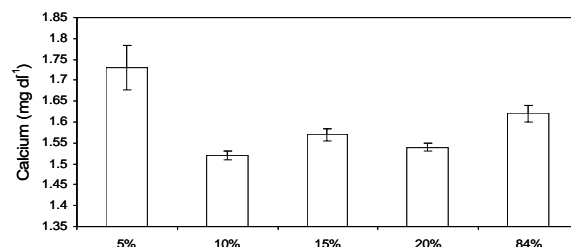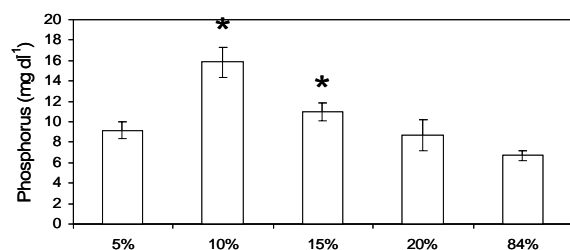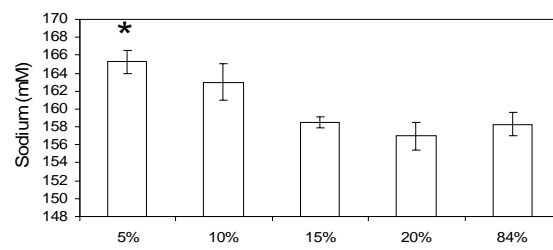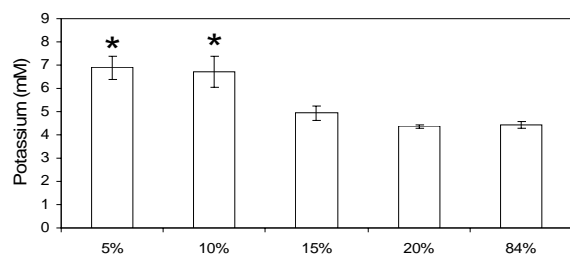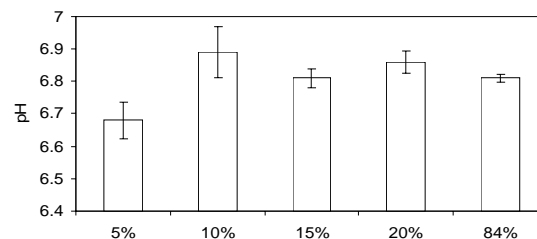
Fig. 1. Comparison of blood parameters measured in Atlantic menhaden during acute hypoxia. Oxygen saturations of 5%, 10%, 15%, and 20% were compared to 84% (control). Asterisks (*) indicate significant difference between the hypoxia-exposed and the control group ($P < 0.05$). Mean values ± SE; $n = 5$ for all groups except 84% ($n = 3$).
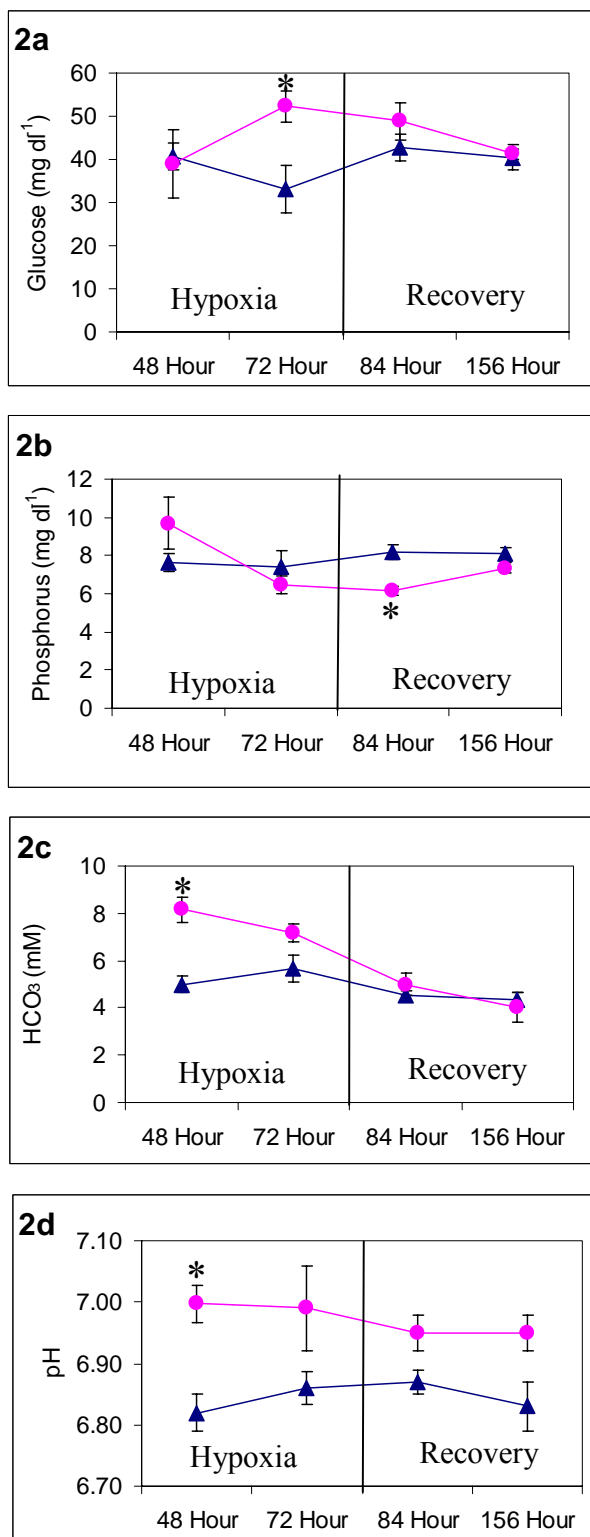
Fig. 2. Blood parameter measurements for Atlantic menhaden exposed to oxygen concentrations of 20% (●) and 84% (▲) saturation for 48, 72, 84, and 156 hours. Treatment group was exposed to 20% oxygen saturation for 72 hours. During the recovery period, oxygen saturation was the same as the control group (84%). Time points for which significant differences between hypoxia-exposed and control menhaden were observed are denoted by asterisks (*). Mean values ± SE; $n = 6$.