ABSTRACT

UDOMKUSONSRI, PAREEYA. Pathogenesis of the Acute Ulceration Response (AUR) in Fish. (Under the direction of Dr. Edward J. Noga and Dr. Nancy A. Monteiro-Riviere.)

We demonstrated that rapidly confined hybrid striped bass (*Morone saxatilis* male x *M. chrysops* female) developed a syndrome characterized by the immediate and dramatic loss of their skin. We have named this phenomenon the Acute Ulceration Response (AUR). AUR is characterized by the rapid onset of severe epidermal erosion, ulceration and degeneration on the body skin and fins, as well as corneal ulceration, in stressed hybrid striped bass. Grossly, the distal edges of the fins became obviously ragged and blanched. The earliest microscopic change in the fins occurred within 15 min, with swelling of the outermost layers of the epidermis and epidermal erosion. After 30-min stress, the epidermis at the distal edges of the fins became ulcerated. Both apoptotic and necrotic epithelial cells were observed at 30 min confinement stress. The middle and basal epidermis developed severe spongiosis, and the dermis and hypodermis became edematous. Epidermal ulceration appeared on all fins of stressed fish and was significantly greater compared to fins from unstressed fish. A time-course study of the response to acute confinement stress showed a significant correlation between confinement period and severity of AUR.

In separate experiments, the size of the acclimation space, temperature during acclimation and confinement, and exogenous adrenergic modulators were shown to influence the risk of developing AUR. Acute confinement can also cause AUR in a wide array of fish species, including guppy (*Poecilia reticulata*), freshwater angelfish (*Pterophyllum scalare*)

and channel catfish (*Ictalurus punctatus*), but not in rainbow trout (*Oncorhynchus mykiss*). AUR might be expected to predispose fish to secondary microbial infections, and this was supported by a study in which high mortality (87.5%) from severe water mold (*Saprolegnia*) infection developed in hybrid striped bass having AUR that were subsequently exposed to a low concentration of *Saprolegnia* zoospores. However, when fish with AUR were placed into a healthy environment (no pathogens added), microbial load (as reflected by bacterial concentration) in AUR lesions remained low throughout the recovery period. In conclusion, AUR might play a critical role in skin ulcer epidemics of many fish species after an acute stress. Furthermore, manipulation of the physical environment might reduce the risk of fish developing AUR and environmental pathogen load seems to play a critical role in determining whether AUR lesions will lead to devastating disease losses.

PATHOGENESIS OF THE ACUTE ULCERATION RESPONSE (AUR) IN FISH

by

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To My Family and My Parents

BIOGRAPHY

Pareeya Udomkusonsri grew up in Bangkok, Thailand. She was awarded the DVM degree with the second honor in 1992 from the Kasetsart University, Bangkok. She served as a veterinarian for the Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Kasetsart University for 6 months. In 1992, she became a faculty member of the Department of Pharmacology and Toxicology, College of Veterinary Medicine, Kasetsart University. In 1997, the Royal Thai Government awarded her a fellowship to pursue doctoral studies at North Carolina State University, Raleigh. During the course of her studies, together with her principal thesis advisor Dr. Edward J. Noga, she studied the pathogenesis of the Acute Ulceration Response (AUR) in fish. She also awarded Dr. Monica Menard Award for Excellence in Veterinary Pathobiological Research by North Carolina Veterinary Medical Foundation, Inc, in 2002. In 2003, she achieved her Ph.D. degree in Comparative Biomedical Science at North Carolina State University, United State of America.

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I.

LITERATURE REVIEW

FISH SKIN: Normal Structure and Role in Host Defense

The skin of fish is a primary barrier against the external environment and preserves the constancy of the internal *milieu*. The layers of fish skin, like those of all vertebrates, consist of epidermis and dermis, which are separated by the basement membrane, or basal lamina, which is visible under the electron microscope.

EPIDERMIS

Fish epidermis is a typical stratified, squamous, epithelium; however, unlike mammalian skin, it is metabolically active, with mitotic capacity throughout all epidermal layers (Bullock et al 1978). In all but a few fish (Mittal and Banerjee 1974), the outer surface of the epidermis is not keratinized. The epidermis covers the body surface and is continuous throughout the fins and cornea. Its thickness varies with body site, age, sex, condition, and degree of maturation of fish (Ellis et al 1978; Whitear 1986b), and is frequently thicker in the dorsal body areas in pelagic fish species and in the ventral surfaces of benthic fish. In salmonids, the epidermis in non-scaled areas, such as the head and fins, is normally thicker than in scaled areas (Harris and Hunt 1975). In addition, many factors, such as environmental conditions, handling, nutritional status and other stressors, influence the structural and cellular response of the epidermis (Blazer et al 1997; Ferguson 1989; Iger et al 1995; Iger et al 1994a, b; Pickering and Richards 1980; Quiniou et al 1998). For example, the skin of cutthroat trout (*Oncorhynchus claski henshawi*) exhibited sunburn damage; sloughing of mucus cells, necrosis and edema in the epidermis and dermis, after exposure to

ultraviolet-B radiation (Blazer et al 1997). Fish epidermis also plays an important role in wound healing and antigen uptake (Kiryu et al 2000; Ototake et al 1996).

The epidermal cell is known by many names, including malpighian cell, epithelial cell, filament-containing cell, filamentous cell, polygonal cell, polyhedral cell, keratocyte, keratinocyte, principal cell, and common cell. Epidermal cells in fish and mammals originate from ectoderm. In mammals, the skin consists of five epidermal layers: stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum and stratum basale; however, fish epidermis is generally separated into outer, middle and basal layers (stratum germinativum) (Groman, 1982; Hawkes 1983). The epidermis consists of a non-vascularized stratified squamous epithelium and has no stratum corneum which is normally found in the terrestrial vertebrates. In primitive, jawless fish (Agnatha), the surface of the epithelium is pitted like a sponge (Whitear 1986b). However, the exterior surface of the superficial epithelial cells of teleosts is characterized by microridges or micropapillae, which often form regular, fingerprint-like patterns. The function of microridges is unknown; however, they may provide defense against trauma and might hold mucus secretions on the surface. They also might increase gas exchange (Hawkes 1974) and might be involved in wound closure since they can move by the contraction of basal actin microfilaments (Bereiter Hahn 1971).

Mucus cells are found in the epidermis, but the numbers vary with site, species and sexual stage (Roberts and Bullock 1978). They usually originate from the middle layers of epidermis, although they may be seen in the basal layer of a very thin epidermis. Immature mucus cells are rounded, and become flattened laterally and increase in size when they

approach the surface. Mature mucus cells are filled with numerous mucosomes, with the nucleus displaced to the periphery of the cell. When a mucus cell reaches the surface, the plasma membrane ruptures at the apex, releasing its cell contents; then the cell dies. Mucus secretions contain mucopolysaccharides (sialomucin, sulfomucin) that decrease friction with water.

Club cells are large, oval or round cells and have one or two nuclei with prominent nucleoli. They are found in the middle layers of epidermis in some fish species, and normally are called alarm cells since they secrete potent "alarm" substances. Club cells release fright pheromones into the surrounding water when the epidermis is damaged or the cells are lysed (Smith 1992). Alarm substances also play a role in wound healing, and are possibly protective agents against parasites, other pathogens, or irritants (Shiomi et al 1988; Smith 1982).

BASEMENT MEMBRANE

The basement membrane in fish is similar to that of other vertebrates, acting as a lining between the epidermis and dermis (Whitear 1986a). A lucent adepidermal space under the basal epidermal layer contains fibrillar material (anchoring filaments) connecting hemidesmosomes of the basal epidermal cells to the electron-dense basement membrane (Ferri 1982; Whitear 1986a). The thickness of the basement membrane depends upon the species and location on the body. It is generally less well-developed in scaled areas compared to the head or fins (Roberts and Bullock 1978). The periodic acid Shiff reaction (PAS) stains the basement membrane that the color penetrates between the basal layer cells.

Anchoring filaments connect the basement membrane to the collagenous tissues beneath. Under the basement membrane, an electron-lucent layer is supplied with nerves and capillaries.

The basement membrane acts as a barrier and controls the passage of cells and chemicals. It also regulates morphogenesis and wound healing, serving as an attachment site for epithelial cells or other cells.

DERMIS

The dermis consists of two major layers, stratum spongiosum (stratum laxum) and stratum compactum. The upper dermis (stratum spongiosum) is a loose network of collagen and reticulin fibers. The stratum spongiosum contains vascular and neural components, and scales are anchored in the scale pockets in this layer. It also includes fibroblasts, pigment cells, leucocytes and scale-synthesizing tissues (Whitear 1986a). The stratum compactum consists of collagen bundles that form a dense matrix above the hypodermis. The collagen fibers in these layers are high ordered at right angles to each other. The stratum compactum may be reduced in fin tissue (Sharples and Evans 1996). Dermal fibroblasts are distributed between collagen bundles. This layer is important in locomotor activity (Whitear 1986a) and acts as a tendon in parallel with the muscles. The dermis in juvenile striped bass (*Morone saxatilis*) has only a narrow layer of stratum compactum, composed of dense collagen fibers, elastic fibers, melanocytes and a small amount of connective tissue (Groman 1982). In adult striped bass, the dermis is composed of 2 layers; a papillary layer (stratum compactum) containing dense collagen fibers, and an outer stratum spongiosum of loose connective tissue.

The fish mast cell, or eosinophilic granular cell (EGC), was first described in the skin of plaice (*Pleuronectus platessa* L.) (Roberts et al 1971). Mast cells are common in the dermis around blood vessels, and appear in many other tissues such as gill, oral epithelium, swim bladder and alimentary tract (Powell et al 1993; Reite and Evensen 1994; Sire and Vernier 1995; Zaccone 1980). Teleost mast cells are motile, mononuclear cells with prominent granules and may play an important role in host defense (Silphaduang and Noga 2001). Teleost mast cells contain a number of mediators (e.g., histamine) and can be stimulated by bacteria, parasites and chemical stimuli, resulting in their mobilization and degranulation (Matsuyama and Lida 1999, 2000; Powell et al 1993; Sire and Vernier 1995).

Recently, Silphaduang and Noga (2001) discovered a novel family of peptide antibiotics, named piscidins, from mast cells in hybrid striped bass (*Morone saxatilis* x *M. chrysops*). Piscidins have potent, broad-spectrum activity against important bacterial pathogens of both fish and mammals, including multi-drug resistant bacteria. Hybrid striped bass mast cells in gill, skin, stomach, intestine, and pyloric ceca are immunoreactive for piscidins, including those lining blood vessels in the viscera. The mast cells of other fish are also positive for piscidins, including white bass (*M. chrysops*), striped bass (*M. saxatilis*), spot (*Leistomus xanthurus*) and croaker (*Micropogonias undulatus*). Those fish are all in the Suborder Percoidei, Order Perciformes. This suggests that piscidins may be evolutionarily conserved in this group.

In teleosts, chromatophores (pigment cells) are normally found in the stratum spongiosum of the dermis, and in the hypodermis. They are located close to the basement

membrane: in the upper portion of the stratum spongiosum in non-scaled areas, or under the scales in scaled skin. Epidermal chromatophores are classified according to the color of their pigment. Five different chromatophores are known, melanophores (black or brown pigment), erythrophores (red or yellowish pigment), xanthophores (primarily yellow pigment), leucophores and iridophores (colorless, reflecting pigment). The difference between leucophores and iridophores is based upon the translocation of pigment-containing organelles. Leucophores have fewer dendritic processes, but leucosomes can move centripetally or centrifugally similar to other dendritic-chromatophores (Fujii 1993). Leucophores reflect light in all directions. Iridophores are non-dendritic or occasionally dendritic, and contain large crystalline platelets that form stacks in the cytoplasm with uniform spacing between adjacent platelets within a stack (Fujii 1993).

Most vertebrate pigment cells originate from neural crest, and then migrate, differentiating in the integument. Many fish do not develop a distinct neural crest; however, fish chromatoblasts appear to migrate from a location close to the neural keel, which is equivalent to the neural crest. Because chromatophores share an ontogenetic origin with neurons, most of them are dentritic cells with multiple branched or unbranched processes extending from the cell body. Chromatosomes, pigment-containing organelles, translocate centripetally (aggregation) or centrifugally (dispersion) in response to various neuronal or hormonal signals. The movement of chromatosomes involves two possible hypothesized mechanisms (Fujii 1993). In the first model which has been widely accepted, pigment granules are moved by interaction with microtubules that radiate from the center of the cell toward the periphery of the dendritic processes. The second model, which is a microtubule-

independent motility system, actin filaments provide the motive force for granule movement. It is also possible that pigment granules may be enmeshed in a filamentous network which passively drags the granules (Rodionov et al 1998).

The pigments in chromatophores are melanins, carotenoids, pteridines, and/or purines. The black color of melanins is formed by polymerization of DOPA-quinone, synthesized from tyrosine. In xanthophores, carotenoids (a yellowish color, highly unsaturated hydrocarbon) are formed from four isoprene units and two ionone rings. Pteridines are associated with red color and consist of a pyrimidine and a pyrazine ring. Carotenoids and pteridines make yellowish to red color in erythrophores. Since animals cannot synthesize carotenoids, pigmentation in xanthophores and erythrophores is influenced by diet. The colorless pigments in leucophores and iridophores are purines. Guanine is the main pigment, but other purines such as hypoxantine and uric acid are found in both chromatophores.

Melanophores are the most common and usually the largest chromatophore in fish (Fujii 1993). Melanophores usually respond to nervous and hormonal stimuli with rapid aggregation or dispersion of melanosomes (Fujii 1993). Xanthophores and erythrophores are generally smaller than melanophores, but the morphological features are similar to melanophores. In contrast to the light-absorbing chromatophores (melanophore, xanthophore and erythrophores), leucophores and iridophores are light-reflecting chromatophores that are found in whitish or silvery areas of skin.

Chromatophore activity is regulated by humoral and neuronal controls. In the humoral control, it requires stimulation of cells releasing hormones into the circulation. Hormone is transported via blood and binds to receptors on the chromatophores. Chromatophores are also directly innervated, which provide an instant response and specific change. Neuronal control is dominant in higher teleosts allowing rapid body color change, while humonal control is the rule in cyclostomes and elasmobranchs.

Color change in fish can occur via two different mechanisms. First, chromatophores can redistribute the pigment-containing organelles within the cells. Color change often appears immediately, through the activity of neuronal stimulation directly on the chromatophores. Examples include adaptive skin color in response to background color or lighting conditions and color change related to the physiological state of the fish. These phenomena reflect the physiological control of the pigment cells, and therefore are called physiological color changes. The other mechanism of color change, morphological color change, results from changes in the total number and size of pigment cells, and or the amount of pigments in the cells. This color change is gradual and much slower, taking several days or weeks to be completed. However, both physiological and morphological color changes occur simultaneously, with the latter providing the base coloration.

FIN

Fin consists of epidermal folds of skin which is supported by lepidotrichia, segmental rays formed of giant fibers of collagen. Lepidotrichia first appear at the base of the fins, and later are more deeply embedded and associated with those on the opposite side of the fin to

form an incomplete tube which is linked by connective tissue. Actinotrichia persist at the distal end of each lepidotrichium to the margin of the fin (Beccera et al 1983). Each lepidotrichal segment is composed of a pair of hemisegments, plate-like elements separated by an intrasegmental region. The hemisegment or demiray consists of an acellular matrix or has very few cells. The intrasegmental region contains nerve bundles, blood vessels and connective tissues (Montes et al 1982). Lepidotrichia are divided lengthwise, and each segment is connected with intersegmental joints. Fins are covered by a stratified squamous epithelium that is continuous from the body epidermis. The dermis in fin has a reduced stratum compactum and thicker hypodermis (Groman 1982; Whitear 1986a).

PATHOLOGICAL RESPONSES OF THE SKIN TO DAMAGE

The pathological responses to stress were well documented in which skin is capable of expressing: degeneration, erosion, ulceration, leukocyte infiltration (reviewed in Bodammer 2000; Iger et al 1992; Iger et al 1994a, b, c, d; Iger and Wendelaar Bonga 1994; Noga 2000). Epidermal degeneration was defined as swollen epidermal cells (intracellular edema) with pyknotic nuclei; epidermal erosion was the sloughing of epithelial layers, but with the basement membrane still intact; ulceration was defined as complete loss of all epithelial layers and the basement membrane. Leukocytes are normally found in the dermis but in stressed skin, leukocytes may also infiltrate the basal epidermal layers.

Two modes of cell death can occur in skin cells, apoptosis and necrosis. They can be distinguished based on differences in the morphological, chemical and molecular changes in dying cells. Necrosis is a cellular response to an extensive trauma and triggers the

inflammatory response in damaged tissue, while apoptosis is a noninflammatory, programmed cell death that is associated with embryogenesis, metamorphosis, and normal cell turnover (Wyllie 1997; Wyllie et al 1980), but also with certain pathologies as well. Necrosis typically occurs in response to toxins, hypoxia, or ischemia, and affects cells in groups rather than singly (Anilkumar et al 1992; Wyllie et al 1980). Necrotic cells show destruction of organelles, chromatin flocculation, mitochondrial swelling, rupture of the plasma membrane, and release of cytoplasmic contents (Darzynkiewicz et al 1994; Lin et al 1996). Apoptosis is subjected to control by genetic and normal physiological stimuli, such as endocrine changes (e.g., cortisol, ACTH) (Iger et al 1995; Iger et al 1992; Wyllie 1997), as well as certain toxic agents such as radiation and chemotherapeutic agents (Anilkumar et al 1992). Apoptosis is morphologically characterized by cell shrinkage, nuclear condensation, membrane blebbing and internucleosomal fragmentation of DNA into units of 180-200 base pairs (DNA laddering).

Apoptotic cells are usually phagocytosed and digested by resident cells (Anilkumar et al 1992). Iger et al (1994a) reported that the autophagocytic vesicles, that appeared inside the epidermal cells of both unstressed and stressed fish, were eliminated by the epidermal cells. Thus, the autophagocytic vesicles in the epidermal cells may actually contain apoptotic cells. Furthermore, apoptotic cells in unstressed fish are indicative of the normal turnover of epidermal cells.

STRESS IN FISH

Stress refers to a condition in which organism equilibrium is disturbed due to a stimulus (Wendelaar Bonga 1997). The definition of stress is difficult to describe to fit in all

areas. For example, physiologists describe stress as an increase of plasma hormones and metabolites. Toxicologists describe stress as an induction of mixed-functional oxidase enzymes, while fish culturists are concerned with growth and mortality of fish. In aquaculture, a stimulus or stressor can place constraints on fish health. These constraints are from changes in the physical environment (e.g., water pH, temperature, salinity), animal interaction (e.g., competition for food, space and sexual partners), water pollution (e.g., organic chemicals and heavy metals) and aquaculture practices (e.g., handling, transport and crowding) which cause stress to the fish. The acute stress response is an adaptive response that promotes the best chance of survival in the threatening situation; thus, acute stress does not necessarily harm an organism. However, if the stress is severe enough, or continuous and prolonged, the response can result in severe damage to growth, reproductive capacities or immune defense systems.

The stress response generally occurs in three stages. First is an initial alarm reaction from the activation of stress-related hormones. This initiates energy production, helping to maintain homeostasis and survival. Second is the resistance stage, which occurs when physiological systems have successfully compensated and the organisms are acclimated. There is an energy cost for compensation and thus growth is decreased. Third is the exhaustion stage, when prolonged or severely stressful conditions exceed the ability of acclimation to maintain homeostasis; this is maladaptive. In aquaculture, stress is often, if not usually, a predisposing factor to an infectious disease (bacterial, fungal, parasitic, or viral infection) outbreak (Pickering and Duston 1983). Thus, reducing stress is a major goal in aquaculture.

HORMONAL BASIS OF THE STRESS RESPONSE

Stress causes neuro-hormonal changes (primary response) and results in physiological consequences of this neuro-hormonal stimulation (secondary response). The primary response to stress in fish involves many hormones, e.g., catecholamines (CA), glucocorticoids (GC), growth hormone and prolactin (Wendelaar Bonga, 1997); however, CA and GC are generally recognized as the dominant stress hormones (Mazeaud and Mazeaud 1981; Wendelaar Bonga 1997). In the secondary response to stress, both CA and GC increase oxygen uptake, blood flow, cardiac output and energy consumption. It is difficult to distinguish between the secondary effects of GC and CA (Mazeaud and Mazeaud 1981) since both CA and GC are usually activated in response to stress. When stress is continuous or chronic, fish develop the tertiary response, with decreased growth rate, modified behavior and increased susceptibility to diseases. In populations or ecosystems, a higher order response (the quaternary response) is a result of alteration in species composition which is caused by the disruption in energy flow through trophic levels (Wedemeyer 1996).

GLUCOCORTICOIDS

Release of GC is caused by an activation of the hypothalamic-pituitary-interrenal (HPI) axis (functionally similar to the hypothalamic-pituitary-adrenal axis in mammals). Cortisol is the predominant glucocorticoid in most teleosts (Schreck et al 1991). In addition, cortisone, a metabolite of cortisol, has been reported as a primary GC in unstressed coho salmon (*Oncorhynchus kisutch*) (Patino et al 1987). Cortisol is produced by the interrenal

tissue, which originates from mesoderm and is located on the walls of the posterior cardinal veins (PCV). Stress stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary gland by the activation of corticotropin-releasing factor (CRF) from the hypothalamus. The circulating ACTH stimulates the interrenal cells to produce cortisol.

Cortisol has broad activity and is important in regulating hydromineral balance and metabolism in fish (Table 1.1). Other effects include reduction of growth and suppression of immune function (Mazur and Iwama 1993; Schreck et al 1991; Wendelaar Bonga 1997). For example, fish have increased susceptibility to water mold infection after prolonged oral administration of cortisol, and implantation of the steroid increases fish mortality due to furunculosis (Pickering and Duston 1983). Furthermore, prolonged elevated cortisol caused brown trout (*Salmo gairdneri*) to die from a combination of water mold (*Saprolegnia*) infection, severe bacterial fin-rot and furunculosis (Pickering and Pottinger 1989).

Elevated cortisol is one of the most widely used indicators of stress in fish (Wendelaar Bonga 1997). After exposure to stress, the plasma cortisol concentration rises rapidly in a few minutes. In fish, resting plasma cortisol levels are very low, 2-42 ng/ml (reviewed in Gamperl et al 1994a). Acute and chronic stress causes an increase of plasma cortisol which can vary from 20-740 ng/ml (Barton and Iwama 1991; Gamperl et al 1994a), depending upon fish species, strain within a species, sexual maturation, season, temperature, environment, and sampling time after stress (Barton and Iwama 1991; Pickering and Pottinger 1989; Wendelaar Bonga 1997). For example, the plasma cortisol in catfish (*Heteropneustes fossilis*) subjected to 2-3 min chasing was unchanged (Sherwani and Parwez

2000). It was suggested that this catfish was very tolerant of stress.

Increased plasma cortisol was reported in response to many stressors, including transport, increased water temperature, net confinement, or poor water quality (Davis and Parker 1990; Fevolden et al 2003; Iger et al 1994b; Mazur and Iwama 1993). Cortisol levels will return to normal if the stress is discontinued to allow the fish to recover. However, the recovery period varies, depending on the type, intensity and duration of stress, as well as the fish strain (Noga et al 1994; Pickering and Pottinger 1989; Pickering et al 1982). Acute stress (1-hr confinement or 30-sec emersion), caused a significant increase of cortisol in rainbow trout and brown trout; cortisol levels returned to control values within 24-48 hr (Pickering and Pottinger 1989). The cortisol peak in rainbow trout was lower than that in brown trout. In addition, chronic confinement caused a prolonged increase of cortisol in rainbow trout that required 3 weeks for complete acclimation, while in brown trout it required more than 7 weeks. Noga et al (1994) found that plasma cortisol concentration increased significantly after exposure of striped bass and hybrid striped bass (*Morone saxatilis* x *M. chrysops* and *M. saxatilis* x *M. americana*) to an acute net confinement for 45 minutes. Plasma cortisol in striped bass increased faster (from 3 ng/ml to 742 ng/ml) and reached a higher level than in hybrid striped bass (from 212 ng/ml to 490 ng/ml). Plasma cortisol concentration was still high in striped bass for at least 48 hours after the net confinement. Conversely, plasma cortisol in hybrid striped bass decreased to normal or near normal levels within this period. The mortality of stressed striped bass and its hybrids increased markedly when fish were crowded at the high density. Domesticated sea trout (*Salmo trutta)* displayed an increase of plasma cortisol that was lower than wild sea trout

after exposure to stress (Lepage et al 2000). These studies suggested that strains of fish respond differently to stress.

CATECHOLAMINES

The adrenergic function of fish is sensitive to stress and results in an increase of plasma CA (Randall and Perry 1992; Thomas and Perry 1992). Epinephrine (E) and norepinephrine (NE) are the predominant CAs in fish; however, epinephrine is usually the predominant CA in teleosts while NE is dominant in elasmobranchs (Reid et al 1998; Wendelaar Bonga 1997). The release of CA into the circulation is mediated by the preganglionic-cholinergic innervation of the chromaffin tissues (Nilsson 1984; Thomas and Perry 1992). The arrangement of chromaffin cells is quite diverse in fish. In the primitive cyclostomes, chromaffin cells are associated with the systemic and portal hearts. In elasmobranches, these cells are located near paravertebral autonomic ganglia. In teleosts, the chromaffin tissues are equivalent to the adrenal medulla in mammals, which originates from neuroectoderm. They are normally located within the wall of the posterior cardinal veins (PCV) and in small clusters in the head kidney. In Perciformes and Salmoniformes, chromaffin cells are also present in the posterior kidney in contact with the caudal veins (Milano et al 1997). The chromaffin cells are closely associated with the interrenal cells (steroidogenic cells) around the walls of the PCV.

Sympathetic stimulation by preganglionic nerve fibers causes the release of acetylcholine, which stimulates cholinergic receptors, and ultimately initiates a series of Ca^{2+}-dependent events leading to increased CA secretion by exocytosis. A summary of the

cholinergic receptors on the chromaffin cells of various animals is shown in Table 1.2. In teleosts, the cholinergic receptors on teleost chromaffin cells are predominantly nicotinic receptors (Abele et al 1998; Al-Kharrat et al 1997); thus, hexamethonium (a nicotinic receptor antagonist) can prevent the secretion of CA in at least some fish (e.g., American eel, *Anguilla rostrata*; hagfish, *Myxine glutinosa*; dogfish, *Squalus acanthias*) (Bernier and Perry 1996; Opdyke et al 1983a; Reid et al 1998; Reid and Perry 1995). Muscarinic receptors also have an inhibitory effect, especially on norepinephrine-secreting cells (Al-Kharrat et al 1997). Muscarinic receptors on rainbow trout chromaffin cells are involved in CA secretion (Montpetit and Perry 1999). Stimulation of muscarinic receptors increased the intracellular Ca^{2+} concentration via activation of phospholipase C, but it was insufficient to trigger CA secretion. However, it may be possible that the increase of intracellular Ca^{2+} concentration enhanced the nicotinic receptor Ca^{2+} events and increased nicotinic-induced CA secretion. CA are also synthesized and released from adrenergic nerve endings by adrenergic stimulation (Nilsson 1984; Perry et al 1991).

In fish as in other vertebrates, the chromaffin cells contain a high CA content and are stained dark by dichromate solution due to the oxidation of catecholamine stores to adrenochromes. E and NE are stored in separate cells (Abelli et al 1996). Norepinephrine-containing cells normally have spherical, strongly electron-dense secretory granules, and the granules are evenly distributed. In epinephrine-containing cells, the granules are electron-lucent, spherical or elongated, and distributed homogeneously. In all vertebrates, E and NE are synthesized from tyrosine via the Holtz-Blaschko pathway (Randall and Perry 1992). Tyrosine in the cells is hydroxylated to dihydroxyphenylalanine (DOPA) by tyrosine

hydroxylase (TH) and subsequently decarboxylated to dopamine by dopamine decarboxylase (DH). Dopamine is taken into the storage vesicles and is hydroxylated by dopamine-β-hydroxylase (DβH) to NE. NE is released from the vesicles into the cytosol and is then methylated by phenylethanolamine-*N*-methyl transferase (PNMT) to E. E is then moved to storage vesicles. Normally, E and NE are produced in the different cells depending upon the presence of PNMT and both CAs are released into the circulation by exocytosis. TH, DH and PNMT are cytoplasmic enzymes while DβH is associated with the storage vesicles. The rate-limiting step in the epinephrine biosynthesis pathway is normally the TH step in mammals, but is the PNMT step in fish (Abrahamsson and Nilsson 1976; Senthilkumaran and Joy 1995). Circulating CAs are catabolized by two enzymes, catechol-*O*-methyl transferase (COMT) and mitochondrial monoaminoxidase (MOA) which are responsible for ortho-methylation and deamination, respectively. COMT is mainly in the liver, which plays a more important role in the catabolism of circulating CAs, while MOA is found mainly is the kidney, as well as the brain (Randall and Perry 1992).

In teleosts, release of CA into the circulation (referred as a primary effect of stress) is induced by a number of stimuli, such as preganglionic sympathetic nerve fibers, localized changes in blood chemistry, activation of the renin-agiotensin system, serotonin, and adrenocorticotropic hormone (Bernier and Perry 1996, 1997; Nilsson et al 1976; Opdyke et al 1981, 1983a; Perry et al 1991; Reid et al 1996). CA is released rapidly by the simulation of various stresses, such as hypoxia (Perry et al 1991, 2000; Randall and Perry, 1992; Reid et al 1993; Thomas et al 1994), crowding and handling (Fløysand et al 1992; Gerwick et al 1999), exposure to low pH water (Witters et al 1991), or exercise (Bulter et al 1989; Postlethwaite

and McDonald 1995). The resting levels of plasma epinephrine range from 1 to 10 nM and can be elevated up to 300-fold under acute stress (review in Gamperl et al 1994a; Reid et al 1998; Thomas and Perry 1992). The magnitude of CA release is related to the intensity and type of stress; only severe stress (e.g., exhaustive exercise, severe hypoxia, air exposure) causes an increase in circulating CA while mild or moderate stresses (e.g., sustained aerobic exercise) usually do not change the CA levels (reviewed in Randall and Perry 1992; Perry and Bernier 1999). In tilapia (*Oreochromis aureus*), an increase of CA levels to a 30-min cold stress was detected earlier than an elevation in cortisol, but the duration of plasma CA elevation was shorter than that of cortisol (Chen et al 2002).

While several factors are known to initiate CA secretion, neuronal activity of preganglionic sympathetic nerve fibers is the predominant mechanism for CA secretion in response to stress (Reid et al 1998). Furthermore, the pituitary-interrenal hormones can modulate CA storage and release in rainbow trout (Reid et al 1996). It was demonstrated that ACTH elicited CA release by stimulating the production of cAMP, then increasing CA release via exocytosis. Prolonged plasma cortisol increased CA storage in the kidney and tissue around the PCV. In mammals, cortisol increases transcription of the genes encoding for PNMT; however, this effect did not occur in rainbow trout (Reid et al 1996).

Generally, CA are released much quicker than GC because chromaffin cells are innervated directly with sympathetic ganglionic fibers and already store CA in their secretory vesicles (Barton, 1988; Nilsson 1984). The secondary effects of CA are mainly on circulation, osmoregulation and energetics (Randall and Perry 1992; Wendelaar Bonga 1997)

(Table 1.1). CA stimulates gluconeogenesis and glycogenolysis, causing an increase of blood glucose released from the liver (McKinley and Hazel 1993; Moon and Mommsen 1990). Thus, many studies have used blood glucose as an index for the stress response (Lepage et al 2000; Reubush and Heath 1997). Increased CAs during stress causes hyperventilation and increased stroke volume of the heart (Mazeaud and Mazeaud 1981; Wedemeyer 1996). Epinephrine is linked to decreased blood PO_2 and blood pH. CA stimulates Na^+/K^+ exchange and inhibits CO_2/HCO_3^- exchange across the plasma membrane of red blood cells, which causes blood plasma acidification and cytoplasmic alkalinzation and increases the affinity of hemoglobin for oxygen, suggesting that CA plays a role in the optimization of oxygen transport (Perry et al 1991; Randall and Perry 1992; Witters et al 1991).

ADRENERGIC RECEPTORS (Adrenoceptors)

The activity of CA is a result of binding to specific receptors located on the membrane of the target cell. In 1948, Ahlquist classified adrenergic receptors as α- and β-adrenoceptors (AR). CA affects the circulatory system by interacting with α- and β-adrenoceptors to increase oxygen uptake of the gill and increase oxygen transport capacity of the blood. Increasing blood pressure and vasoconstriction is mediated by α-ARs to control vascular resistance (Einstein et al 1994; Randall and Perry 1992; Wahlqvist and Nilsson 1980, 1981; Wood 1976).

Adrenoceptors are classified based on the responses to various CAs, including epinephrine, norepinephrine and isoproterenol (a synthetic CA). The α-and β-ARs are defined in termed of agonist potencies as follows:

Receptor	Order of agonist potency
α	Norepinephrine = epinephrine >> isoproterenol
β	Isoproterenol > norepinephrine > epinephrine

ARs are classified specifically into several types and subtypes according to their pharmacology and molecular basis: α_1 (A,B,D); α_2 (A,B,C,D); β_1, β_2, β_3 ARs (reviewed in Hieble et al 1995). Most teleost ARs are classified based upon their phamacology (using adrenergic agonists and antagonists) (Table 1.3). All ARs consist of a seven protein, transmembrane-spanning domain of G-protein receptors. The hydrophobic domains are connected by hydrophilic sequences forming loops that protrude out of the membrane. The sequences of all G-proteins have high amino acid homologies; thus, ARs in animals are highly conserved evolutionarily. All β-AR subtypes are in the same transduction pathways, and are coupled with a G-stimulating protein named Gs, while α_1-and α_2-ARs are coupled with Gq and Gi proteins, respectively (Figure 1.1).

Gs-protein means G-stimulating protein in which agonists (i.e., epinephrine, norepinephrine) cause stimulation of the intracellular secondary messenger, cAMP. Gs protein is αβγ heterotrimer in which the α-subunit has a GTP/GDP binding site and responds to stimulation by adenylyl cyclase (AC). The β and γ subunits are tightly associated and

form the βγ complex. The transduction pathway for fish β-AR is coupled with the Gs-adenylyl cyclase system as is present in mammalian cells. When AR agonists or ligands bind Gs, it causes the βγ complex to dissociate and the α subunit stimulates the AC enzyme which is responsible for synthesizing the second messenger molecule, cAMP, from ATP. Fish AC is also stimulated by forskolin which is a classical, direct AC activator in mammals (Moon et al 1997). cAMP acts by activating protein kinases which catalyse the phosphorylation of serine and threonine residues in different cellular proteins, using ATP as the source of the phosphate groups. This mechanism acts to regulate cellular functions. The cellular functions that cAMP can regulate include: cell division and cell differentiation, ion transport, ion channel function which leads to changes in electrical excitability, the contractile proteins in smooth muscle, and regulation of enzymes involved in energy metabolism. Increased cAMP activates various effector systems (e.g., hepatic GPase and triacylglyceraol lipase, cardiac Ca^{2+} channels and red blood cell Na^{+} /H^{+} exchange in many fish [Fabbri et al 1998; Fange 1994]).

α-adrenoceptors exist on peripheral sympathetic nerve terminals. α_1 is found mostly postsynaptically, while α_2 is typically sited presynaptically and also occurs postsynaptically. All α-adrenoceptors use G-proteins as their transduction mechanism (Figure 1.1). α_1-adrenoceptors are coupled through the Gq mechanism; Gq activates phospholipase C which in turn phosphorylates phosphotidyl inositol 4,5, biphosphate (PIP_2) to produce inositol 1,4,5 triphosphate (IP_3), and diacylglycerol (DAG). The secondary messenger IP_3 causes release of calcium from intracellular stores in the sarcoplasmic reticulum which increases

intracellular Ca^{2+}; DAG stimulates protein kinase C (PKC) and activates calcium channels. They both produce their effects by the release of calcium. The α_2-adrenoceptor G-protein, Gi, is negatively coupled to AC and so it reduces the formation of cAMP, which leads to a decreased influx of calcium (the ion responsible for transmitter release) during the action potential. Therefore, lower levels of calcium will correspondingly lead to a decrease in transmitter release.

The effects of catecholamines on fish tissues

Liver

Epinephrine stimulates glucose release from the liver by binding to β-adrenoceptors, typically the β_2-AR subtype, in the hepatic plasma membrane (McKinley and Hazel 1993), stimulating AC activity and producing cAMP. Cyclic AMP modulates the activity of protein kinases and phosphatases, which affect the regulatory enzymes in the glycogenolysis pathway by activating glycogen phosphorylase and inhibiting pyruvate kinase (Fabbri et al 1998; Wright et al 1989). In addition, the increase of blood glucose in goldfish (*Carassius auratus*) hepatocytes, *in vitro*, was stimulated by α-ARs and was inhibited by phentolamine (an α-adrenergic antagonist) (Krumschnabel et al 2001). The stimulation of hepatic α-ARs increased the intracellular $[Ca^{2+}]$, which activated the regulating enzymes in the glycogenolysis pathway. The increase of blood glucose is normally used as a stress response index.

Cardiovascular system

Wood (1976) found a dominant α-adrenergic constrictor mechanism and β-adrenergic dilatory mechanism in the systemic vasculature of rainbow trout. In fish heart, CA can cause eitherpositive or negative chronotropic and inotropic effects (Temma et al 1989; Tirri and Lehto 1984). The stimulation of β-ARs by CA causes positive chronotropic and inotropic effects (Ask et al 1981; Gamperl et al 1998; Gamperl et al 1994b; Tirri and Lehto 1984), while the negative effect is a result of the coexistence of α-adrenoceptor stimulation (Capra and Satchell 1977; Temma et al 1986; Temma et al 1989; Tirri and Ripatii 1982). The subtype of β-adrenergic receptors in the heart is β_2-AR (Ask 1983; Gamperl et al 1994b; Temma et al 1986). Furthermore, the chronotropic effect depends on the concentration of CA; a low concentration of NE caused positive chronotropy, while a high concentration caused either positive or negative chronotropic effects (via the stimulation on β- and α-AR, respectively) in carp (*Cyprinus carpio*) (Temma et al 1989). Tiiri and Lehto (1994) found that CA concentration and temperature also had an influence on the inotropic effect in perch (*Perca fluviatilis*). A biphasic inotropic effect was found when a ventricular strip was incubated at a high temperature (24°C): a high concentration of NE caused positive inotropy, while a low concentration caused a negative effect. Wood (1975) demonstrated that the branchial vascular system in rainbow trout is controlled by an adrenergic mechanism. Adrenergic stimulation caused vasodilatation, predominantly via β-adrenergic receptors, and a more rapid vasoconstriction via α-adrenoceptors.

Red blood cells

The presence of β-ARs on red blood cells has been reported in rainbow trout (Gilmour et al 1994; Reid et al 1993). The activation of β-ARs probably helps to protect red blood cells during hypoxia. The release of CAs during stress causes an increase of intracellular cAMP in the red blood cells. The increase of cAMP stimulates a plasma membrane Na^+ /H^+ exchange antiporter and then results in an increase of intracellular pH. The intracellular acidosis in the erythrocyte increases oxygen's affinity for hemoglobin. This activation can be inhibited by propranolol, a β-AR antagonist (Nikinmaa 1992).

Melanophores

The control of pigment granules (melanosomes) in melanophores is primarily neural, especially via sympathetic nerves. The pigment granule aggregation in melanophores, causing blanching, is an effect of CA stimulation on the α_1- and α_2ARs (Burton and Vokey 2000; Grundstrom et al 1985; Svensson et al 1993), while the activation of β-ARs causes melanosome dispersion (Mayo and Burton 1998a).

THE EFFECTS OF STRESS ON SKIN INTEGRITY

Stress responses can be expressed by the skin after exposure to many stressors, including polluted water, acidified water, heavy metals, high water temperature, hormonal imbalance and toxic algae. There are a number of other stressors associated with skin damage (reviewed in Bodammer 2000; Iger et al 1992; Iger et al 1994a, b, c, d; Iger and Wendelaar Bonga 1994; Noga 2000).

Toxins

Exposure to copper resulted in decreasing of the carp skin thickness which was detectable within 24 hr (Iger et al 1994c). Necrotic, swollen, epidermal cells with disrupted plasma membranes, electron lucent cytoplasm, and fragmentation of nuclear chromatin, were observed during the entire experimental period (43 days). In addition, apoptotic cells (shrunken cells showing condensation of cellular elements, increased electron density of cytoplasm, and loss of junctional complexes) were common during the first 14 days. In carp exposed to industrial pollutants, the skin was covered with a thick mucus, and mucus cells differentiated near the epidermal edge (Iger et al 1992). In lead- or cadmium-exposed carp, the epidermal cells had increased mitotic, necrotic and apoptotic rates. Lymphocytes increased at the basal layers of the epidermis. Iger and Wendelaar Bonga (1994e) found degenerative changes in carp skin after exposure to acidified water for 20 days. The epidermis was initially thinned, but subsequently increased compared to the controls; there was also an increase of mucus secretion during the early period of exposure. Apoptotic cells increased during the first week.

Stress Hormones

After administration of a single meal containing cortisol to rainbow trout (causing elevated plasma cortisol), degenerative cells appeared in the epidermis (Iger et al 1995). This treatment also increased mitosis, secretory activity and apoptosis of the epidermal cells, as well as increasing differentiation rate and apoptosis of normal mucus cells. Similar changes also occurred in the skin of fish exposed to various stressors (e.g., polluted water, elevated

temperature [Iger et al 1994a, b]). However, epidermal cell necrosis and leukocyte migration, not observed after cortisol administration, occurred in fish acclimating to environmental challenges (Abraham et al 1991; Iger et al 1992; Iger et al 1994b), and may represent the direct effect of the stressors or locally controlled processes.

Temperature

Extreme temperature change is a hazard to fish health and causes stress (thermal stress). For example, a difference between the hauling tank water and the receiving water can cause stress to transported fish. The responses to thermal stress include disturbances in growth, reproduction, behavior, metabolism, osmotic and ionic regulation, and ultimately death (Elliott 1981).

Pathological changes in the skin can also occur after temperature stress. The thickness of rainbow trout skin decreased after 3 hours exposure to an elevation of temperature (from 15° to 20°C)(Iger et al 1994a). Many necrotic cells appeared in the epidermal layer. The decreased epidermal thickness in stressed fish was associated with degeneration and shedding of epidermal cells and enhanced mucus secretion. Necrotic cells were prominent, possibly reflecting that the process was associated with accidental cell death and perhaps was a direct effect of the stressor. The skin thickness was restored after 24 hr and from day 4 the thickness was greater than the control. The increased thickness was associated with enlargement of the intercellular space (spongiosis), leukocyte infiltration, and increased mitoses. The higher mitotic rate, together with necrosis and apoptosis, indicated increased turnover rate of the cells.

The exact mechanism responsible for the effect of temperature stress on fish skin is unknown, but temperature stress is known to cause serious changes in stress hormones. Exposure of rainbow trout to chilled water (1°C) caused increased plasma cortisol by 4 hr after exposure (Barton and Peter 1982). Plasma cortisol increased from 85.9 to 160.7 ng/ml when *Tilapia aurea* were exposed to acute cold stress by reducing the temperature from 22°C to 11°-12°C for 60 minutes (Kindle and Whitmore 1986). In addition, when tilapia were chronically exposed to cold water for 5 weeks, cortisol levels were significantly higher (119.6 ng/ml) compared to controls (50.3 ng/ml). Carmichael et al (1984) found that raising the water temperature from 10° to 22°C in 20 minutes caused a rapid increase of plasma corticosteroids in largemouth bass, while plasma corticosteroids only slightly increased if the temperature was raised from 16° to 22°C in 18 minutes. Mazeaud et al (1977) found that plasma epinephrine increased when sockeye salmon (*Oncorhynchus nerka*), acclimated to 11°C, were transferred into 21°C water. Plasma epinephrine levels increased during the first 10 minutes, and then remained at a steady level during a longer (3 hr) stress period.

Davis et al (1984) found that acclimation temperature affected the resting concentration of cortisol in channel catfish (*Ictalurus punctatus*). The highest resting cortisol concentrations were in fish acclimated to 5° or 10°C (25-29 ng/ml), intermediate in fish maintained at 25°, 30° or 35°C (12-13 ng/ml) and lowest in fish held at 15°, 20° or 21°C (5-9 ng/ml). After confinement stress, cortisol levels increased most quickly in fish at the higher temperatures, had a more delayed elevation at the intermediate temperatures, but were hardly changed in fish held at 5° or 10°C. Basal concentrations of plasma cortisol (near 50 ng/ml) were higher in yearling channel catfish acclimated to 10°C than in fish acclimated to 20° or

30°C (near 25 ng/ml) (Strange 1980). However, fish at 20° and 30°C rapidly increased plasma cortisol levels within 0.5 hr (average 75 ng/ml) of initial confinement stress, while fish at 10°C had a slower increase in cortisol levels beginning after 1 hour of confinement and reaching over 100 ng/ml at 6 hr.

Davis and Parker (1990) found that acclimation temperature (5°,10°,16°,21°,25° or 30°C) significantly affected plasma cortisol in yearling striped bass. A 12-minute confinement stress increased cortisol levels in fish held at 16°C and higher. Cortisol levels were highest in fish held at 30°C. Cortisol did not change significantly in fish held at 10°C and a delayed increase at 6 and 24 hour of recovery occurred in fish held at 5°C. Cortisol recovered rapidly in fish held at 10° and 16°C. They suggested that acclimating and moving striped bass within this temperature range (10°-16°C) should decrease stress-related responses. Furthermore, fish acclimated to different temperatures responded to acute and chronic stress differently (Barton and Schreck 1987). It was demonstrated that cortisol peaks at 1 hour after a 30-second handling stress in juvenile chinook salmon (*Oncorhynchus tshawytscha*) acclimated to low (7.5°C), medium (12.5°C) or high (21°C) temperature. Also, plasma cortisol response to continuous confinement peaked in 6 hours in the high temperature group, as compared with 12 hours in the other 2 groups.

Besides increasing plasma epinephrine or cortisol, fish exposed to abrupt temperature change might develop AUR. However, acclimation temperature may affect AUR development after exposure to confinement stress in which fish acclimated in cold water should develop AUR less than those acclimated in high temperature.

Psychology

The psychological aspects of stress appear important in determining the severity of a stress (Schreck 1981). Some investigations have used an elimination of visual perception and trained conditioning to elucidate the psychological aspects of stress in fish (Woodward and Smith 1985; Young and Cech 1993). Exercise conditioning for 60 days significantly improved physiological responses to and recovery from handling stress (capture, net confinement and crowding) in cultured and wild young striped bass (Young and Cech 1993). Plasma cortisol increased within 0.5 hr after acute handling in both unexercised and exercise-conditioned fish. However, cortisol levels in exercise-conditioned striped bass returned to prestress levels in 4 hr after handling, but not in the unexercised fish. Cortisol levels in exercise-conditioned fish were significantly lower than those in the corresponding unexercised fish at 4 hr after handling. In addition, faster clearance of plasma lactate following handling stress was shown in exercise-conditioned fish, compared with unexercised fish. These experiments suggest that fish can be conditioned to avoid or reduce the stress response. Exercise conditioning may be useful in changing the perception of the stressor (handling) and improve the psychological condition of the fish. Woodward and Smith (1985) found that exercise-trained rainbow trout had lower resting levels of epinephrine, norepinephrine and cortisol, compared with non-exercised control fish during the last 3 weeks of a 6-week swimming training program. Initially, trained fish that were swimming had higher epinephrine levels than resting, trained fish. After 2 weeks of exercise, trained fish did not significantly elevate epinephrine levels during swimming. Presumably, familiarity with exercise training could reduce the stress response as fish become accustomed

to the training routine. The decrease of plasma epinephrine might reflect an adaptation to the stress.

Crowding

In intensive aquaculture, crowding can adversely affect health and physiological condition (Schreck 1981; Wedemeyer 1996). Crowding is often used to describe a high fish-loading density (weight of fish/unit of water). High fish density causes stress, reduced growth, disease problems such as fin erosion, and mortality (Ruane et al 2002; Wendelaar Bonga 1997). Crowding probably has a significant psychological component, but is a complex event that also may comprise several other stressors. Increased fish density causes increased biomass production, which can cause elevated ammonia and CO_2. Failure to remove dissolved CO_2 results first in hypercapnia and acidosis, then tissue hypoxia and eventually CO_2 narcosis and death. Failure to allow sufficient areal space (kg fish/ m^2 bottom surface area) caused fin erosion in Atlantic salmon (*Salmo salar*) and fin erosion included partial or complete loss of the dorsal, pectoral and pelvic fins in 8°-10°C water (Wedemeyer 1996). Crowding stress in fancy carp (*Cyprinus carpio L.*) increased plasma cortisol and glucose, and decreased non-specific immunity, such as phagocytosis, serum lysozyme and bactericidal complement activity (Yin et al 1995). However, there was no difference in resistance to *Aeromonas hydrophila* between day 7 and day 30. They suggested that the chronically stressed carp might have an adaptation to survive with a lower level of non-specific immune defense mechanisms. After Atlantic salmon were exposed to crowding stress, plasma epinephrine and cortisol increased significantly (Fløysand et al 1992; Mazur

and Iwama 1993). Besides the indirect effects of crowding described above, we suspect that crowding may directly affect the development of AUR.

THE ACUTE ULCERATION RESPONSE (AUR): AN EXTREME RESPONSE TO ACUTE STRESS

Noga et al (1998) found that striped bass (*Morone saxatilis*) and hybrid striped bass (*Morone saxatilis* female x *Morone chrysops* male) displayed epidermal ulceration on the fins after exposure to an acute, 2 hour, confinement stress. Striped bass and hybrids striped bass showed skin ulceration on all fins. The epidermal damage began to develop on the distal edge of the fins, progressing towards the base of the fins. Because we discovered that this ulceration response due to acute stress occurred not only on the fins, but also could affect the ventrum and the cornea (see Chapter II, p. 77), we have named this syndrome the Acute Ulceration Response (AUR).

The ulcerative lesions in striped bass appeared more severe than in hybrid striped bass. AUR lesions expanded rapidly with various degrees of epidermal erosion and ulceration. The epidermis had ballooned cells with nuclear debris, indicating epidermal degeneration. Ulceration presented with dermal and hypodermal edema and necrosis of the remaining stromal tissue and tips of bone in the fin rays. AUR-like lesions were induced by high doses of exogenous epinephrine (>1 μg epinephrine/kg body weight). The blood concentrations of epinephrine associated with acute stress in fish are within the range of 500-1500 nM (about 100-300 μg/kg) (Mazeaud and Mazeaud 1981). It may have been necessary to use high doses of epinephrine because the kinetics of tissue distribution and degradation of

administered epinephrine may have decreased the epinephrine levels in blood and tissues. Intraperitoneal epinephrine injection of unstressed fish induced lesions similar to AUR in hybrid striped bass, suggesting that these lesions are a physiological adrenergic response of the fish to an acute stress. It also suggests that epinephrine plays an important role in the development of AUR. It is unknown why AUR lesions are specific for the fin epidermis. A site-specific shutdown of peripheral vascular perfusion might lead to tissue hypoxia or the release of cytotoxins from acute inflammation (Noga et al 1998).

PROBING THE POSSIBLE HORMONAL BASIS FOR AUR

As it has been shown that CA (i.e., epinephrine) could induce lesions similar to AUR in hybrid striped bass (Noga et al 1998), stress hormones probably play an important role in the development of AUR. Thus, inhibition or suppression of the stress hormone response may prevent or reduce this damage. The experimental suppression of stress hormone release in Atlantic cod (*Gadus morhua)* and eel has been accomplished using surgery or pharmacological blockers (reviewed in Gamperl et al 1994a; Randall and Perry 1992; Wendelaar Bonga 1997). Sectioning of sympathetic nerves was used to suppress or reduce CAs (Bulter et al 1989). However, sectioning of the spinal nerves to the head kidney did not completely abolish the increase of plasma CA in Atlantic cod during 10 min of air-exposure (Wahlqvist and Nilsson 1980) or hypoxia (Perry et al 1991). They suggested that mechanisms other than neural stimulation of head kidney chromaffin tissues were contributing to the rise in plasma epinephrine, such as stimulation of adrenergic nerve terminals and direct stimulation of chromaffin cells by hypoxemia. Removal of interrenal

tissues in the head kidney and posterior cardinal veins suppressed cortisol release during stress (Olivereau and Olivereau 1991). However, the diffuse nature of the interrenal tissues makes complete surgical removal almost impossible (Pickering et al 1987). Furthermore, interrenalectomy also causes osmotic stress. Surgical removal of the pituitary gland suppresses interrenal activity, but also interferes with release of many other hormones (e.g., prolactin, growth hormone), making results often difficult to interpret.

A decrease of CA levels in the circulation can also be accomplished by inhibiting CA synthesis at the adrenergic nerves and chromaffin cells, or inhibiting the response of adrenoceptors by adrenergic antagonists. Diethyldithiocarbamate and amylxanthate reduced head kidney epinephrine and norepinephrine in rainbow trout by inhibiting dopamine β hydroxylase, one of the enzymes responsible for CA biosynthesis, but these drugs are cytotoxic (Nilsson and Block 1991). Opdyke et al (1983) found that hexamethonium partly inhibited CA secretion in exercised dogfish shark (*Squalus acanthias*). Hexamethonium, a non-depolarizing, nicotinic ganglionic blocking agent, selectively competes with acetylcholine, a neurotransmitter at sympathetic and parasympathetic nerve ganglia, for nicotinic receptors on the postsynaptic membrane. In Opdyke et al's (1983) investigation, infused hexamethonium (0.83 mg/kg/min, 10 min) reduced the epinephrine and norepinephrine peak, but failed to alter blood pressure response after exercise. As neural control of catecholamine secretion from chromaffin tissues involves both nicotinic and muscarinic cholinergic receptors in teleosts (Montpetit and Perry 1999), hexamethonium could probably reduce catecholamine levels after stress but not completely. Montpetit and

Perry (1999) also suggested that the circulation of dogfish was supported by a non-neurologically mediated adrenergic mechanism.

In fish, adrenergic agonists and antagonists have been used to investigate the types, specificity, and activity of adrenoceptors. Alpha- and beta-adrenergic antagonists (phentolamine, propranolol, respectively) and ganglionic antagonists (hexamethonium) can abolish the effects of epinephrine and/or reduce the release of catecholamines during stress in fish (Narnaware and Baker 1996; Opdyke et al 1983b; Peyraud-Waitzenegger 1979; Wahlqvist and Nilsson 1981). Peyraud-Waitzenegger (1979) studied the effect of epinephrine after blockade of α- and β-ARs (phentolamine, propranolol) in eel and found that 1 mg/kg of either propranolol or phentolamine effectively inhibited the hypertensive effect of epinephrine. However, it is important to realize that most adrenergic antagonists that are active on mammalian tissues are often not suitable for specific interactions with fish tissues. For example, yohimbine, an α_2-adrenergic antagonist in mammalian systems, antagonized both α_1- and α_2-ARs in fish *in situ* and *in vitro* (Fabbri et al 1999; Moon and Mommsen 1990), including β-adrenergic ligand-binding at high concentrations.

STRESS AND FISH DISEASE

Fish disease is one of the most important problems and challenges encountered by fish culturists. Disease reduces hatchery efficiency and production, which in turn, increases costs and reduces profits. Fish disease does not occur as a single event but is the end result of interactions of the pathogen, the host (fish) and the stressful environment (Figure 1.2). Even

if the pathogen is present, a disease outbreak will not occur unless the environment becomes too stressful for the fish (Pickering 1998; Plumb 1999; Sniezko 1974).

Fish in intensive culture are continuously affected by environmental fluctuations and management practices such as handling, crowding, transporting, fluctuating temperatures, and poor water quality (Wedemeyer 1996). All of these factors can impose considerable stress on the homeostatic mechanisms of fish, rendering them susceptible to a wide variety of pathogens. Parasites, water molds, viruses, and bacteria are all causes for concern to aquaculturists (Plumb 1999). Stress compromises the fish's natural defenses so that it cannot effectively protect itself from invading pathogens (Wendelaar Bonga 1997). A fish disease which may lead to death is a state of imbalance between the fish and its environment.

A disease treatment is an artificial way to slow down the invading pathogen so that the fish has time to defend itself with an immune response. Aquaculture practices should be designed to minimize stress on fish in order to decrease the occurrence of disease outbreaks. When disease outbreaks occur, the underlying cause of mortality should be identified, as well as underlying stress factors that may be compromising the natural survival mechanisms of the fish. Correction of stressors (i.e., poor water quality, excessive crowding, etc.) should precede or accompany disease treatments. Prevention of disease outbreaks is more rewarding and cost-effective than treatment of sick fish.

WATER MOLD INFECTION

Water molds or oomycetes are the most important group of fish fungal pathogens that affect salmonids and other teleosts. They are ubiquitous saprophytes in soil, freshwater and brackish water. Water molds were once thought to be true fungi, but they do not contain chitin in their cell walls as do true fungi. Their cell walls are mainly composed of β-1-3-glucans, a mixture of cellulosic compounds and glycan. Epithelial damage on the skin, gills, and gut, due to trauma or other pathogens, can provide a route of entry for oomycetes (Roberts 2001). Water mold infection is easily detected by identifying its broad, aseptate hyphae in the diseased tissue.

Classification of water molds

The most important water molds affecting fish are members of the Family *Saprolegniaceae*, with 3 important genera in aquaculture; *Saprolegnia*, *Aphanomyces*, and *Achlya*. Taxonomic classification of *Saprolegnia* is described in (Bruno and Wood 1999):

Kingdom: *Protoctista*

Division: *Oomycota*

Phylum: *Heterokonta*

Class: *Oomycotea*

Order: *Saprolegniales*

Family: *Saprolegniaceae*

Genus: *Saprolegnia*

Species: *Saprelegnia parasitica*, *S diclina* complex, e.t.c.

Saprolegniosis

Water molds cause a disease commonly called saprolegniosis, while their infection is termed saprolegniasis (Roberts 1989). Saprolegniosis affects all species and ages of freshwater fish, and also affects many estuarine fish (Noga 1993a; Pickering and Willoughby 1982). Water molds usually feed saprophytically on soil and dead organic matter, includingdead plants and animals. Oomycetes are normally considered opportunists (Neish 1977; Pickering and Willoughby 1982), but sometimes have been reported to be primary causes of disease since they can at times apparently infect undamaged skin of healthy fish (Pickering and Christie 1981). Water mold transmission does not require an intermediate host (Noga and Dykstra 1986; Singhal et al 1987). Saprolegniosis is clinically characterized by growth of cottony-appearing mycelia, particularly on the gills, body skin, and/or fins (Willoughby 1994). The newly formed cottony mass is white, but when hyphae trap sediment, algae or debris, they become red, brown or green. Saprolegniosis is commonly observed superficially in the epidermis, dermis and occasionally the superficial musculature, but rarely in the internal organs (Pickering and Willoughby 1982; Roberts 1978). Superficial skin or gill damage from water mold infection is often fatal, due to loss of osmoregulation.

Outbreaks of saprolegniosis are frequently associated with many kinds of stress, such as adverse water temperature, poor water quality, handling, or crowding (Bailey 1984; Copland and Willoughby 1982; Whisler 1996). Temperature is an important factor in the development of water mold infection. Most epidemics occur when temperatures are low. Winter saprolegniosis in channel catfish, *Ictalurus punctatus*, was associated with

immunosuppression induced from low temperature (Bly et al 1992, 1993, 1996). The susceptibility to water mold infection increases in stressed fish, since stress can induce skin damage and immunosuppression (Iger et al 1995; Pickering and Duston 1983). Hypercortisolemia can also increase the susceptibility to saprolegniosis in brown trout (Pickering and Duston 1983; Pickering and Pottinger 1985). *Saprolegnia* infection itself can also cause more stress since plasma cortisol is increased in infected fish (Pottinger and Day 1999). Increased susceptibility to saprolegniosis after damage to the epidermis has been shown in fish under experimental conditions (Howe et al 1998; Howe and Stehly 1998; Singhal et al 1987; Xu and Rogers 1991). In virtually all studies, the skin had to be injured experimentally by scraping, descaling or physical abrasion prior to exposing fish to the water mold zoospores, in order to induce an infection. Those experiments concluded that natural *Saprolegnia* infections occurred primarily in association with skin abrasions; therefore, wounded fish are much more susceptible to water mold infection.

Biology of water molds

Water molds have a complex life cycle which includes both sexual and asexual reproduction. Sexual stages appear in the form of antheridia (male) and oogonia (female) on the hyphae. Sexual reproduction occurs by contact of gametangia, resulting in the fusion of the sperm nucleus and the oosphere, and producing the oospore which forms mycelia. The oosphere is produced in the oogonium; sperm reaches them via antheridia branches.

Asexual reproduction of *Saprolegnia* occurs on modified hyphae, that produce asexual sporangia which release motile primary zoospores. Primary zoospores are active for

a short period (a few minutes) before they encyst, germinate and release a secondary zoospore. Secondary zoospores, an infective stage, are normally motile for a longer period of time than primary zoospores. Water molds are believed to be transmitted primarily via motile zoospores. Polyplanetism is a term that describes the repeated cycles of zoospore encystment and release, and allows secondary zoospores to make an attempt to locate a suitable substrate (Bruno and Wood 1999). Pathogenic *Saprolegnia* species often have bundles of long, hooked hairs (sometimes called boathooks), while saprophytic species often have short, single, hooked hairs or no hairs. These hairs are speculated to facilitate infection, to be used for buoyancy, or for host-water mold recognition (Hatai and Hoshiai 1993; Pickering and Willoughby 1982).

Different species of water molds cangerminate under different environment and nutrient levels (Willoughby 1985). Many pathogenic water molds (e.g., *S. parasitica*) can grow on dilute nutrient media such as mucus (Pickering et al 1982). Water molds infect eggs by adhesion and penetration of the egg membrane (Willoughby 1994). Dead eggs are first infected by zoospores carried by the water supply or attracted by chemotaxis (Smith et al 1985). The dilute nutrients from dead eggs may encourage the germination of zoospores and enhance the colonization of dead eggs.

Diagnosis and treatment of water molds

Diagnosis of water mold infection requires an examination of live, affected fish. Since water molds are ubiquitous, dead fish are rapidly colonized, resulting in misdiagnosis. Water molds are commonly opportunistic pathogens that can invade wounds caused by other

pathogens, such as bacteria and parasites; thus, the initiating cause should be carefully explored for proper diagnosis. Water mold infection is easily diagnosed by the presence of broad, aseptate hyphae in wet mounts. Histological diagnosis is based upon observing lesions containing broad, aseptate hyphae in tissue sections. Water mold hyphae are most seen easily with silver stains, such as Gomori's methenamine silver (GMS). The structure of asexual zoosporangium formation is used to identify oomycetes to genus (reviewed in Noga, 1993b). Identification of *Saprolegnia* isolates to species is difficult since fish-lesion isolates usually do not produce any sexual structures, which is required for identification to species (Hughes 1994; Wood et al 1988). DNA fingerprinting is now becoming an important method for identification of *Saprolegnia* isolates (Bangyeekhun et al 2003; Bangyeekhun et al 2001; Whisler 1996).

Water molds are very difficult to treat and legally approved therapeutic agents are limited; thus, preventing or limiting water mold outbreaks by reducing stress from husbandry practices (such as good water quality, avoidance of overcrowded, handling) is very important. Malachite green is a synthetic dye that has been used to treat water molds affecting fish. In water, malachite green exists in two forms, the dye ion which is water soluble and the non-ionic pseudo-base (commonly a carbinol) which is insoluble in water. The anti-microbial activity is from the colored cations of malachite green. Malachite green is more effective against water molds than formalin (Treves-Brown 2000). It has been used for prevention and treatment of water molds by immersion. However, malachite green is a mutagen and suspected carcinogen (Meyer and Jorgenson 1983); thus, many countries have banned on its use in food-fish production. Potassium permanganate, copper sulphate and

formalin are also used for treatment of water mold infection. Furthermore, salt dips are also used to treat water mold and helps to counteract the osmotic stress in the infected fish with damage skin.

HYPOTHESES AND RESEARCH OBJECTIVES

Fish skin covering the entire body and fins responds rapidly to stresses. Since fish skin directly contacts the environment, epidermal damage may occur via either direct contact with toxicants or as an indirect response to physiological changes. Epidermal damage, especially skin ulceration, is one of the most common biomarkers of polluted or stressful environments. Fin ulceration in striped bass and their hybrids was described as an acute stress response (Noga et al 1998). The hypotheses of this study were that:

- Morphologically distinct features can be identified in AUR lesions via light microscopy and ultrastructure

- Fish acclimated to high temperature or a large acclimation space are more susceptible to AUR

- Adrenergic modulation influences the development of AUR

- Other fish species are susceptible to confinement-induced AUR

- AUR increases the susceptibility of fish to microbial infections

The specific objectives of this project were:

1) To reproduce AUR in hybrid striped bass and determine the pathology of skin damage due to AUR by light and electron microscopy.

2) To determine whether AUR affects other fish species.

3) To determine the effect of acclimation space on risk of developing AUR.

4) To determine the effect of water temperature during stress and acclimation temperature on risk of developing AUR.

5) To determine the susceptibility of fish with AUR to bacteria and water mold infections

6) To determine the involvement of CAs in AUR pathogenesis by examining the effect of: an adrenergic agonist (epinephrine), adrenergic antogonists (phentolamine and propranolol) and a ganglionic blocker (hexamethonium) on AUR development.

Table 1.1. The effect of corticosteroid and catecholamine release during stress in fish.

Agent	Effect
Catecholamines (epinephrine and norepinephrine)	Increase cardiac output
	Positive inotropic and chronotropic effects
	Vasodilatation or vasoconstriction
	Increase blood glucose (stimulate hepatic glycogenolysis, gluconeogenesis, lipolysis)
	Increase blood lactic acid
	Inhibit insulin release
	Increase glucagon release
	Hemodilution in freshwater fish
	Hemoconcentraiton in marine fish
	Higher the GC production
Corticosteroids (Cortisol, cortisone)	Increase protein mobilization
	Increase blood glucose
	Increase blood fatty acid
	Immunosuppression
	Inhibit growth, reproduction

Table 1.2. Summary of the cholinergic receptor types in various animals.

Animal	Cholinergic receptors	Reference
Bovine	Nicotinic	Forsberg et al 1986
Canine	Muscarinic	Tobin et al 1992
Feline	Nicotinic and muscarinic	Ballesta et al 1989; Uceda et al 1992
Chicken	Muscarinic	Knight and Baker 1986; Ledbetter and Kirshner 1975
Hamster	Nicotinic	Liang and Perlman 1979
Porcine	Nicotinic and muscarinic	Xu et al 1991
Teleost	Nicotinic and muscarinic	Al-Kharrat et al 1997; Fritsche et al 1993; Reid and Perry 1994

Table 1.3: Summary of alpha- and beta-adrenoceptors: type and subtype characteristics in fish.

Adernoceptor	Tissues	Functions	Antagonists	References
α	Coelic and mesenteric artery (Red Irish Lord, *Hemilepidotus hemilepidotus*)	Increase gastrointestinal blood flow	PHE	Axelsson et al., 2000
	Liver: hepatocyte (Goldfish, *Carassius auratus*)	Increase cellular glucose, and acid secretion via Na^+/H^+ exchange,	PHE	Krumschnabel et al., 2001
	Heart (Perch, *Perca fluviatilis*)	Negative chronotropic effects	PHE	Tirri and Lehto, 1984
	Heart (Carp, *Cyprinus caprio*)	Negative chronotropic effects	PHE	Temma et al., 1989
	Tail: blood vessel (Atlantic cod, *Gadus morhua*)	Vasoconstriction	PHE	Wahlqvist and Nilsson, 1981
	Tail: blood vessel (Eel, *Anguilla anguilla*)	Vasoconstriction	PHE	Davie, 1981
	Gill: branchial vasculature (Atlantic cod)	Arterio-venous vascular constriction	PHE	Nilsson and Pettersson, 1981
	Gill: branchial artery (Rainbow trout)	Vasoconstiction	YOH	Wood, 1975

PHE = Phentolamine, PRO = Propranolol, PRA = Prazosin, YOH = Yohimbine

Table 1.3: Continued.

α	Eel, *in vivo*	Increase arterial Po_2	PHE	Peyraud-Waitzenegger, 1979
α_1	Blood vessel: dorsal aorta	Increase dorsal aortic pressure	PHE, PRA	Xu and Olson, 1993
	Liver (Rainbow trout)	Increase e? intracellular $[IP_3]$ and $[Ca^{2+}]$ which activates glycogenolysis	PRA	Fabbri et al., 1995
α_{1B}	Liver: hepatocyte (Catfish, *Ictalurus puntatus*)	Modulate $[Ca^{2+}]_i$ and phosphoinositide turnover	PHE, PRA, WB4101, Benoxathian, 5-methl-urapidil	Garcia-Sainz et al., 1995
α_2	Scale: melanocytes (Cuckoo wrasse, *Labrus ossifagus*)	Pigment (melanosome) aggregation, causing blanching	YOH	Grundstrom et al., 1985; Martensson and Anderson, 1997; Svensson et al., 1993
	Scale: melanocytes (Cuckoo wrasse, *Labrus ossifagus*)	Pigment (melanosome) aggregation, causing blanching	PRA,YOH	Andersson et al., 1984

PHE = Phentolamine, PRO = Propranolol, PRA = Prazosin, YOH = Yohimbine

Table 1.3: Continued.

α_2	Eye: melanophore (Flounder, *Pleuronectes americanicus*)	Pigment (melanosome) aggregation,	PHE, PRA, YOH	Mayo and Burton, 1998b
	Chromaffin cell (Rainbow trout)	Inhibit CA release	PHE (and used clonidine as agonist)	Montpetit and Perry, 2002
α_1, α_2	Skin (Flounder)	Pigment (melanosome) aggregation	PRA, YOH	Burton and Vokey, 2000
β	Eel, *Anguilla anguilla, in vivo*	Hyperventilation	PRO	Peyraud-Waitzenegger, 1979
	Heart (Rainbow trout; Chinook salmon, *Oncorhynchus tshawytscha*)	Positive innotropic and chronotropic effects	CGP-12177	Gamperl et al., 1998; Gamperl et al., 1994b
	Heart: atrium (Carp, *Cyprinus caprio*)	Positive chronotropic effects	PRO	Temma et al., 1989

PHE = Phentolamine, PRO = Propranolol, PRA = Prazosin, YOH = Yohimbine

Table 1.3: Continued.

β	Gill: branchial artery (Rainbow trout)	Vasodilatation	PRO, Dichloroisoproterenol	Wood, 1975
	Gill: branchial artery (Atlantic cod)	Vasodilatation, increase branchial venous flow	Sotalol	Sundin, 1995
β	Leukocyte (Goldfish, *Carrasius aurata*)	Neuro-immuno communication	CGP-12177, PRO, Dihydroalprenolol, Atenolol, Butoxamine	Jozefowski and Plytycz, 1998
	Red blood cell (Rainbow trout)	Increase blood oxygen transport	CGP	Gilmour et al., 1994; Reid et al., 1993
	Liver (Catfish, *Ictalurus melar*)	Increase enzyme adenylate cyclase	Alprenolol	Fabbri et al., 1992
			PRO	Ottolenghi et al., 1988
	Liver (Rainbow trout)	Increase blood glucose (via glycogenolysis and gluconeogenesis)	PRO	Weber and Shanghavi, 2000; Wright et al., 1989

PHE = Phentolamine, PRO = Propranolol, PRA = Prazosin, YOH = Yohimbine

Table 1.3: Continued.

β	Liver (Rainbow trout)	Increase enzyme adenylate cyclase and cAMP	CGP-12177	Fabbri et al., 1995
	Liver (Eel)		CGP-12177, PRO, Alprenolol, Butoxamine, Atenolol	Fabbri et al., 2001
	Melanophore (Flounder)	Melanosome dispersion	PRO	Mayo and Burton, 1998b
β_1	Coronary artery (Rainbow trout)	Vasodilatation	Atenolol	Small et al., 1990
β_1	Heart, ventriculum (Carp)	Positive innotropic and chronotropic effects	PHE, Atenolol, PRO, Resperpine, Practolol, Carteolol	Temma et al., 1986
β_2	Heart: atrium (Trout, *Salmo gairdnieri*; flounder, *P flesus*; shark, *Squalus acanthias*)	Positive innotropic and chronotropic effects	PHE, phenoxybenzamine, PRO, Practolol	Ask, 1983
	Chromaffin cell (Rainbow trout)	Inhibition CA release	Nadolol (and used salbutamol as agonist)	Montpetit and Perry, 2002

PHE = Phentolamine, PRO = Propranolol, PRA = Prazosin, YOH = Yohimbine

Table 1.3: Continued.

β_2	Liver (Rainbow trout)	Increase glucose via glycogenolysis and gluconeogenesis	PHE, PRO, Metoprolol	McKenna and Hazel, 1993
	Liver, white muscle, red blood cell (Rainbow trout)	-	(use RT-PCR analysis)	Nickerson et al., 2001

PHE = Phentolamine, PRO = Propranolol, PRA = Prazosin, YOH = Yohimbine

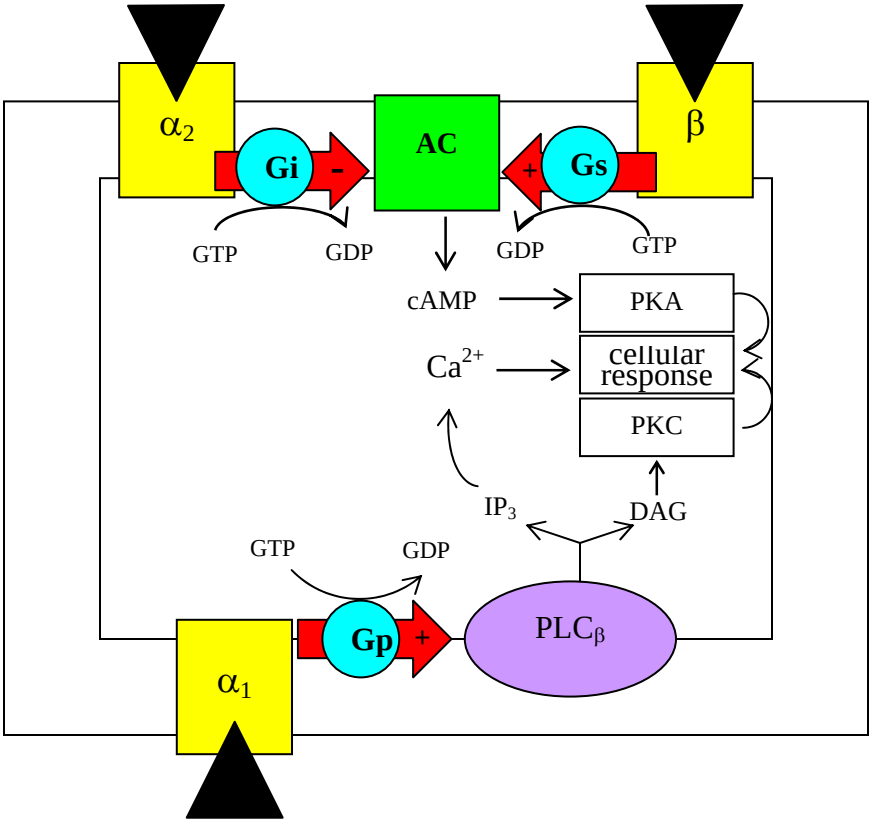

Figure 1.1: Generalized scheme for signal transduction of catecholamine (CA) binding to an α-and β- adrenergic receptor on a target cell. + and - refer to positive and negative effects, respectively. Abbreviations: G, G-protein; AC, adenylyl cyclase; cAMP, cyclic AMP; PLC_{β}, phospholipase β; IP_3, inositol 1,4,5 triphosphate; DAG, diacyl glyceraol; PKA and PKC, PKA enzymes. (from Fabbri et al 1998).

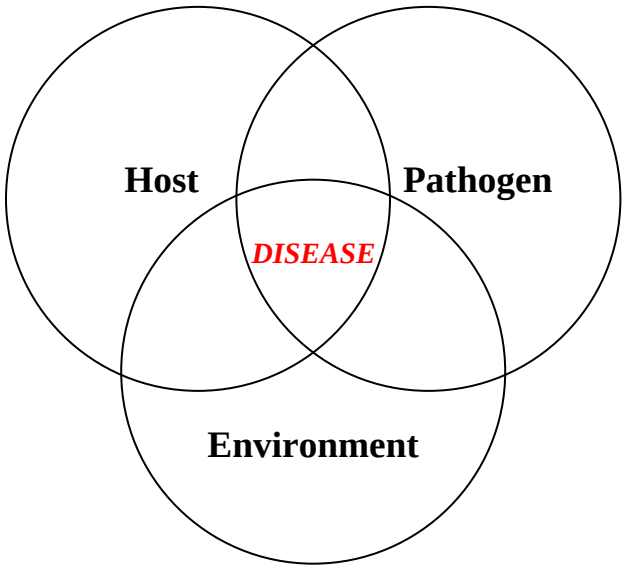

Figure 1.2: Disease occurs when hosts are exposed to pathogens under certain environmental (i.e., stress) conditions. (from Sniezko1974).

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II.

PATHOGENESIS OF THE ACUTE ULCERATION RESPONSE (AUR)

IN HYBRID STRIPED BASS

ABSTRACT

Previously, we discovered that acute confinement stress causes rapid ulceration of the fins of hybrid striped bass (*Morone chrysops* female X *Morone saxatilis* male). In this paper, we report the development of a reproducible model for studying this phenomenon in juvenile hybrid striped bass. We also determined how quickly ulceration could develop in acutely stressed fish and documented the sequential light microscopic and ultrastructural changes associated with this response. When hybrid striped bass were subjected to a standardized confinement protocol, the pathological response was extremely rapid (fin ulceration began to develop within 15 min of confinement). Grossly, the distal edges of the fins became ragged and blanched, and melanophores aggregated near the basement membrane and dermis after 15 min of confinement. Microscopically, the earliest detectable change in the fins, which occurred within 15 min of exposure to the acute confinement stress, was swelling and loss of microridges of the outermost epidermal cells; this was followed by epidermal erosion. After 30 min of stress, the epidermis at the distal edges of fins developed epidermal ulceration. At this time, both necrotic and apoptotic epidermal cells were present. The middle and basal epidermal layers were severely spongiotic and the dermis and hypodermis were edematous. At later time periods (up to 2 hrs), lesions were similar but increasingly more severe, progressing from the distal edge of the fin towards the base. The response to acute stress showed a significant correlation between confinement period and severity of the pathological changes (epidermal degeneration, epidermal ulceration and leukocyte infiltration). Also, we demonstrated that epidermal damage is not restricted to the fins but also affects the body skin

and eyes. The ventral area of the body and the corneal epithelium of stressed fish were ulcerated; however, skin on the head and operculum was not affected, suggesting a site-specific mode of damage. In stressed fish, epidermal ulceration was found in 67-97% of all the fins, 88% of skin on the ventrum, and 67% of the corneas, while control fish had only had very mild epidermal ulceration in the few fish that it was present on 5-10% of the fins, but none at the ventral skin or corneas. Due to the widespread damage to epidermal tissues of the body surface, we have named this reaction the Acute Ulceration Response (AUR). Our study indicates that acute confinement can rapidly cause significant damage to epidermal and ocular epithelium. AUR might be a primary cause of morbidity in acutely stressed fish.

INTRODUCTION

Hybrid striped bass (*Morone saxatilis* female X *M. chrysops* male) have great aquaculture potential and have become one of the fastest growing segments of U.S. aquaculture (Harrell and Webster 1997). The hybrid striped bass industry is now fifth in volume, and fourth in value, of all food fish in the U.S (Carlberg et al 2000). Hybrid striped bass demonstrate hybrid vigor, such as higher early growth rates, better survival and more resistance to disease than their parental species (Anderson et al 2001). However, they are very susceptible to stress, which is often followed by an infectious disease outbreak (Plumb 1997).

In intensive aquaculture, stress can be induced in many ways, including changes in the immediate physical environment (pH, temperature, water quality), animal interaction, and aquaculture practices (handling, transport and crowding). Acute confinement is one of the most common stressors in routine culture practices, such as grading, transport and vaccination. Acute

stressors such as confinement often lead to an infectious disease epidemic. In a previous study, we discovered that a major reason that hybrid striped bass might be susceptible to infections after confinement was because confinement for as little as 2 hr caused rapid sloughing of the epidermis on their fins (Noga et al 1998). As the epidermis is a major source of host defenses, such as immunoglobulins, lectins, complement-like proteins, lysozyme, and antimicrobial polypeptides (Robinette and Noga 2001; Yano 1996), this response could have serious effects on fish health.

In the present study, we closely examined the histological and ultrastructural changes associated with this acute ulceration response (AUR) and documented that AUR affects not only the fins but also skin on the body as well as the cornea.

MATERIALS AND METHODS

Experimental stress model

Hybrid striped bass (*Morone saxatilis* male X *M. chrysops* female), 6-18 months old and 70-110 mm total length, were maintained in a 760-liter holding tank at 14°C. Fish were fed a commercial feed at approximately 2% of their body weight daily and maintained on a 12-hour light:12-hour dark photoperiod. The low temperature and restricted feed provided a constant supply of small, similar-size fish. For experiments, fish were acclimated to a 1500-liter freshwater tank; over a 2-week period, the water temperature was gradually raised to 27°C. Food was withheld on the day of the experiment. In an initial experiment 4 unstressed fish were sacrificed with anesthetic overdose (200 mg/l tricaine buffered with 400 mg/l

sodium bicarbonate) as controls immediately after gentle removal with a soft net from the acclimating tank. Another 4 fish were gently removed individually without anesthesia and individually confined in plastic mesh boxes (size 3.5 x 14 x 12 cm). Boxes were agitated every 15 sec per min during 2 hr of confinement. Stressed fish were sacrificed with anesthetic overdose. In a separate time-course study, 8 fish were sacrificed with anesthetic overdose after 30, 60, 90, or 120-min of confinement. Ten control (unstressed) fish were immediately sacrificed after removal from the acclimating tank. In another study, to determine the minimum amount of time required for ulcers to develop, we exposed 4 fish to the same acute stress for 0, 1, 5, 10, 14, or 15 min. Fish were first examined for ulcers using fluorescein (see below) and then examined for skin ulcers. Water quality during all experiments was: dissolved oxygen 6.8-7.5 mg/l, temperature 27°C, pH 6.65-6.87, unionized ammonia <0.001 mg/l and nitrite <0.10 mg/l.

Localization of ulcers

Recently, we have developed a means of rapidly locating ulcers on fish (Noga and Udomkusonsri 2002). By staining fish with fluorescein, a nontoxic fluorescent dye, we can precisely identify even microscopic ulcers, which are not visible to the naked eye. In selected fish, we used fluorescein to localize skin ulcers. Briefly, fish were placed in a solution of 0.20 mg fluorescein (AK-Fluor®, 10% fluorescein sodium injection, 100 mg/ml, Akorn, Inc., Decatur, IL) per ml of water for 6 min, after which they were immediately rinsed with freshwater for 3 min. The fish were then euthanized with buffered tricaine and immediately examined under ultraviolet light (Mineralight model UVGL-58, Upland, CA)

for skin or ocular damage. Fluorescein-positive areas were fixed for histology and examined histologically for ulceration.

Histopathology

For light microscopy, eyes, fins, and skin tissues of the head, operculum and ventrum were fixed in 10% neutral buffered formalin, decalcified in 10% ethylenediaminetetraacetic acid (EDTA) in 0.1M phosphate buffer (pH 7.2), embedded in paraffin, and processed routinely. All sections were stained with hematoxylin and eosin (H&E) or with periodic acid Schiff (PAS). All tissues were evaluated blindly as described in (Noga et al 1998). Briefly, lesions were scored on a scale of 1-5, with (1) being minimal damage (1-20% of area affected by the lesion), (2) being mild damage (21-40% of area affected by the lesion), (3) being moderate damage (41-60% of area affected by the lesion), (4) being severe damage (61-80% of area affected by the lesion) and (5) being extremely severe/high damage (81-100% of area affected by the lesion). Eyes were not scored but rather simply examined for presence or absence of corneal ulceration.

All fin and skin tissues were oriented in the longitudinal plane and then evaluated for pathological changes, including epidermal degeneration, epidermal erosion, epidermal ulceration, and leucocyte infiltration. Eyes were cross-sectioned and examined for corneal ulceration. Epidermal degeneration was defined as swollen epidermal cells (intracellular edema) with pyknotic nuclei. Epidermal erosion was identified by the sloughing of the epidermal layers, but with the basement membrane still intact. Epidermal ulceration was noted as complete loss of all epidermal layers as well as the basement membrane. Leucocyte

infiltration was defined as the presence of a greater number of leukocytes in the epidermal and dermal layers than those present in unstressed controls.

Electron microscopy

For transmission electron microscopy (TEM), caudal fins were cut into small pieces (~1.0 x 2.0 mm) and fixed in McDowell's and Trump's fixative (4% formaldehyde and 1% glutaldehyde buffered in monobasic sodium phosphate, pH 7.2-7.4) for at least 1 hr. Fin tissues were then decalcified in 0.1M EDTA at 4°C for 1 week (Quilhac and Sire 1999). Tissues were then rinsed three times with 0.1M phosphate buffer (pH 7.2-7.4) for 15 min and post-fixed in 1% osmium tetroxide in the same buffer for 1 hr. Tissues were dehydrated in 50% ethanol for 15 min, 75% ethanol for 15 min, 95% ethanol for 15 min (twice), 100% ethanol for 30 min (twice), and 100% acetone for 30 min (twice). Tissues were infiltrated in 50% Spurr resin (1 part Spurr in 1 part acetone) for 30 min and 100% Spurr for 60 min (twice), embedded in fresh Spurr, and polymerized at 70°C for 8 hr. Semi-thin sections, 0.5 μm thick, were stained with 1% toluidine blue in 1% sodium borate. Ultrathin-sections, 90 nm thick, were post-stained with uranyl acetate and lead citrate for examination with a Philips 208S transmission electron microscope.

For scanning electron microscopy (SEM), caudal fins were fixed in McDowell's and Trump's fixative for at least 1 hr. Tissues were rinsed in 0.1M phosphate buffer for 30 min, dehydrated in a graded series of alcohols and dried in a CO_2 critical point dryer (Ladd Research Industries, Williston, VT). Tissues were mounted on stubs and sputter-coated. Observations were made with a Jeol JSM-35CF scanning electron microscope.

Statistical analyses

The exact unconditional test was used to analyze whether the proportion of epidermal damage in stressed fish was statistically greater than in control fish (Berger 1996). The test was performed for each type of epidermal damage and used the binomial one-sided model with Fisher's Exact-Boschloo as the test statistic with a 99.9% confidence interval. The one way ANOVA and Cochran-Mantel-Haenszel (CMH) test in SAS (Version 8, SAS Institute, Cary, NC) was used to analyze the time course of confinement stress at 0, 30, 60, 90 and 120 min. The one-way ANOVA tests for significant differences of AUR severity in stressed fish compared to the control. The purpose of the CMH test is to determine whether each type of epidermal damage for each fin, skin and corneal tissue is conditionally independent of the confinement period when adjusting for the control variable (the variation between replications) (Agresti 1996). We concluded that there was a linear association between treatment and epidermal damage when the p-value of the correlation statistic was < 0.05.

RESULTS

When hybrid striped bass were exposed to our acute confinement protocol, the pathological responses were highly reproducible. We have observed a similar response to this acute confinement protocol after replicating this procedure over 8 times.

Grossly visible lesions

Initially, fish responded to confinement by immediately becoming excited after being placed into the individual confinement boxes. Within 15 min, the body skin darkened and a

dark band appeared along the edge of the fins. Thereafter, the fish became fatigued with slowed opercular movements, and remained on the bottom of the confinement box. Extensive epidermal erosion and ulceration of all fins was clearly evident under stereomicroscopy after 15 min of stress. Mild fin splitting was detectable at the distal edges after 15 min of confinement and progressed intensely with time (Figures 2.1a-f, 2.2). After 30 min of confinement, the edge of each fin became pale and exposed lepidotrichia (fin rays) were grossly visible in severely damaged fins. With fluorescein treatment, an intense green fluorescence was detected on the fins after 15 min; later, both the ventral skin and cornea of the stressed fish also fluoresced (Figure 2.3). Epidermal ulceration was confirmed histologically in these fluorescein-positive areas. No fish died during the 2-hr experimental stress.

Histopathology

Normal epidermis and corneal epithelium

Like other fish, the skin of hybrid striped bass consists of two basic layers, the epidermis and dermis. A glycocalyx matrix usually covers the outer surface of the epidermis. Mucus cells, when present, stain positively with PAS. Fin epidermis consists of a nonkeratinized, stratified, squamous epithelium that is continuous with the epidermis of the body (Figures 2.4a-b). The outermost surface of the epidermal cells has microridges (Figure 2.4c) that are often arranged concentrically, forming maze-like patterns. Major structural components are desmosomes and tonofilaments (Figure 2.4d) that firmly adhere the cells to

each other. The basement membrane, lying between the epidermis and the dermis, is formed by densely packed fibrillar material and stains positive with PAS.

Like other teleosts, the dermis of hybrid striped bass fins has an outer stratum spongiosum and a reduced stratum compactum compared to the dermis on the body, which is composed of dense collagen fibers, elastic fibers, blood vessels and chromatophores (Groman 1982). Hybrid striped bass chromatophores include melanophores (containing melanin granules), xanthophores (containing pterin and carotenoid granules), and iridophores (with guanine or hypoxanthine platelets) (Groman 1982). The fins of hybrid striped bass, like those of other teleosts, are supported by lepidotrichia, parallel skeletal structures of dermal origin (Beccera et al 1983; Sharples and Evans 1996) (Figures 2.4a, b). The lepidotrichia are segmented along their length, with segments linked to each other by intersegmental joints of dense, fibrous connective tissue. Each lepidotrichium is composed of paired hemisegments, between which lies the intrasegmental region with nerves, blood vessels and loose connective tissue. Autophagocytic vesicles are present in the epidermal cells. Eosinophilic granular cells (mast cells), that stain metachromatically with toluidine blue, are distributed in the dermis and the basal layers of the epidermis. Leukocytes are sometimes present in the dermis and the basal epidermal layers of the fins.

The skin on the body of hybrid striped bass, like other teleosts, consists of epidermis, dermis and hypodermis (subcutaneous layer) (Groman 1982). Scales are anchored within the dermal scale pockets, which lie between the stratum spongiosum and the basement membrane and are covered with epidermis. Scales are absent on the head. The corneal

epithelium is contiguous with the skin (Groman 1982) and covers the substantia propria (corneal stroma). The iris extends under the cornea and is a continuation of the pigmented cell layers (choroidea) which supply oxygen to the retina and inner pigmented cell layers.

Quantification of Damage due to AUR

The incidence of different degrees of pathological changes in control and 2-hr stressed fish are quantitatively compared in Figures 2.5a-d. The most dramatic difference was epidermal ulceration, which was found in 67-97% of each fin type of stressed fish, while ulceration was only present in 0-10% of each fin type from control fish. In addition, the degree of ulceration in the unstressed fish was much less than in any of the stressed tissues. For example, 97% of the caudal fins from the stressed fish were ulcerated. These changes were significantly different compared to the unstressed control for all fins, ventral skin and cornea of the eyes ($p < 0.05$) (Figure 2.5c). Incidence of leukocyte infiltration was also considerably greater in fins of stressed fish, occurring in 52-78% of various fins, compared to 0-15 % of fins of unstressed fish; these changes were significantly different for all fins ($p < 0.05$). Changes in epidermal degeneration or erosion were less dramatic and in some cases were more common in control fish. For example, epidermal erosion was found in 0-40% of control fish and in 0-31% of stressed fish. The latter findings were not unexpected, as severely ulcerated fins would have relatively little erosion due to complete loss of the epithelium.

The severity of epidermal ulceration and leukocyte infiltration in all the fins of the stressed fish was also greater than in the fins of the control, unstressed fish (Figures 2.6a-d).

Changes in epidermal degeneration or erosion were less dramatic and in some cases were more common in control fish. In addition to the fin damage, significant ulceration was observed in the ventral skin and corneal epithelium of stressed fish (Figure 2.6c).

The incidence of various degrees of pathological changes in stressed fish examined in the time-course study was also much greater than in control fish (Figure 2.7a-d). However, the incidence of epidermal erosion did not increase since the stressed fish developed epidermal ulceration rather than erosion.

Statistical analysis of the mean severity scores of pathological changes showed a positive linear correlation between severity of pathological changes (epidermal degeneration, epidermal ulceration and leukocyte infiltration) and confinement period; thus, increasing confinement time lead to more severe AUR (Figures 2.8a-d).

Qualitative Changes due to AUR

Fish stressed for up to 14 min did not exhibit AUR, as evidenced by a negative response with the fluorescein test. This was further confirmed by histological examination of fins of fish stressed for 13, 14, or 15 min. Melanophores aggregated near the basement membrane and upper dermis of stressed fins, which caused fin blanching. The earliest microscopically detectable AUR lesions were observed at 15 min after initiation of the acute stress (Figures 2.9a-d), where there was swelling of the superficial epidermal cells, with affected cells at the distal edges of the fins appearing round with loss of microridges (Figures 2.9b, c, d). The outermost swollen epidermal cells were sloughed, and rounding epidermal

cell were attached to the adjacent cells by desmosomes (Figure 2.9d). There also had to be some ulceration. At 30-min of confinement, the swollen epidermis became erosive, and the epidermis beneath the erosive area was devoid of microridges (Figure 2.9a, b). Intercellular edema was present in the middle and basal layers of the epidermis. The basement membrane was still normal at the 30-min stress period. Some epidermal cells had detached desmosomes, large cytoplasmic vacuoles, nuclear shrinkage, loss of cytoplasmic organelles and swollen mitochondria.

Both necrotic and apoptotic cells were present in the affected areas after 30 min confinement (Figure 2.9c, d). Necrotic cells were characterized as swollen, with disruption of the cell membrane, increased electronlucency of the cytoplasm, and fragmentation of the nuclear chromatin. A large number of necrotic cells were found along the outer layers of epidermis. Apoptotic cells were characterized by cellular shrinkage and condensation of the cellular components (Figure 2.9c). Autophagocytic vesicles were also present in the stressed epidermis. After 60 min, epidermal ulceration became more prominent at the distal edges of the fins (Figure 2.10). After 90 min, the most severely damaged areas, at the distal tips of the fins, were depicted by severe epidermal erosion and ulceration (Figures 2.11, 2.12). Fin ulcers demonstrated complete loss of epidermis without an attached basement membrane; this was confirmed by PAS-staining and TEM. Apoptotic cells were focally scattered in the epidermis, while necrotic epidermal cells were present in much larger numbers, especially on the outer surface of the epidermis.

After 2 hr of stress, the hypodermis became edematous (Figure 2.13c). Most of the 2-hr stressed fish developed severe epidermal ulceration at the distal edge of the caudal fin ($p < 0.05$) (Figures 2.13a, b). The epidermis was normal toward the peduncle. In stressed fins, lymphocytes appeared in the basal layers of the eroded epidermis and in the ulcerated areas of dermis, as well as in the dermis. Lymphocytes had a typical appearance, being round with a high nuclear-to-cytoplasmic ratio and intensely basophilic nucleus surrounded by a homogenous, basophilic, cytoplasm. Neutrophils, round cells with an eccentric, indented nucleus, as well as eosinophilic granular cells, were also found in the dermis. While the epithelium on the head and the operculum appeared normal, the area above the anus was highly affected (Figure 2.14). Some stressed fish also developed corneal ulcers; in such cases, the substantia propria remained intact ($p < 0.05$) (Figure 2.15).

DISCUSSION

Distribution of AUR Lesions on the Body Surface

With the fluorescein technique (Noga and Udomkusonsri 2002), we could locate skin ulceration in stressed hybrid striped bass rapidly and accurately. In addition to readily documenting the extent of damage to all affected fins, this technique lead us to discover that certain areas of the skin on the body proper were also affected by AUR (Figure 2.3). In our preliminary study of AUR lesions (Noga et al 1998), we did not detect any damage on the body. However, we were limited by having to examine the skin by classical histological methods and did not happen to choose some sites that were affected by AUR.

In our present study, we clearly observed ulceration on a number of areas of the body in most fish exposed to acute confinement. The most severely affected area on the body appeared to be the ventrum just dorsal to the anus (Figure 2.14). Interestingly, this is the most common site of ulcers in Atlantic menhaden (*Brevoortia tyannus*) affected with Ulcerative Mycosis (UM) (Noga et al 1988). Ulcerative mycosis lesions are typically deep, aggressive ulcers with a prominent water mold (usually *Aphanomyces*) component. Speculation as to why UM lesions are so common in this region has included possible chemo-attraction of water molds to this area due to release of waste products from the anus (Noga et al 1988). However, if the menhaden epidermis at this site is more susceptible to stress-related damage, environmental factors (stressors) may be the major reason for this phenomenon.

Another dramatic response to acute stress that we detected with the fluorescein test was corneal damage. The corneal epithelium is very important for maintenance of corneal transparency and ocular osmotic balance. Removal of the corneal epithelium results in an increase of aqueous humor osmolality in marine fish and a decreased osmolality in freshwater fish; it also can cause corneal edema and cataracts within 24 hr (Ubels and Edelhauser 1987). Such sequelae can severely impair vision, affecting behavior and the ability to feed. In previous studies, we observed that striped bass (*Morone saxatilis*) and hybrid striped bass (*M saxatilis x M chrysops* and *M saxatilis x M americana*) developed corneal cloudiness (presumably edema) when subjected to a 45-min confinement (Noga et al 1994). Largemouth bass (*Micropterus salmoides*) transported under a high density (120g

fish/L water) also developed corneal cloudiness (Brandt and Jones 1986), although the tissues were never examined histologically in either of these studies to confirm the exact lesions present. Brandt and Jones (1986) speculated that the cause of this damage was abrasion of the corneal epithelium by contact with other fish, nets and the sides of tank under crowded conditions, but our findings indicate that such trauma is not needed to cause severe corneal damage.

In previous studies, we found that striped bass and hybrid striped bass (*M. saxatilis* female X *M. chrysops* male), 24 months and 310-390 mm total length, developed the most severe fin ulceration on the pectoral and caudal fins, followed by the dorsal fins, then the anal fins, after a 2-hr stress (Noga et al 1998). This is somewhat similar to our current findings using much smaller fish, where incidence of fin ulceration was greatest in the caudal fins, followed by the dorsal, pectoral, pelvic, and anal fins, in that order (Figure 2.5c). Sharples and Evan (1996) also found that goldfish, *Carassius auratus*, showed fin erosion and ulceration mostly on the pectoral and caudal fins after being chronically subjected to pulp mill effluent. The severity of damage on different fin types is probably affected by physiological differences relating to the microenvironment of the different fins, which in turn is probably influenced by fish species and other factors.

Skin Damage Caused by Stress: AUR vs. Other Reported Lesions

Pathological changes in fin and body skin have been reported in response to a number of stressors such as stress hormones, temperature change and polluted water (Table 1) (Bodammer 2000; Iger et al 1995; Iger et al 1994a, b, c, d). When rainbow trout

(*Oncorhynchus mykiss*) were fed a cortisol-treated diet, plasma cortisol levels increased and was associated with increased shedding of epidermal cells, pigment dispersion, and increased apoptosis of lymphocytes, mucous cells and epidermal cells (Iger et al 1995). Water temperature can affect epidermal thickness and the size and number of mucous cells. Rainbow trout exposed to temperature elevated from 15° to 22°C had decreased skin thickness within 3 hr (Iger et al 1994b). After 24 hr, skin thickness was restored and continued to increase by day 4. Both necrotic and apoptotic cells appeared in the stressed epidermis. When minnows (*Phoxinus phoxinus* L.) were exposed to high temperature (22°C), skin thickness and the number of mucous cells decreased, but club cell numbers increased (Fantin et al 1984). In contrast, when minnows were exposed to low temperature (12°C), the skin increased in thickness, the size and number of mucous cells increased, and the club cells were hypertrophied. Rainbow trout had significantly decreased epidermal thickness after exposure to polluted Rhine water for 7 d (Iger et al 1994a) while he epidermis was hyperplastic after 24 days of exposure. The most severe lesion observed was erosion; however, in none of the above studies was skin ulceration reported.

Epidermal erosion and ulceration has also been observed in goldfish chronically exposed to bleached mill effluent (Lindesjöö and Thulin 1994; Sharples and Evans 1996). Goldfish presented with dermal hyperemia, necrosis and edema in the chronic fin lesions. Melanophores and lymphocytes were also found in the erosive fins. They suggested that a vascular change in the dermis possibly affected blood supply and caused ischemia, leading to degenerative changes in the epidermis, and that the spongiosis probably resulted from an

inflammatory response in the chronically stressed skin (Lindesjöö and Thulin 1994; Murchelano 1975; Roberts 1989).

In contrast to all of the above studies, where skin damage was relatively mild (nonulcerated) or required long periods (at least days) to develop, the Acute Ulceration Response (AUR) causes extremely rapid and very severe damage, and affects all body surfaces, including the fins, body proper, and eyes. Ulcerative lesions were detectable within 15 min of the acute stress (Figure 2.9), which is an incredibly short time for such lesions to develop. The rapidity of this response has important implications for the risk of fish becoming sick after an acute stress. Apparently, even an extremely transient stress can lead to skin ulceration. Thus, even very brief stresses such as confining, transporting or handling a fish for only 15 min has the potential to lead to AUR; furthermore, we have evidence that AUR dramatically increases the susceptibility of fish to some infectious diseases (P Udomkusonsri and E Noga, unpublished data).

Possible Mechanisms Responsible for AUR

The amazing speed with which AUR developed in experimentally stressed fish suggests that a very severe perturbation of homeostasis is needed for this response. The earliest detectable lesion in affected fish was swelling and sloughing of the outer epidermal layer (Figures 2.9c, d). The cytoplasm of necrotic cells at the outer epidermal layer appeared transparent (Figures 2.10c, d). This typically results from the inability of the cells to control the influx of water and from outflow of cellular proteins (Frenkel et al 1999; Wyllie et al

1980). While both necrosis and apoptosis appeared to play a role in epidermal loss with AUR, necrosis was by far more common.

Necrosis is a cellular response to an extensive trauma and triggers the inflammatory response in damaged tissue, while apoptosis is a noninflammatory, programmed cell death that is associated to embryogenesis, metamorphosis, and normal cell turnover (Wyllie et al 1980). Necrosis typically occurs in response to toxins, hypoxia, or ischemia and affects cells in groups rather than singly (Anilkumar et al 1992; Wyllie et al 1980). Apoptosis is controlled by genetic and normal physiological stimuli, such as endocrine changes (e.g., cortisol, ACTH) (Iger et al 1995; Iger et al 1992; Wyllie 1997) and toxic agents such as radiation or chemotherapeutic agents (Anilkumar et al 1992). We observed apoptosis in scattered, single cells in the epidermis, while necrotic epidermal cells were found in much larger numbers, especially on the outer surface of the epidermis. These data suggest that AUR is mainly a necrotic event.

Apoptotic bodies are usually phagocytosed and digested by resident cells (Anilkumar et al 1992). Iger et al (1994d) reported that autophagocytic vesicles having apoptotic cells, that appeared inside the epidermal cells of both unstressed and stressed fish; were eliminated by the epidermal cells. Thus, the autophagocytic vesicles in the epidermal cells in our study support or confirm the occurrence of apoptosis in both unstressed and stressed fish. Apoptotic cells in control fish indicate the normal turnover of epidermis.

In our study, leukocytes were found in the basal layer of the epidermis and close to the basement membrane in some control, unstressed fish. We used the term “leukocyte”

since we found 2 types of leukocytes, mainly lymphocytes, but also neutrophils, in the epidermis. In stressed fins, we found leukocytes not only close to the basement membrane, but also in the upper layers of the dermis. Lymphocyte infiltration probably represented a non-specific immune response to the acute stress (Iger et al 1994b); however, the mechanisms regulating this lymphocyte infiltration are unknown. It is possible that the necrotic cells in stressed fish may release their cytoplasmic contents into the intercellular space, signalling the migration of inflammatory cells into the affected areas. Although there is no information on whether fish leukocytes can infiltrate into the epidermis of skin within 2 hr, it is possible that those leukocytes migrated from the dermis to the basal layer of the epidermis. The appearance of melanophore aggregation in epidermis was reported and may be a response to stress (Iger et al 1994d). This phenomenon may represent an additional defense mechanism since melanin pigments have bactericidal activity (Ellis 1977).

During stress, epinephrine is released from chromaffin cells and causes physiological changes to help the fish survive in a stressful environment (Wendelaar Bonga 1997). Epinephrine affects vascular resistance by acting on alpha-adrenoceptors, causing vasoconstriction. Epinephrine causes vasoconstriction in the tail fin of eel (*Anguilla australis*) and Atlantic cod (*Gadus morhua*) (Davie 1981; Wahlqvist and Nilsson 1981). Also, hybrid striped bass injected with epinephrine develop a mild form of AUR (Noga et al 1998), suggesting that the adrenergic response may play a role in AUR. We previously suggested that peripheral blood vessels may constrict and then affected tissues became hypoxia even they were in adequate oxygenated water (Noga et al 1998). Klontz (1995) also

suggested ischemia induced tissue necrosis in AUR-liked lesion after transport. Since the epidermis is usually avascular (Ferguson 1989; Roberts 2001), any vascular disturbances must be due to pathological changes in the dermis.

It is possible that when hybrid striped bass are acutely stressed, the peripheral vasculature is constricted by the effect of epinephrine, causing the epidermis to become hypoxic or ischemic, resulting in degenerative changes in the epithelial tissues, including the corneal epithelium. Skin ulcers have been found to be one of the most useful biomarkers of polluted or stressful environments (Bernet et al 1999; Noga 2000; Sindermann 1990). Since the skin of fish covers the entire body and fins, and is metabolically active and rapidly responds to stressors (Iger et al 1994a; Whitear 1986), this epidermal damage may occur via either direct contact with toxicants or indirectly due to physiological changes. Furthermore, the skin of hybrid striped bass and other fish serves a primary defense mechanism that contains specific and nonspecific defense factors, such as immunoglobulins, lectins, complement-like proteins, lysozyme and antimicrobial polypeptides (Robinette and Noga 2001; Yano 1996). After fish lose their protective skin barrier, opportunistic bacteria (e.g., *Aeromonas*, *Pseudomonas*), parasites, viruses and water molds (*Saprolegnia)* can invade the skin and cause infection (Plumb 1997). Skin loss also causes a serious osmotic stress due to loss of the protective barrier. Thus, AUR might predispose fish to many explosive epidemics of opportunistic skin pathogens, which can cause morbidity and mortality in acutely stressed fish.

Table 2.1. Stressors linked to ultrastructural changes in fish skin.

Stressor	Fish Species	Stress Period	Skin Lesion Reported	Reference
Hypercortisolemia	Rainbow trout (*Oncorhynchus mykiss*)	3.5 hr – 7 days	Apoptosis of mucous and epidermal cells; shedding of epidermis; lymphocyte infiltration; melanosome migration	Iger et al 1995
Hyperthermia	Rainbow trout	3 hours	Decreased the skin thickness; apoptotic and necrotic epidermal cells; lymphocyte infiltration and migration of macrophage in both epidermis and dermis; hyperplasia of skin later.	Iger et al 1994a
Hyperthermia	Minnow (*Phoxinus phoxinus*)	3-6 months	Decreased the skin thickness and mucous cells number; increased club cells number.	Fantin et al 1984
Hypothermia	Minnow	3-6 months	Increased the size and number of mucous cells, and the skin thickness; hypertrophy of club cells.	Fantin et al 1984
Polluted water	Rainbow trout	4 hr – 24 days	Decreased skin thickness and later skin hyperplasia; apoptotic and necrotic epidermal cells; lymphocyte infiltration in the epidermis and the dermis.	Iger et al 1994b
Pulp mill effluents	Goldfish (*Carassius auratus*)	Chronic	Necrosis of epidermis; disrupted basement membrane; edema, necrosis and hyperemia of dermis	Lindessjoo and Thulin 1994
Pulp mill effluents	Goldfish	Chronic	Loss of the cuticle; necrosis and sloughing of epidermis; epidermal and dermal edema	Sharples and Evans 1996

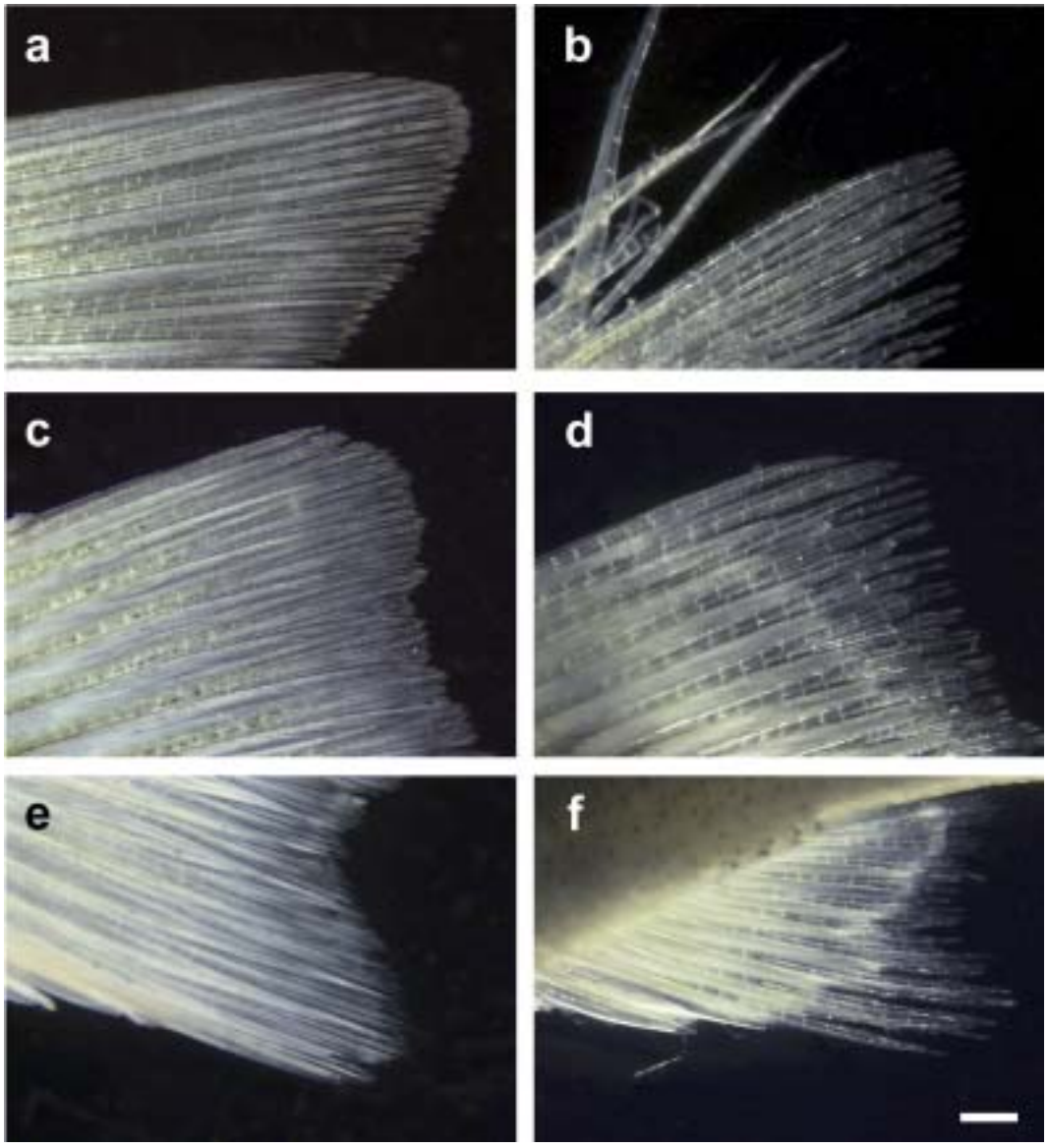


Figure 2.1. Hybrid striped bass fins of control and 2-hr stressed fish under a stereomicroscope. Stressed fins were ragged and split at the distal edges. a) Control caudal fin. b) Stressed caudal fin. c) Control dorsal fin. d) Stressed dorsal fin. e) Control anal fin. f) Stressed anal fin. Bar = 0.227 cm.

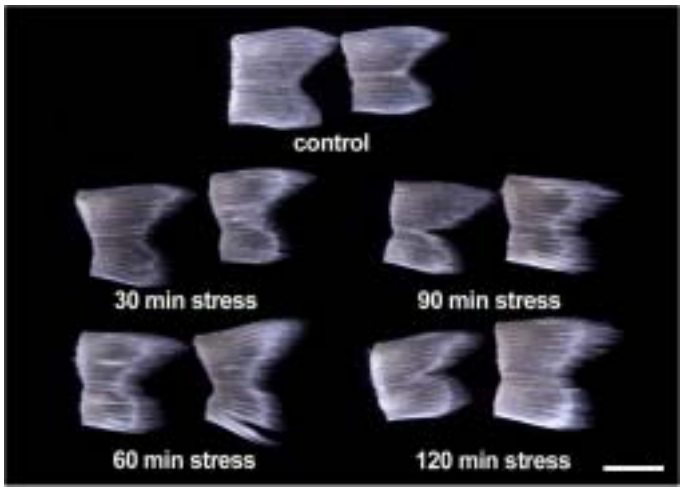


Figure 2.2. Caudal fins of hybrid striped bass showing time-course response of the acute ulceration response (AUR) after 0 (control), 30, 60, 90 and 120 min of confinement, where fin tissue showed blanching and was ragged at its distal edge. Epidermal erosion and ulceration developed in all fin tissues, which was confirmed by histology. Bar = 1 cm.

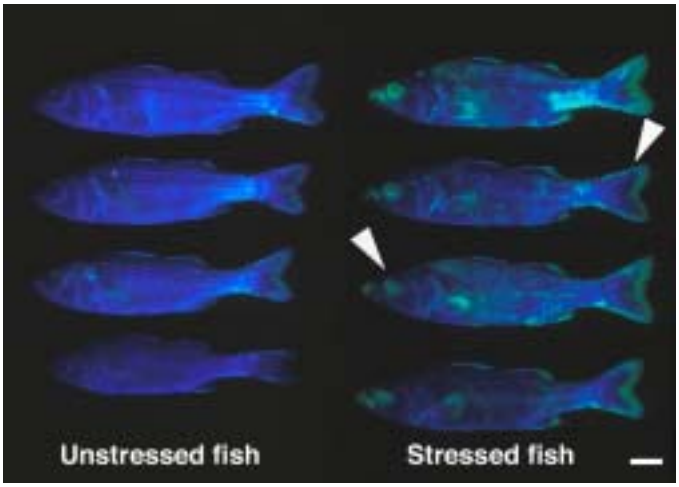


Figure 2.3. Hybrid striped bass treated with fluorescein and photographed under short wavelength ultraviolet light with no filter. Column Left: Control (unstressed) fish. Column Right: Fish stressed for 120 min. Note the ulcers on the fins, cornea and ventrum (visible as intense green fluorescence). Bar = 1 cm.

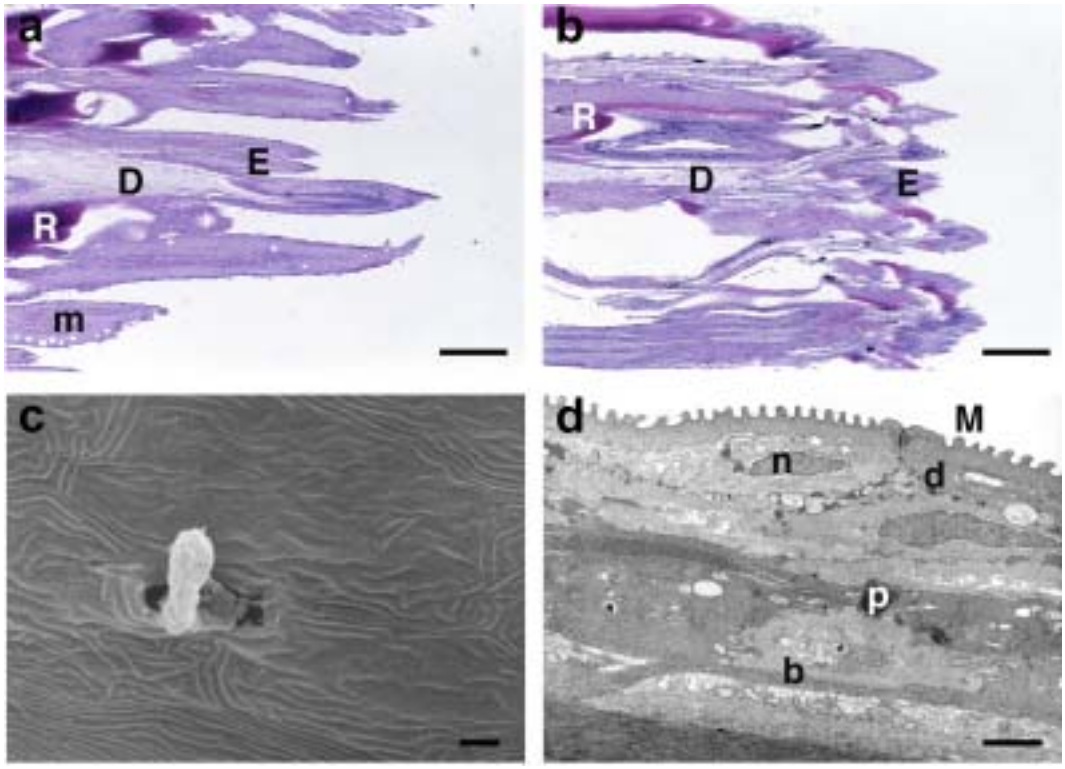


Figure 2.4. Control, unstressed fins of hybrid striped bass. E epidermis; D dermis; R fin ray; m mucous cells; M microridges; d desmosomes; b basement membrane; n nucleus of epidermal cell; p autophagocytic vesicle. a) Normal caudal fin. H&E. Bar = 200 μm. b) Normal anal fin. H&E. Bar = 200 μm. c) SEM of normal caudal fin showing microridges on the surface of epidermis and opening of mucous cell which is releasing the mucus secretion. Bar = 1.5 μm. d) TEM of epidermis of control caudal fin showing outer epidermis with numerous microridges. Bar = 3 μm.

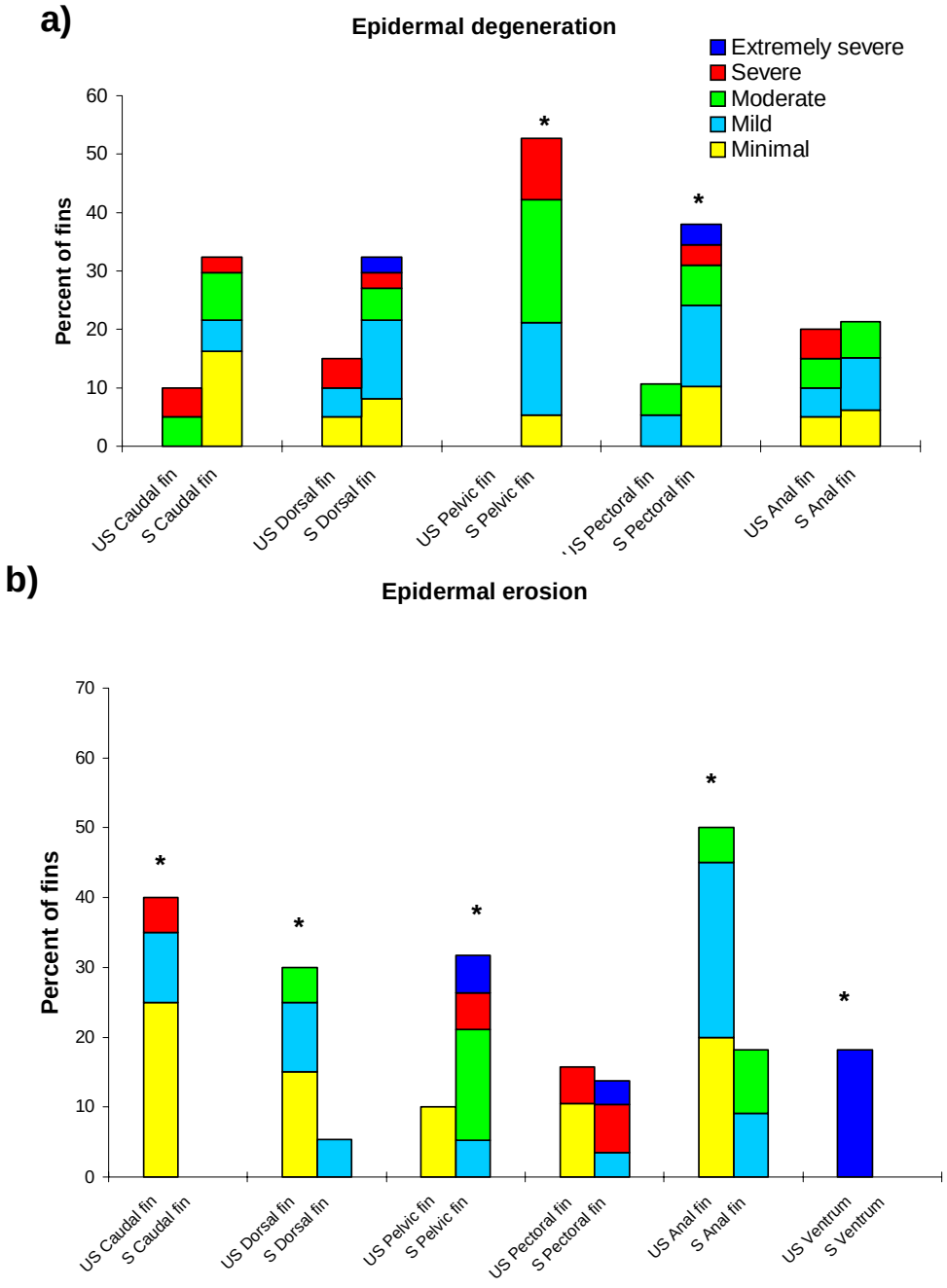
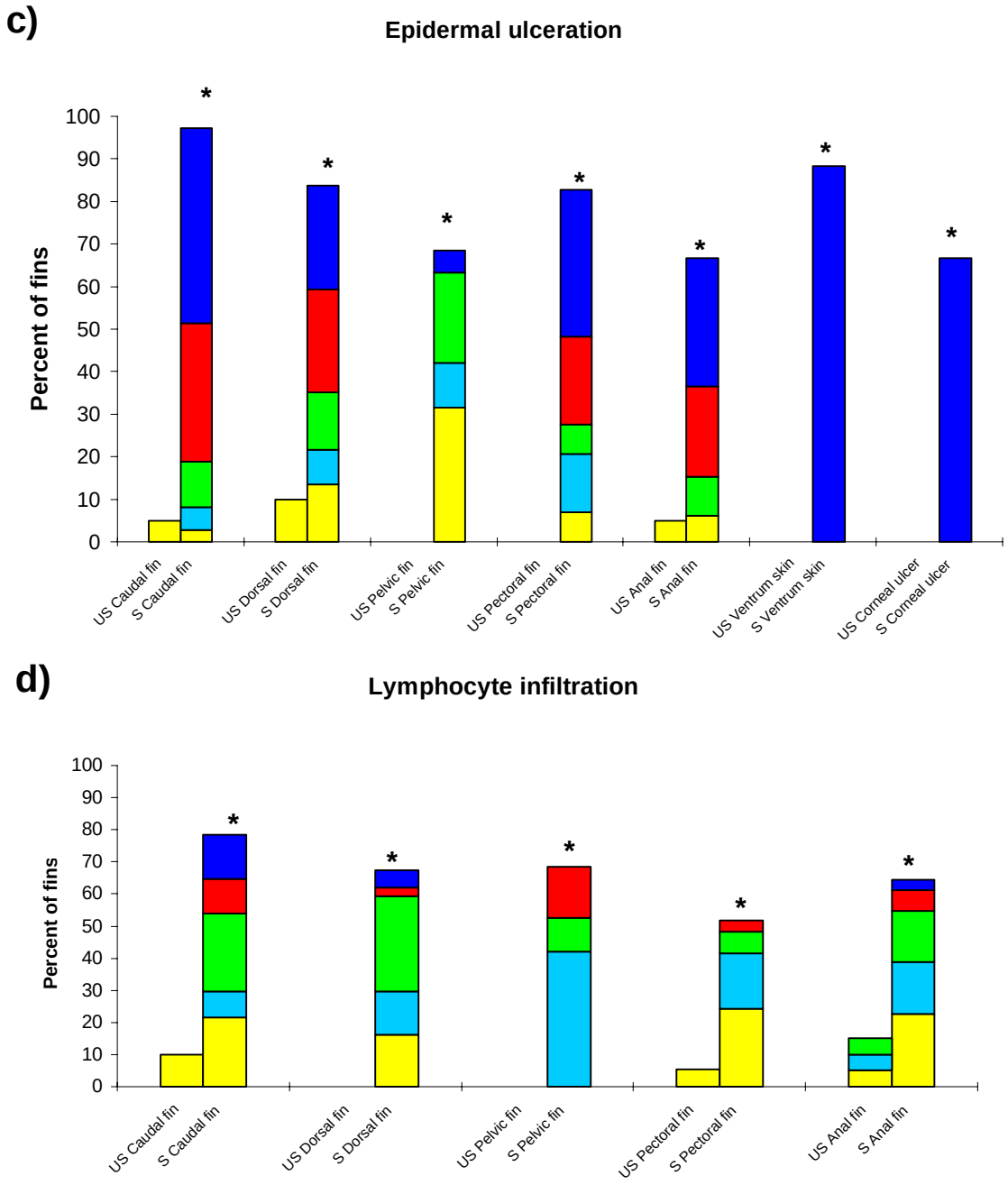


Figure 2.5. Incidence of various degrees of epidermal degeneration, epidermal erosion, epidermal ulceration, and leukocyte infiltration on each fin type of control and 2-hr stressed hybrid striped bass. * indicates that the mean incidence of epidermal damage was significantly greater in stressed than unstressed fish as determined by Fisher's Exact-Boschloo test, $p < 0.05$. $N = 32$.



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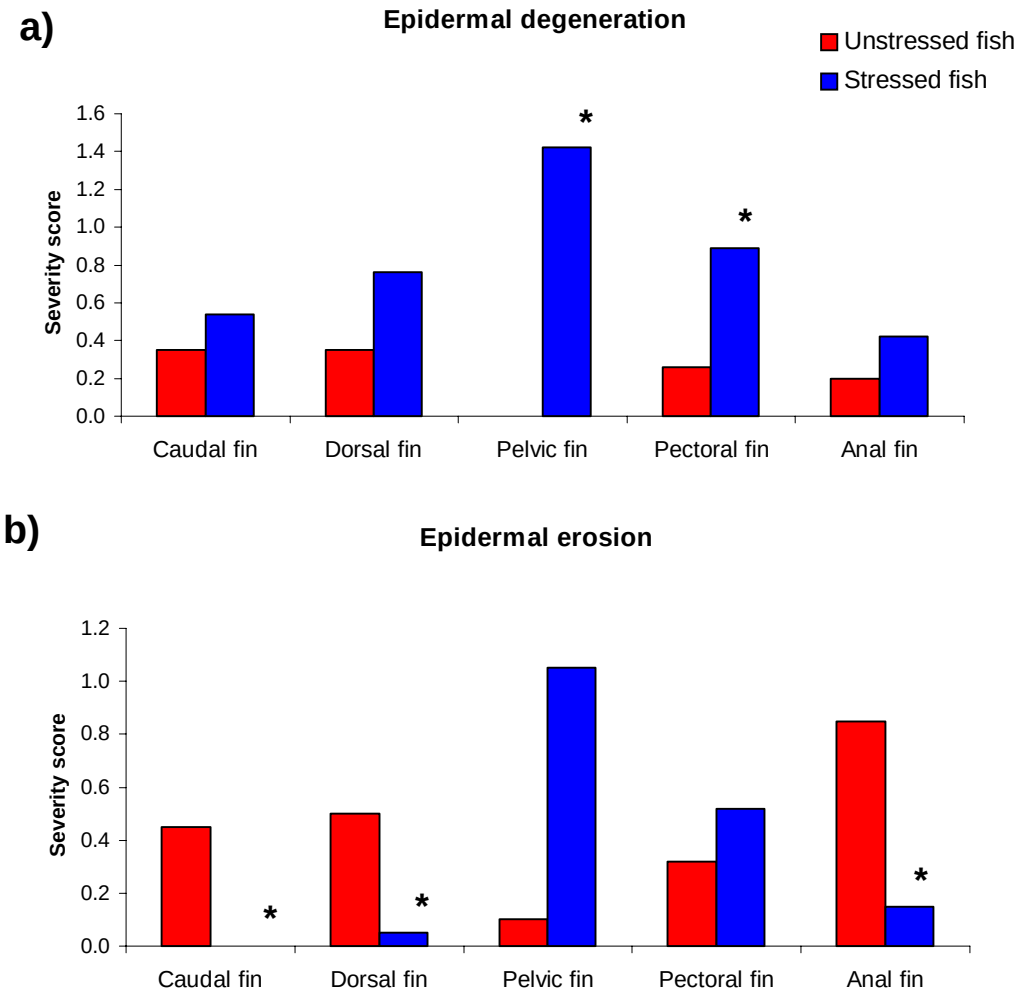
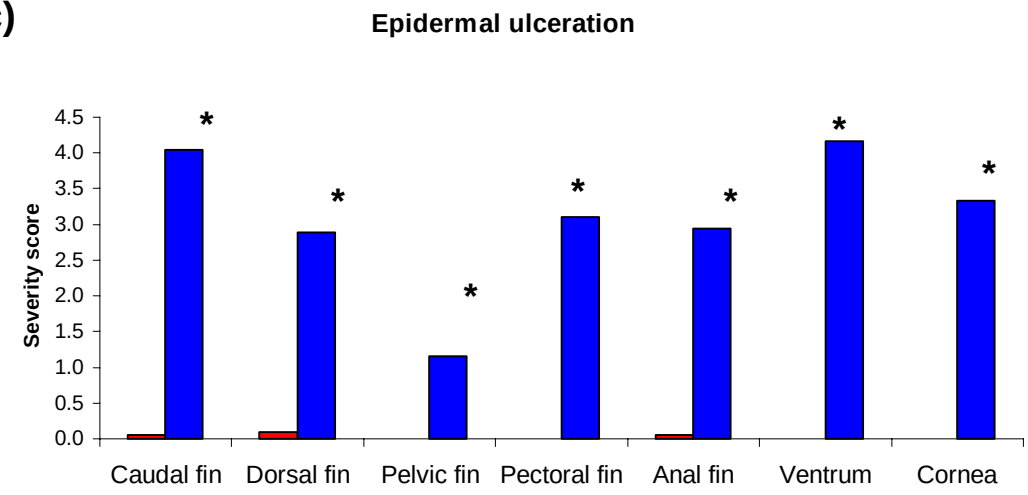


Figure 2.6. Mean severity score of epidermal degeneration, epidermal erosion and leukocyte infiltration on each fin type, and epidermal ulceration on the fins, ventrum and cornea, of control and 2 hr stressed hybrid striped bass. * indicates that there was a significant difference between the fin damage of stressed fish and control, unstressed fish as determined by ANOVA, $p < 0.05$. There were 8 replications and a total of 32 fish for each group. Data for all 8 experiments were combined for the analysis.

c)



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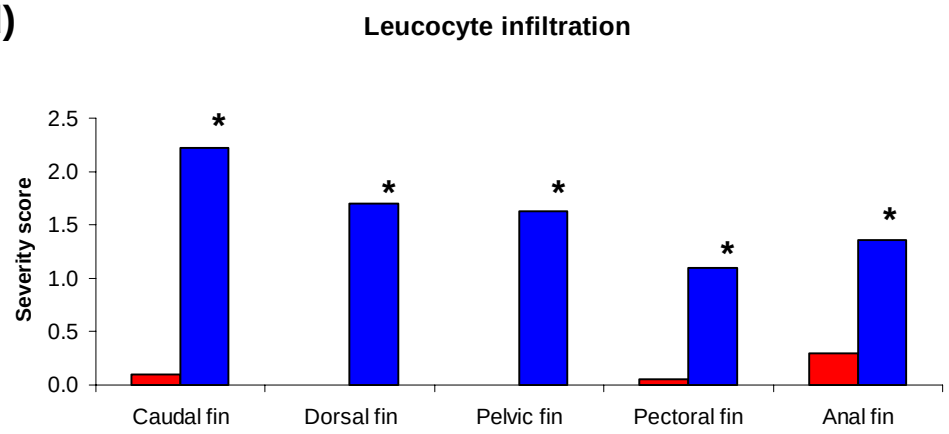

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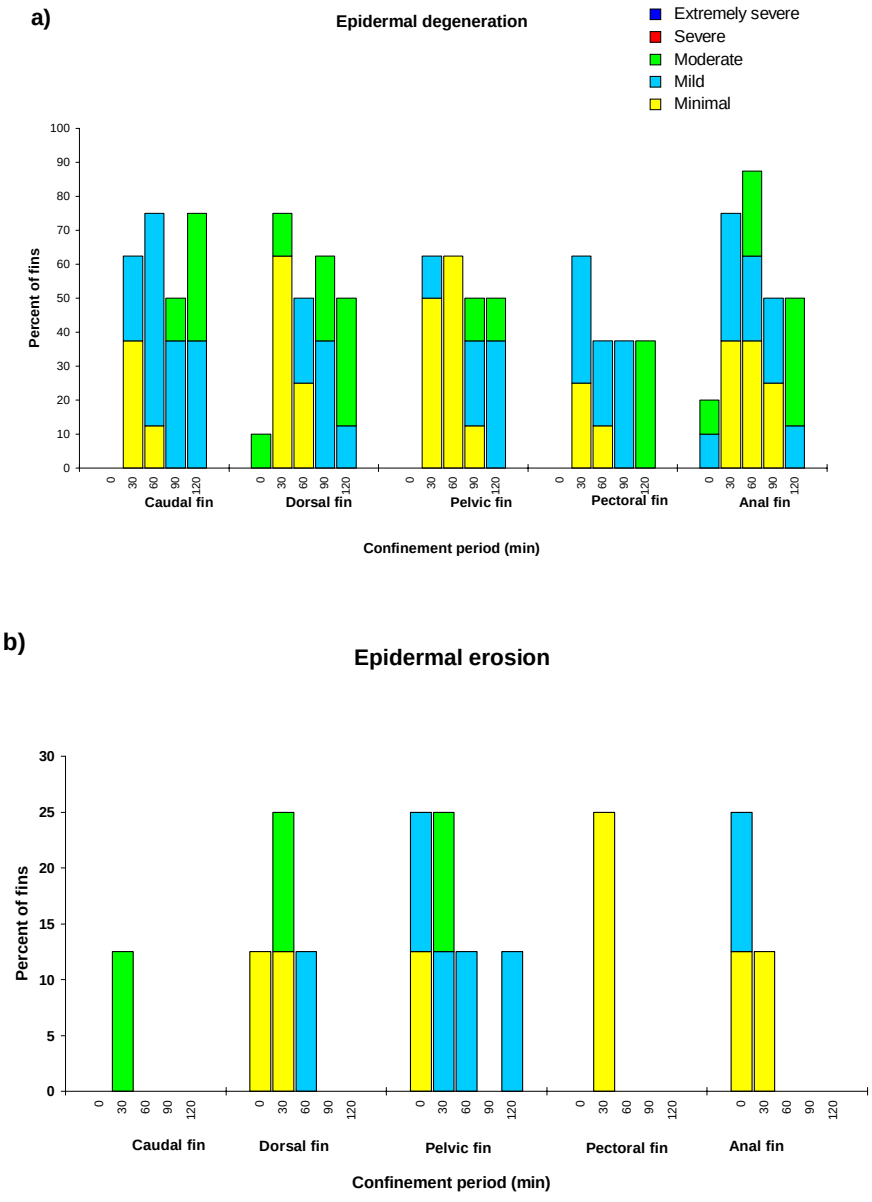
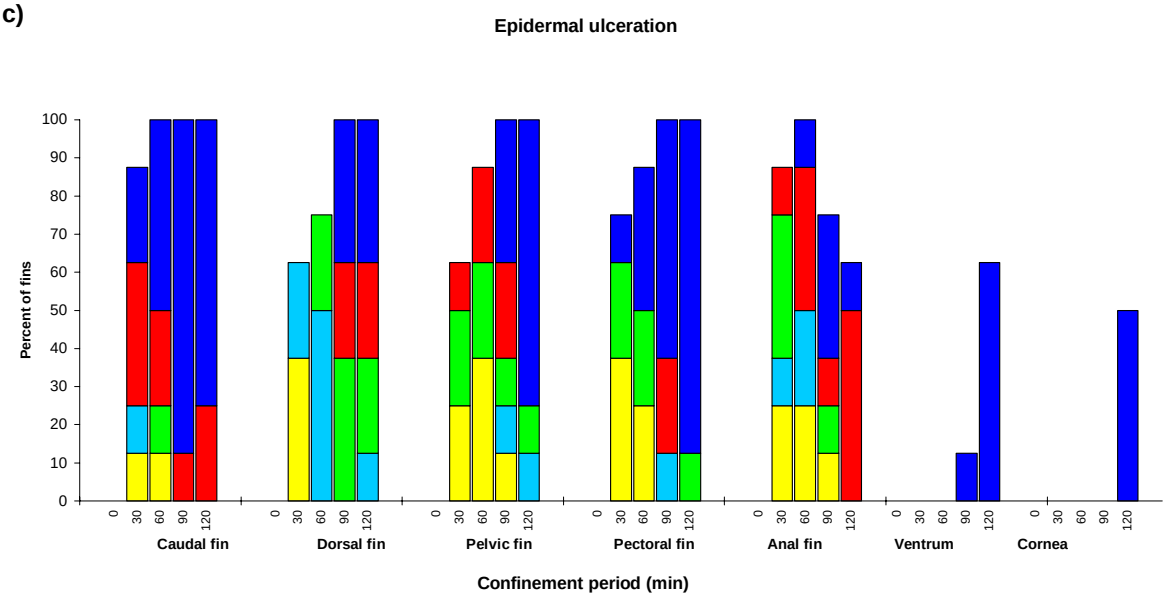


Figure 2.7. The incidence of epithelial degeneration, epithelial erosion, epithelial ulceration, and leukocyte infiltration on each fin type of control hybrid striped bass fish and fish stressed for 30, 60, 90 or 120 min. * indicates that the pathological change in the fin of the stressed fish was significantly different than in the same fin of the unstressed fish, as determined by ANOVA ($p<0.05$). There were 4 replications and a total of 8 fish for each group. Data for all 4 experiments were combined for the analysis.

c)



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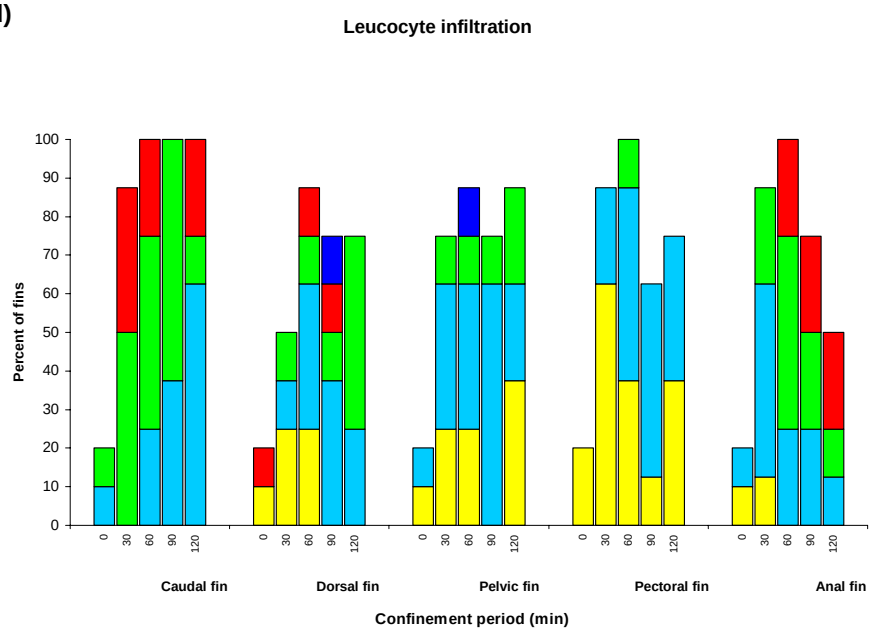

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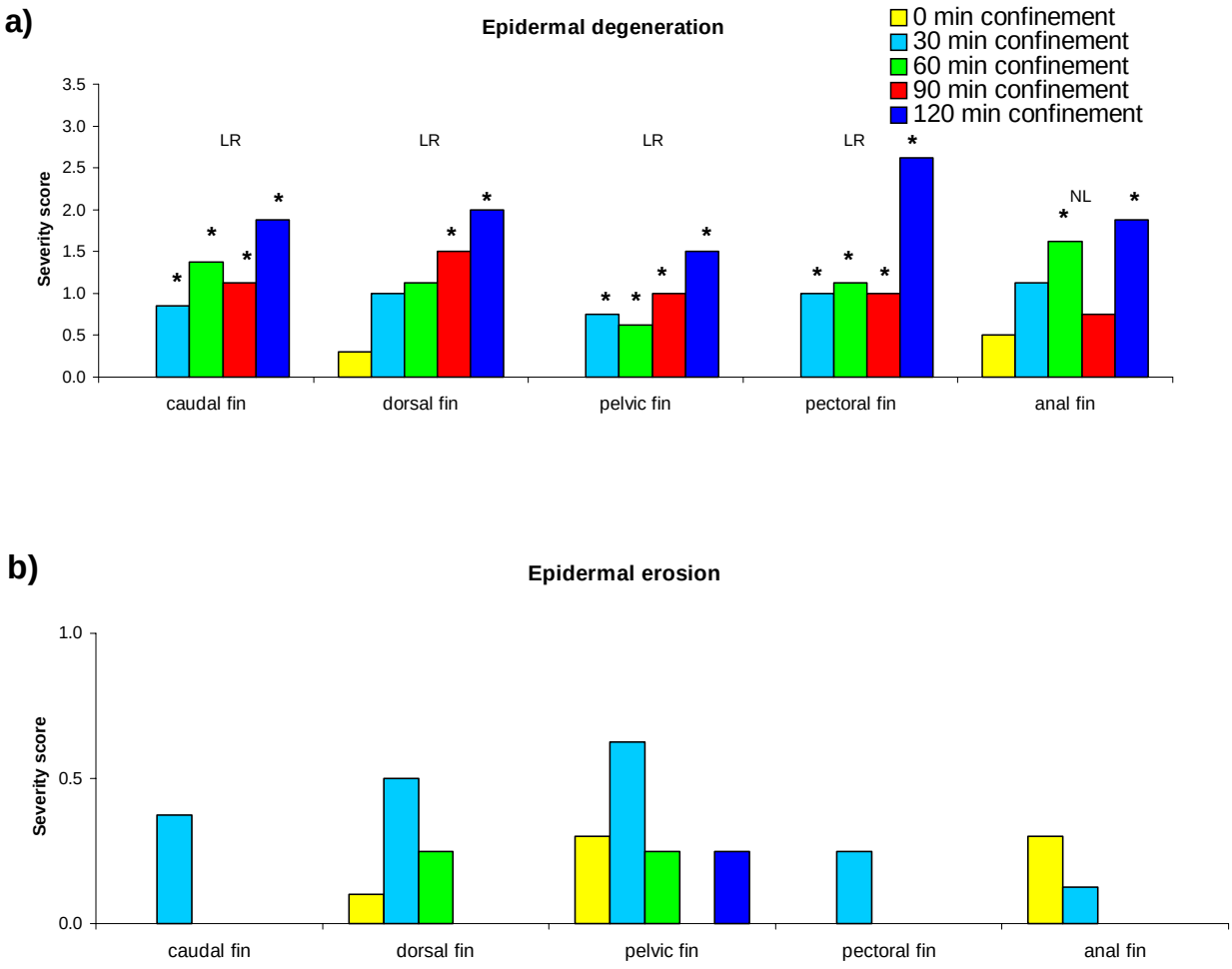
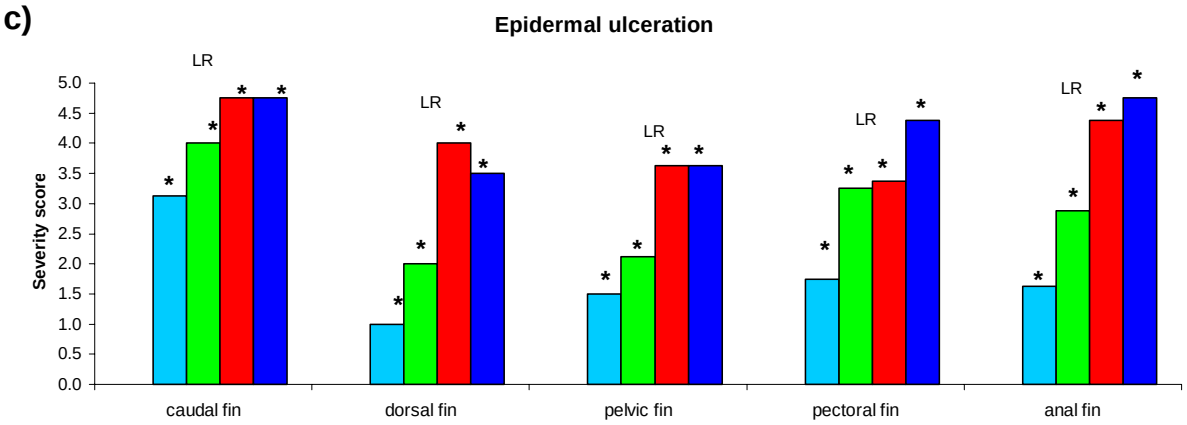
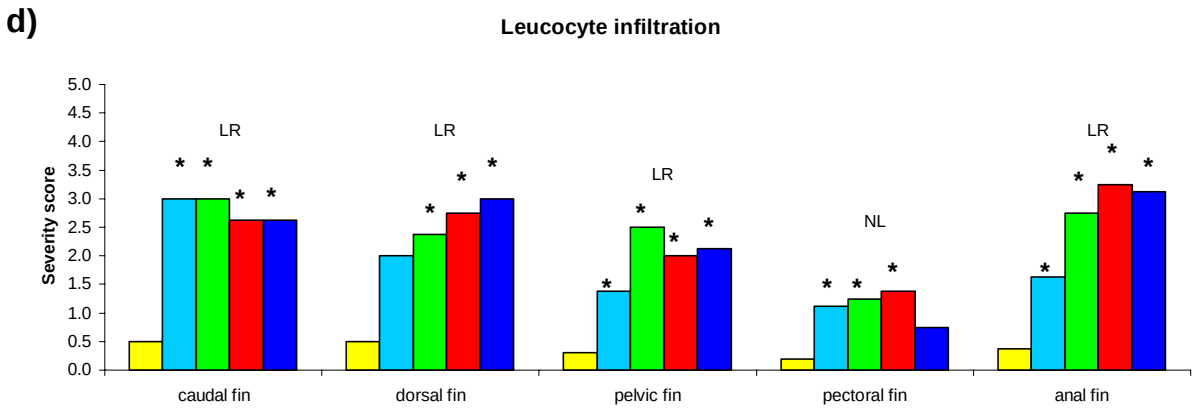


Figure 2.8. Mean severity of fin degeneration, erosion and ulceration, and leukocyte infiltration on each fin type of control fish and hybrid striped bass stressed for 30, 60, 90 and 120 min. LR indicates that there was a linear association between mean severity and temperature using the Cochran-Mantel-Haenszel statistical analysis ($p < 0.05$). NL indicates that there was no linear association. * indicates the significant difference of fin damage in stressed fish compared to control, unstressed fish which determined by ANOVA, $p < 0.05$.





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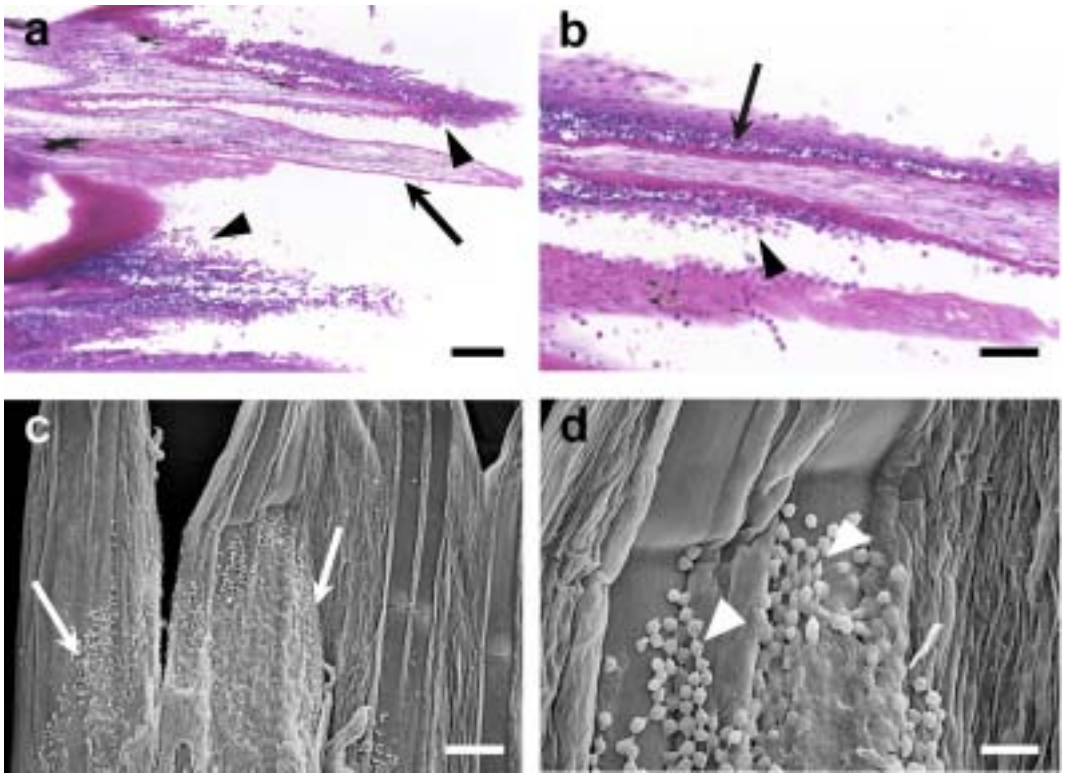

Figure 2.9. Caudal fins of hybrid striped bass confined for 15 min. E epidermis; D dermis; b basement membrane. a) Caudal fin showing epidermal erosion (arrow head) and epidermal ulceration (arrow) at the distal edge of fin. H&E. Bar = 100 μm. b) Outer epidermal cells were swollen and sloughed (arrow head). Leucocytes appeared at the basal layers of epidermis and dermis (arrow). Bar = 260 μm. c) SEM of caudal fin showing epidermal erosion and ulceration at the distal edge (arrow). Bar = 100 μm. d) SEM of caudal fin showing rounding epidermal cells (arrow head). Bar = 26 μm.

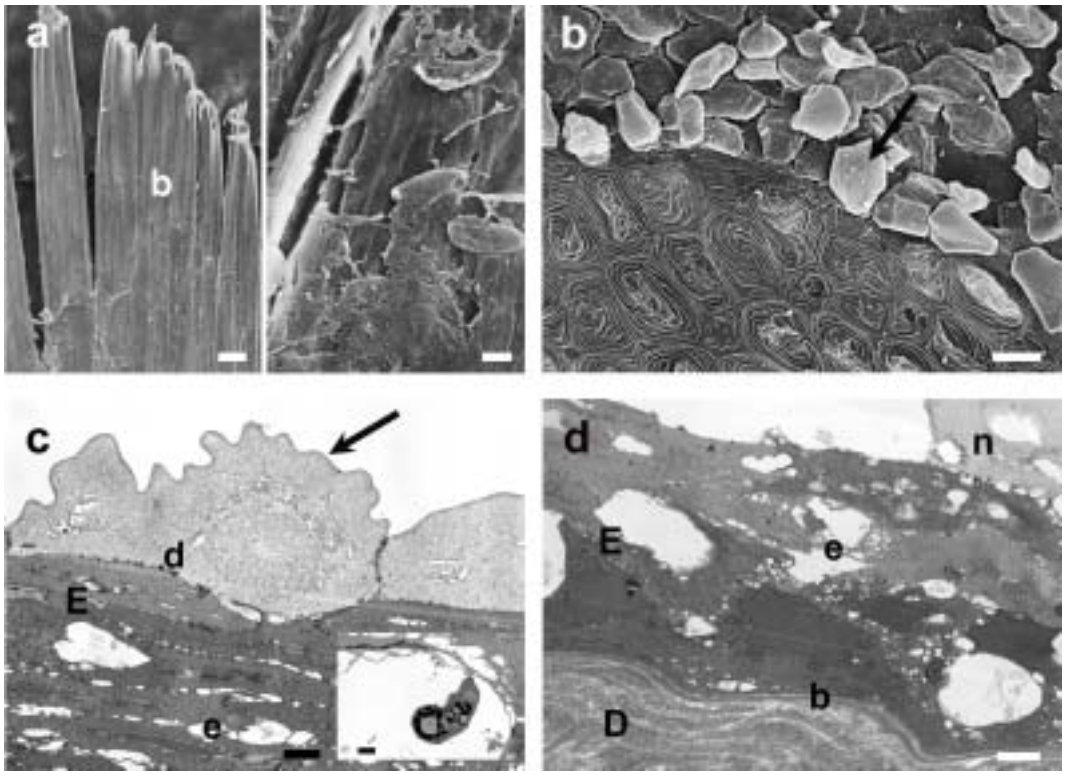

Figure 2.10. Caudal fins of hybrid striped bass confined for 30 min. E epidermis; D dermis; b basement membrane; e intercellular edema; n necrotic cell; d desmosome. a) SEM of caudal fin showing epidermal erosion and ulceration near the distal tip of the fin. Bar = 350 μm. Right: showing epidermal erosion and ulceration, and exposure of the basement membrane (b) beneath the sloughing epidermis. Bar = 6 μm. b) SEM of fin showed swelling epidermal cells lacking microridges (arrow). This area was adjacent to the epidermal ulcer. Bar = 30 μm. c) TEM of epidermis, note swollen epidermal cells (arrow) and intercellular edema at the middle layers of epidermis. Bar = 1.6 μm. Inset: apoptotic body present in the basal layers. Bar = 1.1 μm. d) TEM of the epidermis, note necrotic cell (n) and loss of microridges at the outer surface. Bar = 2 μm.

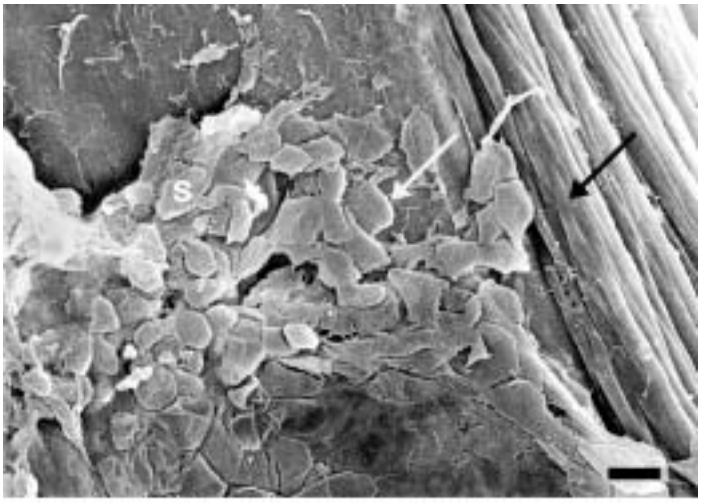

Figure 2.11. Caudal fins of hybrid striped bass confined for 60 min. SEM of fin showing areas of epidermal erosion (white arrow), ulceration (black arrow), and swollen epidermis (S). Bar = 8 μm.

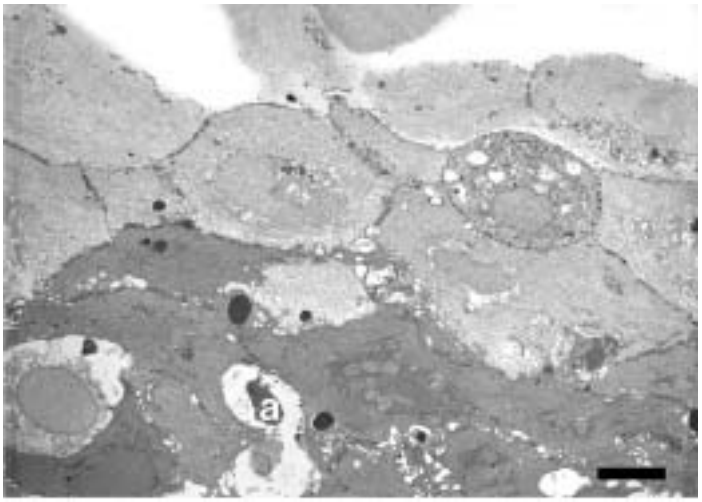

Figure 2.12. TEM of caudal fin of hybrid striped bass confined for 90 min showing epidermal erosion. Note epidermal cells were swollen, and loss of microridges. The middle layers of epidermis show intercellular edema. (a) apoptotic cell. Bar = 4 μm.

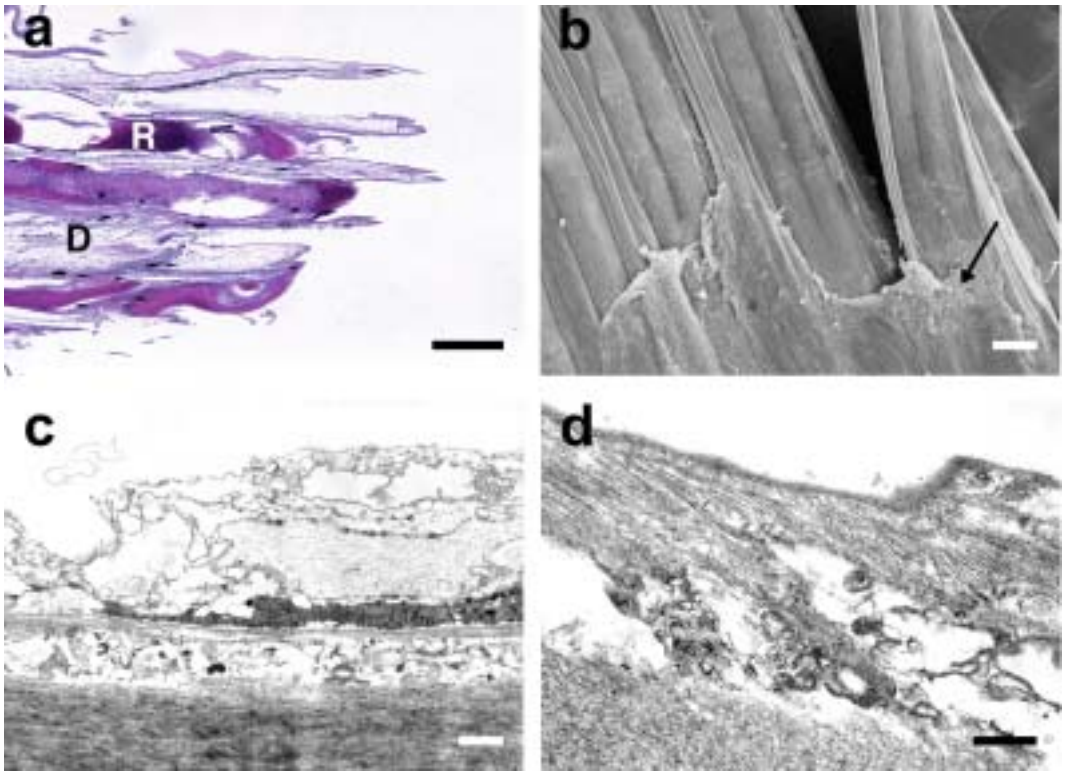

Figure 2.13. Fins of hybrid striped bass confined for 120 min. D dermis; R fin ray. a) Caudal fin after 120 min of stress showing ulceration of the distal fin tissues, and dermal / hypodermal edema. H&E. Bar = 200 μm. b) SEM of caudal fin showing severe sloughing (arrow). Bar = 500 μm. c) TEM showing epidermal degeneration, necrosis, and hypodermal edema. Bar = 1 μm. d) TEM of severe epidermal ulceration showing total loss of epidermis and basement membrane. Bar = 1.5 μm.

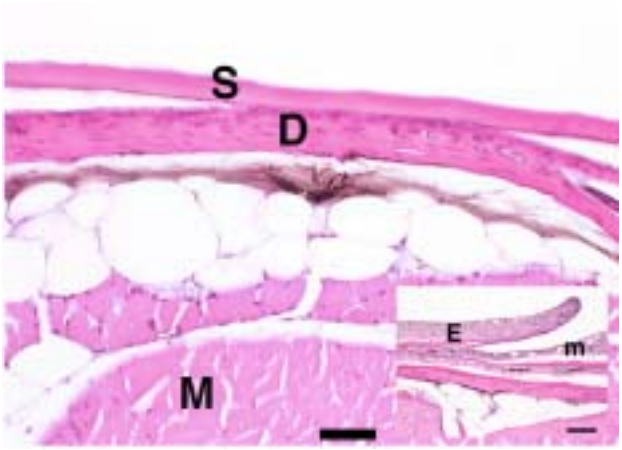

Figure 2.14. Ventrum skin of hybrid striped bass. After 2 hr of stress, note epidermal ulceration; E epidermis; D dermis; M muscle; S scale. Inset: Control (Unstressed) fish. m mucous cell. Bar = 100 μm.

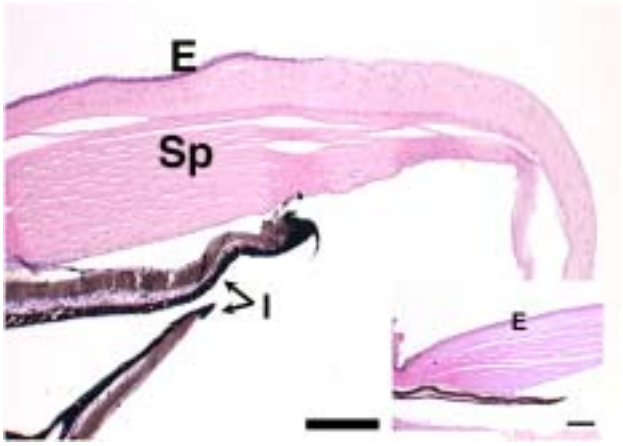

Figure 2.15. Cornea of the eye in hybrid striped bass depicting corneal ulceration after 120 min of confinement stress. C corneal epithelium; I iris; Sp substantia propria. Bar = 200 μm. Inset: Cornea of control, unstressed hybrid striped bass showing transparent cornea with several epidermal layer. Bar = 100 μm.

ACKNOWLEDGEMENTS

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III.

ENVIRONMENTAL AND PHYSIOLOGICAL FACTORS AFFECTING DEVELOPMENT OF THE ACUTE ULCERATION RESPONSE (AUR)

ABSTRACT

Previously, we demonstrated that juvenile hybrid striped bass (*Morone saxatilis* male X *M. chrysops* female) developed rapid pathological changes (epidermal erosion/ulceration and leucocyte infiltration on fins, skin and cornea), after being subjected to acute confinement stress. We have named this syndrome the Acute Ulceration Response (AUR). The present study shows that size of the acclimation space (aquarium size), temperature during acclimation, temperature during confinement, and exogenous adrenergic modulators influenced the risk of developing AUR. While fish acclimated to both small (2.3 liters) and large (l500 liters) environments developed AUR, fish that were stressed after acclimation to the smaller space developed less severe AUR, suggesting that gradual acclimation to confinement reduces the risk of developing AUR. When fish were acclimated to a high temperature (27°C), stressing them at a lower temperature (13°C) resulted in significantly less AUR on the dorsal and pectoral fins compared to fish stressed at a higher temperature (27°C). However, fish acclimated to 13°C and then stressed at 13°C had significantly less severe AUR on all fins, as well as the ventrum. These data suggest that while lowering the temperature during an acute stress can reduce the severity of AUR, the acclimation temperature prior to stress might be even more important in predisposing fish to AUR. Intraperitoneal administration of epinephrine (1-10,000 μg/kg body weight, BW) caused a dose-dependent epidermal ulceration of the caudal, dorsal and pectoral fins. In contrast, adrenergic antagonists reduced the severity of AUR when administered before stressing the fish. However, these effects were not seen on all fins in every treatment. Phentolamine, an alpha-adrenergic antagonist, at doses from 10-1,000 μg/kg BW, did not decrease the number

of damaged fins, but the severity of epidermal ulceration on the caudal and pectoral fins was significantly reduced in some treatments. Propranolol, a beta-adrenergic antagonist, at doses from 10-1,000 μg/kg BW, significantly reduced the severity of epidermal ulceration in the pelvic fins. A low dose (10 μg/kg BW) of hexamethonium, a nicotinic ganglionic antagonist, significantly reduced the severity of epidermal ulceration on the dorsal, pelvic and pectoral fins. Our data suggest that adrenergic stimulation is an important factor in AUR development during acute confinement stress, and that adrenergic blockers have the potential to reduce the severity and consequences of AUR.

INTRODUCTION

Acute stress is probably one of the most common instigators of epidemics in aquaculture. One of the most common sources of acute stress is confinement, such as for the movement/handling of fish on the farm or for transport to distant sites. While crowding/confinement is an extremely common cultural stress, and many studies have characterized various physiological changes associated with it (reviewed in Pickering 1998), we know very little about the mechanisms directly responsible for the adverse consequences of confinement stress (i.e., infectious disease outbreaks). In this regard, we have recently discovered that when striped bass (*Morone saxatilis*) or hybrid striped bass (*Morone saxatilis* female x *M. chrysops* male) were exposed to an acute confinement stress, they rapidly (within less than 2 hr) sloughed the skin on their fins (Noga et al 1998), which might certainly predispose them to infectious agents. We subsequently discovered that this acute epidermal loss, which we have named the Acute Ulceration Response (AUR), could be

induced extremely rapidly (after only 15 min of acute stress) and that it could occur not only on the fins but also the epidermis on the body as well as the corneal epithelium (Udomkusonsri et al, Unpublished Data).

Confinement stress is relatively complex and is comprised of both physical and environmental factors (reviewed in Wedemeyer 1996). Physical stressors include confinement in a small, crowded space (e.g., hauling tank) and injury during handling and hauling. Environmental factors might include low dissolved oxygen, high ammonia, and stressful (usually high) temperature. Prior exposure (acclimation) to a particular stress also has an important influence on the subsequent response of fish to that stress. For example, the ability of fish to adapt to a confined space is influenced by the density to which the fish had been previously exposed (Pottinger and Pickering 1992; Wedemeyer 1976).

Temperature seems to play an especially critical role in the response of fish to confinement and other types of stressors (Carmichael et al 1984b; Davis and Parker 1990). Fish metabolism increases proportionately with higher temperature and higher temperature also affects other critical environmental factors (e.g., lowers oxygen solubility, increases the toxicity of carbon dioxide and ammonia).

Environmental temperature is also intimately involved in the modulation of the stress hormone response. For example, it is well known that increased temperature, which increases a fish's metabolism, can dramatically increase the secretion rate of stress hormones, such as glucocorticoids (i.e., cortisol) and catecholamines (i.e., epinephrine) (Barton and Schreck 1987; Davis and Parker 1990). Consequently, most culturists usually prefer to

transport fish at the lower range of a fish's temperature tolerance to try to mitigate the effects of temperature (Wedemeyer 1996).

In previous studies, we demonstrated that exogenous administration of the stress hormone, epinephrine, could partly reproduce AUR lesions (Noga et al 1998). Epinephrine is released rapidly in response to a wide variety of stresses (Gamperl et al 1994a; Randall and Perry 1992) and modulates many physiological responses that promote cardio-respiratory function and metabolism in order to maintain adequate oxygen in the tissues. Epinephrine increases blood pressure by acting on alpha-adrenoceptors, causing constriction of systemic and peripheral blood vasculature, and increases branchial perfusion by acting on beta-adrenoceptors, causing vasodilation of gill blood vessels (Axelsson et al 2000; Davie 1981; Wahlqvist and Nilsson 1981; Wood 1975; Xu and Olson 1993). We have previously hypothesized that when hybrid striped bass are acutely stressed, the peripheral vasculature might be constricted by the effect of epinephrine, causing the epidermis to become hypoxic or ischemic, resulting in degenerative change in the epidermal tissues, including in the corneal epithelium (Noga et al 1998, Udomkusonsri et al, Unpublished Data).

Alpha-adrenergic antagonists (e.g., phentolamine) and ganglionic antagonists (e.g., hexamethonium) can reduce the release of catecholamines and/or abolish the effects of epinephrine during stress in some fish (Narnaware and Baker 1996; Opdyke et al 1983; Peyraud-Waitzenegger 1979; Wahlqvist and Nilsson 1981). Beta-adrenergic antagonists, such as propranolol, have been shown to decrease the vasodilatation effect of catecholamines in rainbow trout [*Oncorhynchus mykiss* (Walbaum)] (Wood 1976). In addition, Wahlqvist

and Nilsson (1981) demonstrated that phentolamine reduced the vasoconstrictory effect at the tail vasculature of Atlantic cod (*Gadus morhua* Linnaeus) in response to adrenergic stimulation. Thus, administration of an α- or β-adrenergic blocker might decrease or increase, respectively, the risk of developing AUR.

Furthermore, sympathetic stimulation by preganglionic nerve fibers stimulates the release of catecholamine from the chromaffin tissues. In teleosts, hexamethonium (a nicotinic, cholinergic, receptor antagonist) can prevent or decrease the secretion of catecholamines (CA) (Al-Kharrat et al 1997; Bernier and Perry 1996; Reid et al 1998; Reid and Perry 1995). Thus, administration of adrenergic or ganglionic antagonists might affect the development of AUR.

In our present paper, we examined whether acclimation density or temperature influenced the development of AUR, and whether pharmacological modulation of the adrenergic (catecholamine) response affected the severity of AUR in experimentally stressed fish.

MATERIALS AND METHODS

Maintenance of Fish Stocks

Hybrid striped bass (*M. saxatilis* male x *M. chrysops* female, <18 months old, 70-110 mm total length), were maintained in a 760-litre recirculating aquarium at 14ºC. Fish were fed a commercial feed at approximately 2% of their body weight daily and maintained on a 12-hour light:12-hour dark photoperiod. The low temperature and restricted feed provided a

constant supply of small, similar-size fish for experiments. All experiments were performed in freshwater. Water quality during all experiments was: dissolved oxygen 6.8-7.5 mg/l, temperature 27ºC, pH 6.65-6.87, unionized ammonia <0.001 mg/l and nitrite <0.10 mg/l.

Effect of Environmental Conditions on Development of AUR

Effect of acclimation to different sized culture systems

Sixteen hybrid striped bass were placed in a 1500-litre aquarium and the water temperature was gradually raised from 14° to 27ºC in 1 week. Eight of the fish were then moved to a 75-litre aquarium, maintained at 27ºC for 5 days, and then moved to duplicate cages (13 x 15 x 12 cm, 4 fish/cage) at 27ºC for 9 days (acclimated in a small environment for total of 2 weeks). After the 2-week acclimation period, 4 fish (2 fish from each cage)(unstressed controls) were sacrificed with anesthetic overdose (200 mg/l tricaine buffered with 400 mg/l sodium bicarbonate) and then fixed in 10% neutral buffered formalin (NBF) for histological evaluation. Then, 2 fish from each cage (a total of 4 fish) were removed gently with a soft net and individually confined in plastic mesh boxes (3.5 x 15 x 12 cm). The boxes were agitated every 15 sec per min during confinement. After the 2-hr confinement, the fish were euthanized and fixed for histological evaluation.

The remaining 8 fish were kept in the 1500-liter aquarium at 27ºC during these 2 weeks (acclimated to a large environment). After the 2 weeks of acclimation to the large aquarium, four control (unstressed) fish were immediately removed from the acclimating

aquarium, sacrificed with anesthetic overdose, and fixed in NBF for histological evaluation.. Then, the remaining 4 fish were confined individually in confinement boxes as described above at 27ºC for 2 hr, sacrificed with anesthetic overdose, and fixed in NBF for histological evaluation.

Effect of temperature during acute confinement

The optimal growth temperature for juvenile hybrid striped bass is approximately 27ºC (Woiwode and Adelman 1991), and 32ºC is generally the upper temperature limit for the culture of striped bass (Wedemeyer 1996). As lower temperature during transport can mitigate the stress response (Wedemeyer 1996), we examined the effect of two lower temperatures (13ºand 20ºC) on the development of AUR. Twenty–four hybrid striped bass were acclimated in a 1500-litre aquarium at 27ºC. After 2 weeks of acclimation, six unstressed fish were taken from the acclimating aquarium and sacrificed as unstressed controls. Six fish were then individually confined in the confinement boxes at 13, 20 or 27ºC. The confinement boxes were agitated 15 sec/min. After 2 hr of confinement, all fish were sacrificed and fixed in NBF for histological evaluation. This experiment was replicated 2 times.

Effect of temperature prior to acute confinement (acclimation temperature)

Thirty hybrid striped bass were acclimated in 150-litre aquaria to three different temperatures: 13°, 20°, or 30ºC (10 fish per aquarium). After 2 weeks of acclimation, 5 unstressed fish were taken from each acclimating aquarium and sacrificed as unstressed

controls. Then, 5 fish at each temperature were individually confined in confinement boxes, as described above, at the same temperature to which they had been acclimated. The confinement boxes were agitated 15 sec/min. Control (unstressed) and stressed fish were sacrificed and fixed in NBF for histological evaluation. This experiment was replicated 3 times.

Effect of Adrenergic Modulation on Development of AUR

Adrenergic stimulation

Twelve hybrid striped bass were acclimated in a 1500-litre aquarium to 27ºC for 2 weeks. Then, duplicate fish were injected intraperitoneally with one of the following concentrations of epinephrine (1, 10, 100, 1,000 or 10,000 μg/kg body weight [BW]). Two control fish were injected with diluent (0.9% normal saline). After epinephrine or normal saline administration, fish were placed into 75-litre aquaria (2 fish per aquarium) at 27ºC. After 2 hr, all fish were sacrificed and fixed in NBF for histological evaluation. This experiment was replicated 4 times.

Adrenergic inhibition

For each adrenergic blocker tested, eight hybrid striped bass were acclimated to a 1500-litre aquarium at 27ºC for 2 weeks. Fish were then injected intraperitoneally with the blocker (hexamethonium, propranolol or phentolamine), at a dosage of 10, 100 or 1,000 μg/kg BW (2 fish per dose of each blocker). Control fish in each treatment were injected with diluent (0.9% normal saline). After a period of 10 min was allowed for the blockers to

be delivered to the circulation (Pic and Djabali 1982), fish were individually confined in confinement boxes at 27ºC. After a 2-hr confinement period, all fish were sacrificed and fixed in NBF for histological evaluation. Each experiment was replicated 4 times.

Histopathological Analysis

Eyes, fins, and ventrum skin tissues were fixed in NBF, decalcified in 100 g/l ethylenediaminetetraacetic acid (EDTA) in 0.1M phosphate buffer (pH 7.2), embedded in paraffin, and processed routinely for light microscopy. All sections were stained with hematoxylin and eosin (H&E). All tissues were evaluated blindly as described in Noga et al (1998). Briefly, lesions were scored on a scoring scale of 1-5, with (1) being minimal damage (1-20% of area affected by the lesion), (2) being mild damage (21-40% of area affected by the lesion), (3) being moderate damage (41-60% of area affected by the lesion), (4) being severe damage (61-80% of area affected by the lesion) and (5) being extremely severe damage (81-100% of are affected by the lesion).

All fin tissues were oriented in the longitudinal plane, and then evaluated for pathological changes: epidermal degeneration, epidermal erosion, epidermal ulceration, and leukocyte infiltration of affected fins. Skin tissues of the body were also oriented in the longitudinal plane and examined for erosion and ulceration. Eyes were cross-sectioned and examined for corneal ulceration. Epidermal degeneration was defined as swollen epidermal cells (intracellular edema) with pyknotic nuclei; epidermal erosion was the sloughing of epithelial layers, but with the basement membrane still intact; ulceration was defined as complete loss of all epithelial layers and the basement membrane. Leukocytes are normally

found in the dermis but in stressed skin, leukocytes may also infiltrate the basal epidermal layers.

Statistical Analyses

The exact unconditional test was used to analyze the effect of acclimation to different sized culture systems on development of AUR to test whether there was statistical evidence that the proportion of epidermal damage was greater in stressed versus unstressed fish, or in stressed fish acclimated to a large versus a small culture system. This test is available on the NCSU Department of Statistics website (www.stat.ncsu.edu). The test was performed for each type of epidermal damage on each fish and used the binomial one-sided model with Fisher's Exact-Boschloo as the test statistic with a 99.9% confidence interval.

For all other experiments, we used one-way ANOVA and the Cochran-Mantel-Haenszel (CMH) test in SAS (Version 8, SAS Institute, Cary, NC). The one-way ANOVA tests for significant differences between treatments. The CMH tests whether each type of epidermal damage for each tissue is conditionally independent of the treatment (temperature during acclimation or stress, increase of epinephrine or blocker concentration) when adjusting for the control variable (the variation between replication) (Agresti 1996). We concluded that there was a linear association between treatment and epidermal damage when the p-value of the Correlation Statistic was < 0.05.

RESULTS

Effect of Environmental Conditions on Development of AUR

Effect of acclimation to different sized culture systems

When hybrid striped bass were transferred from the 75-litre aquarium to the small cages for acclimation, they showed signs of distress, such as excitation and anorexia, on the first day. The next day, the fish became calm and started to eat. During the acute stress, fish previously acclimated to the small cages were calm, but fish acclimated to the large aquarium were very excited and struggled during the first 15-20 min of confinement. After the 2-hr confinement, all stressed fish (from both the large aquarium and the small cages) displayed gross and microscopic lesions that were typical of AUR (blanching and ragged fins at the distal edge; epidermal degeneration, epidermal erosion, epidermal ulceration, and leukocyte infiltration) (Figure 3.1). However, fin erosion and ulceration was also present in some unstressed fish acclimated to the small cages (Figure 3.1).

Compared to stressed fish acclimated to the small cages, stressed fish acclimated to the large aquarium had a tendency to have more severe epidermal ulceration in most fins, but this was only significantly different for caudal fins ($p < 0.05$)(Table 3.1, Figure 3.2). It is possible that the pooled data from the replicated experiments were various; thus, the statistic analysis could not be shown the significantly different. Ventrum ulceration was also significantly greater in stressed fish acclimated to the large aquarium ($p < 0.05$). Furthermore, all fish acclimated to the large aquarium developed significantly greater

leukocyte infiltration after being subjected to the stress ($p < 0.05$)(Figure 3.2). Stressed fish from the large aquarium also had more epidermal degeneration in the pelvic and pectoral fins than stressed fish acclimated to the small cages (Table 3.1). It should be noted that the caudal fins of stressed fish acclimated to the large aquarium did not display epidermal erosion since all of them developed ulceration.

Effect of temperature during acute confinement

The unstressed hybrid striped bass (all held at 27ºC) had no damage on the fins, ventrum skin, or cornea, while the majority of all tissues in fish stressed at all 3 temperatures had ulceration (Figure 3.3). Fish stressed at 13ºC turned pale and lost equilibrium after the first 15-min of confinement. After 15 min, they regained equilibrium but often stayed on the bottom of the confinement boxes. This sign of disequilibrium was also reported when tilapia, *Tilapia aurea* (Steindachner), were subjected to a sudden temperature drop (from 22 to 11ºC) (Kindle and Whitmore 1986). Fish stressed at 20ºand 27ºC were excited during the early stages of the confinement stress, but did not lose equilibrium. A statistical comparison of the unstressed controls versus fish stressed at 13°, 20º or 27ºC showed that all stressed fish had significantly more severe fin, ventrum skin and corneal ulceration than the controls ($p < 0.05$)(Figure 3.4). Fish stressed at 27ºC developed more severe fin ulceration than fish stressed at either 13º or 20ºC. Fish stressed at 13ºC had significantly less severe epidermal ulceration on the caudal, dorsal and pectoral fins compared to fish stressed at either 20ºor 27ºC ($p < 0.05$). Fish stressed at 20ºC had significantly less epidermal ulceration on the caudal and pectoral fins than fish stressed at 27ºC ($p < 0.05$). Fish stressed at 13ºC had

ulceration of the ventrum and cornea, but it was not significantly less than fish stressed fish at 20º or 27ºC (Figure 3.4). There was no significant difference in leukocyte infiltration among the stressed groups (data not shown).

There was a positive correlation between temperature and severity of epidermal ulceration of the caudal, dorsal and pectoral fins ($p < 0.05$)(Table 3.1, Figure 3.4), where increased temperature lead to more severe epidermal ulceration. A similar trend was apparent for pelvic fin ulceration, but was not statistically significant. However, the incidence of epidermal ulceration of ventrum skin and cornea did not correlate with temperature (Table 3.1).

Effect of temperature prior to acute confinement (acclimation temperature)

Control, unstressed, hybrid striped bass acclimated to either 13º or 20ºC had no ulceration on the fins, ventrum skin, or eyes, but control fish held at 30ºC had a minor amount of ulceration on the caudal fin (Figure 3.5). After the 2-hr stress, fish at 13°, 20°, or 30ºC developed extensive fin ulceration (Figure 3.5), as well as leukocyte infiltration (data not shown). Stressed fish at 13° or 30ºC also developed ventrum or corneal ulcers, but none of the fish at 20ºC did so (Figure 3.5). However, stressed fish previously acclimated to 13ºC had significantly less severe ulceration on all fins compared to fish acclimated to either 20º or 30ºC ($p < 0.05$)(Figure 3.6). There was no significant difference in severity of fin ulceration between stressed fish acclimated to 20º versus 30ºC, except for the caudal fin (Table 3.1, Figure 3.6). There was also a positive correlation between acclimation temperature and the severity of ulceration of all tissues, except for the anal fin and cornea (Figure 3.6).

Leukocyte infiltration on all fins, except the pectoral and anal fins, was also positively correlated with temperature (data not shown).

Effect of Adrenergic Modulation on Development of AUR

Adrenergic stimulation

After intraperitoneal administration of either normal saline or epinephrine, most fish developed various degrees of epidermal degeneration, epidermal erosion (data not shown), epidermal ulceration, and leukocyte infiltration on all fins (Figure 3.7). The incidence (number of affected fins) did not appear to be increased with higher doses of epinephrine (Figure 3.7). Epinephrine treatment caused minimal erosion on the ventrum (Figure 3.7) and did not affect the corneal epithelium (data not shown). However, fish treated with epinephrine developed significantly more severe epidermal erosion and ulceration than the controls (Figure 3.8). There was a positive linear correlation (increase of exogenous epinephrine concentration leading to increased severity) of epidermal ulceration of the caudal, dorsal and pectoral fins ($p < 0.05$)(Table 3.2, Figure 3.8). A similar trend was somewhat suggested for pelvic and anal fin ulceration, but was not statistically correlated with epinephrine concentrations. However, the severity of the response to epinephrine was not as great at any doses as that seen in fish with AUR induced by confinement (Udomkusonsri et al, Unpublished Data).

Adrenergic inhibition

Fish treated with phentolamine, an α-adrenergic antagonist, still developed epidermal erosion, ulceration and leukocyte infiltration after a 2-hr stress (Figure 3.9). Phentolamine treatment did not decrease the number of fins affected with AUR when compared to control, normal saline-treated fish. No control or phentolamine-treated fish developed ulceration on the ventrum or cornea, except for one saline control that had mild ventral skin erosion. The severity of epidermal ulceration in some fins of phentolamine-treated fish was significantly less than in the control (Figure 3.10): fish treated with 10 μg/kg phentolamine had significantly less caudal fin ulceration and fish treated with 100 μg/kg phentolamine had significantly less pectoral fin ulceration ($p < 0.05$). There was a linear correlation between phentolamine concentration and severity of dorsal and pectoral fin ulceration ($p < 0.05$)(Table 3.2), with increasing phentolamine concentration leading to increased severity.

Propranolol, a β-adrenergic antagonist, had a variable effect on the expression of epidermal damage in stressed fish. All control and propranolol-treated fish developed epidermal damage on the fins, including epidermal erosion, epidermal ulceration, and leukocyte infiltration. No fish developed ulceration on the ventrum or cornea. The number of control fins with epidermal ulceration appeared to be no higher than those in propranolol-treated fins (Figure 3.11). Fish treated with 10, 100 or 1,000 μg/kg propranolol had significantly less severe pelvic fin ulceration than control fish ($p < 0.05$)(Figure 3.12). In addition, fish treated with 10 or 100 μg/kg propranolol had significantly less severe dorsal fin ulceration ($p < 0.05$). There were also significant linear correlations between dorsal or pelvic

fin ulceration and propranolol concentration, in which increased propranolol concentration lead to more severe epidermal ulceration ($p < 0.05$)(Table 3.2, Figure 3.12). Treatment with higher propranolol concentrations lead to increased leukocyte infiltration in the caudal fins and decreased leukocyte infiltration in the pelvic and pectoral fins (data not shown).

In the hexamethonium (a nicotinic, ganglionic blocker) study, the incidence of (i.e., number of fins affected with) epidermal erosion and ulceration did not decrease in hexamethonium-treated fish compared to the controls (Figure 3.13). However, epidermal ulceration in some doses of the hexamethonium-treated fish was less severe than in the controls (Table 3.2, Figure 3.14). Hexamethonium at 10 μg/kg significantly reduced the severity of epidermal ulceration in dorsal, pelvic and pectoral fins ($p < 0.05$). Hexamethonium at 100 μg/kg significantly lowered the severity of epidermal ulceration in pelvic fins. There was also a negative correlation between hexamethonium concentration and severity of epidermal ulceration of the dorsal and pelvic fins ($p < 0.05$). In addition, no hexamethonium-treated fish developed ulceration on the ventrum or cornea, while control fish developed both corneal ulceration and epidermal erosion on the ventrum.

DISCUSSION

Effect of Environmental Conditions on Development of AUR

While confinement readily and reproducibly can induce AUR, we suspected that if fish were gradually acclimated to a small, confining space, it would reduce the severity of

AUR. Surprisingly, there was a relatively small effect of this manipulation. Only the severity of caudal fin and ventrum ulceration was significantly reduced in stressed fish previously acclimated to the small cages (Figure 3.1). Others have found that the type of holding facilities (including fiberglass tank, raceway, or pond), as well as different densities in holding facilities (0.14 – 3.9 g fish/liter) had no effect on the resting concentrations of stress indicators such as cortisol, chloride or osmolality in unstressed largemouth bass, *Micropterus salmoides* (Lacépède) (Carmichael et al 1984a). Plasma glucose levels in fish held in ponds were lower, but not significantly so, compared to fish in other facilities. After a 30-min confinement, fish from all the holding facilities had similarly increased plasma cortisol and glucose. Thus, acclimating those fish to different holding environments had no effect on their stress response

Another possible reason for our results is that the period of time that we acclimated the fish might have been less than optimal. Pottinger and Pickering (1992) found that complete acclimation (as defined by low resting blood cortisol levels) of small groups (10 fish/group) of rainbow trout to a confined space required as long as 4 weeks. Fish acclimated as pairs or groups of five developed social hierarchies in which the dominant fish rapidly became acclimated, while the others lagged and developed bacterial infections. Interestingly, acclimation was more rapid in fish segregated singly, and required only 1-2 weeks. A dominant/subordinate interaction was also reported in coho salmon, *O. kisutch* (Walbaum) (Ejike and Schreck 1980), European eel (Peters et al 1980), and other fish (Øverlt et al 1999). Although we know of no specific information addressing social interaction in hybrid striped bass, we have observed dominance hierarchies in holding tanks with small groups of fish

(Noga, EJ, Unpublished Data). Aggressive behavior was also observed in juvenile hybrid striped bass (~1.8 g) held in small groups (11 fish) at 25ºC (Kocabas and Gatlin III 1999). Thus, hybrid striped bass acclimated together in small environments for 2 weeks may be stressed and may need longer than 2 weeks to completely acclimate.

In contrast to the relatively minor effect with different size acclimation environments, we found that temperature had a very important effect on the expression of AUR. Fish acclimated to a high temperature (27ºC) had less severe AUR on the caudal, dorsal and pectoral fins when confined at a low temperature rather than a high temperature (Figure 3.4).

Even more significantly, acclimating fish to a low temperature prior to the acute stress was an even more effective means of reducing the severity of AUR. When fish were first acclimated to a low temperature (13ºC), most tissues from stressed fish had less epidermal damage (Figure 3.6). This is consistent with the great majority of other studies examining the effect of temperature on stress in fish, where lowering the water temperature reducesthe stress response (Carmichael et al 1984a; Carmichael et al 1984b; Milligan et al 1989). Davis and Parker (1990) showed that striped bass had a decreased stress response to confinement when first acclimated to a low temperature. Stressed striped bass, previously acclimated to 10° or 16ºC, had less physiological changes (such as lower blood cortisol, glucose, or chloride) compared to stressed fish acclimated at 21°, 25° or 30ºC. Striped bass acclimated to the highest temperature (30ºC) prior to stressing had the greatest physiological changes after stress and had the highest mortality compared to other acclimation temperatures. However, we do not know why none of the fish acclimated and stressed at

20ºC developed ventral skin and corneal ulceration in our present study. Others have found that resting plasma glucose was greater in fish held at high temperature compared to fish held at a lower temperature (Barton and Schreck 1987; Carmichael et al 1984b); and after an acute stress, plasma glucose was significantly higher in those fish held at the higher temperature, although cortisol levels were similar. Cortisol and epinephrine can both affect blood glucose levels during stress, but the very rapid increase in those studies are more likely to be modulated by catecholamines (Barton and Iwama 1991; Mazeaud et al 1977; Wedemeyer et al 1990); thus, the increase in blood glucose was suggested as the action of catecholamines (especially epinephrine), not glucocorticoids. Previously, we provided evidence that epinephrine plays a role in inducing AUR (Noga et al 1998). In our present study, epinephrine levels in response to confinement are probably much lower and rise much less after stress in hybrid striped bass acclimated to 13ºC compared to those at held at 30ºC, which might explain the more severe AUR in the latter after stress. Resting plasma catecholamines were also significantly greater when catfish, *Heteropneustes fossilis* (Bloch), were acclimated to high temperature (at 28ºC rather than 18ºC) (Senthilkumaran and Joy 1995). This may also explain why unstressed hybrid striped bass acclimated to a high temperature (30ºC) had mild AUR.

One possible reason that we saw a less dramatic reduction in AUR when fish were first acclimated at a higher temperature (27ºC) and then stressed at a lower temperature (13ºC)(Figure 3.4) compared to when they were both acclimated and stressed at the lower temperature (Figure 3.6) is that the rapid temperature decrease may itself be stressful. It is generally recommended that the difference in water temperature between the acclimating and

transporting units should not differ by more than 10ºC (Wedemeyer 1996). For example, plasma epinephrine and cortisol were significantly elevated in tilapia (*Oreochromis aureus*) after exposure to a rapid temperature drop (from 25ºC to 12ºC over 30 min) (Chen et al 2002). It has been recommended that striped bass and hybrid striped bass should be kept between 13°–15ºC during long-term transport, and that the acclimating and transporting unit water temperatures should not differ by more than 3ºC (Weirich 1997).

From our study, we conclude that the physical environment can have an important effect on the risk of developing AUR. Gradual acclimation of fish to a confined space and especially to a lower temperature effectively reduces the severity of AUR. And stressing fish at a lower temperature also decreases the severity of AUR, regardless of their prior acclimation temperature. These findings have important implications in the management of stress in aquaculture and for ultimately reducing the prevalence of disease outbreaks.

Effect of Adrenergic Modulation on Development of AUR

Administration of a single dose of epinephrine to hybrid striped bass induced AUR, but the AUR lesions were mild, even at supraphysiological concentrations (e.g., 10,000 μg/kg), although its effect appeared to be dose-dependent (Figure 3.8). These results were similar to our preliminary data on epinephrine administration using fingerling hybrid striped bass administered similar epinephrine levels intraperitoneally (Noga et al 1998). One possible reason for the relatively weak response is that other physiological changes, including other stress hormones such as cortisol, might also play a role in AUR development and might be needed for its full expression.

Another possible explanation is that exogenous epinephrine administration, even at very high doses, probably only temporarily increases plasma epinephrine levels. Others have also shown that intravenous administration of epinephrine causes only a transient elevation in plasma epinephrine (Gamperl et al 1994a; Nekvasil and Olson 1986; Urgell and Nilsson 1979). Intravenous administration of exogenous epinephrine caused plasma epinephrine to peak rapidly but it decreased >80% within 20 min post-injection in rainbow trout (Gamperl et al 1994a) and >90% within 16-20 min post-injection in cod (*Gadus morhua*) (Urgell and Nilsson, 1979).

A more prolonged epinephrine spike occurs during acute stress (Carmichael et al 1984b; Mazeaud et al 1977) and thus a more physiological, prolonged exposure to epinephrine might be needed to induce typical advanced AUR lesions. Nonetheless, our data further support the hypothesis that epinephrine plays an important role in development of AUR lesions during acute stress.

The vascular system of fish is under adrenergic control (Nilsson 1994; Wahlqvist and Nilsson 1980). In stressful situations, plasma catecholamines (mainly epinephrine) rapidly cause vasoconstriction and negative chronotropy via α-adrenoceptors in the teleost systemic vasculature and heart, respectively (Axelsson et al 2000; Temma et al 1989; Tirri and Ripatii 1982; Xu and Olson 1993). Catecholamines also act on β-adrenergic receptors and cause positive inotropy and chronotropy, increased cardiac output, and decreased gill resistance, thereby increasing gill perfusion (Gamperl et al 1998; Sundin 1995; Wood 1975). Both α- and β-adrenergic receptors were reported to control vascular resistance in the tail vasculature

of cod and eel (Davie 1981; Wahlqvist and Nilsson 1981).

Melanophores in fish skin also respond to adrenergic stimulation by aggregating their melanosomes after stimulation of their α-adrenoceptors (Grundstrom et al 1985). We observed that stressed fish had blanching at the distal edge of the fins as a result of aggregation of melanosomes in the affected areas. This is further indirect evidence for the role of adrenergic action in AUR pathogenesis.

As epinephrine may play a role in AUR development by causing vasoconstriction of the fin tissues during acute stress (Noga et al 1998), we hypothesized that α- and β-adrenergic antagonists should decrease and increase the severity of AUR, respectively. However, we found that not only phentolamine (α-adrenergic antagonist) but also propranolol (β-adrenergic antagonist) could both reduce the severity of fin ulceration in some tissues. But, in both cases, this blocking effect was relatively weak and did not completely abrogate AUR in stressed hybrid bass. This might have been due to a failure to properly interact with target receptors, inadequate delivery to the target sites, or short half-life. However, it might also be due to the often poor response of fish tissues to these pharmacological agents (see below).

Adrenergic agonists and antagonists have been used *in vivo* and *in vitro* to identify the types and subtypes of adrenergic receptors (ARs)(α_1-, α_2-, β_1-, β_2- or β_3-adrenoceptors), to study the sympathetic system in various fish tissues (Fabbri et al 1999; Fabbri et al 1992; Mayo and Burton 1998; Reid and Perry 1991; Wahlqvist and Nilsson 1981; Wood 1975). Davie (1981) demonstrated that epinephrine infusion caused vasoconstriction via α-

adrenoceptors in the eel tail preparation. Peyraud-Waitzenegger (1979) studied the effect of epinephrine after blockade of α-and β-adrenoceptors with phentolamine or propranolol in European eel (*Anguilla anguilla L.*) and found that 1 mg/kg of either drug effectively inhibited the hypertensive effect of epinephrine. However, Davie (1981) found that phentolamine failed to demonstrate β-dilatory activity, and propranolol actually increased vasodilation rather than inducing vasoconstriction, when administered with epinephrine. These results indicated that the β-dilatory effect was very small and propranolol has both α- and β-antagonism in the eel tail preparation. A small β-dilatory effect in cod tail has also been seen during sympathetic stimulation after a high concentration of phentolamine was administered (Wahlqvist and Nilsson 1981). Taken together, it was suggested that β-dilatory activity in the peripheral blood vasculature (i.e., blood vessels supplying the skin) might be minimal. In addition, Abele et al (1998) demonstrated that epinephrine release by electrostimulation was reduced significantly when phentolamine or propranolol were administered to the American eel, *Anguilla rostrata* (Lesueur). This might explain why phentolamine and propranolol could decrease the severity of AUR in only a few skin tissues.

Another possible reason for the relatively minor effects of the phentolamine and propanolol is that most adrenergic antagonists that are active on mammalian tissues are usually not suitable for specific interactions with fish tissues (Fabbri et al 1999; Gamperl et al 1994b). For example, Opdyke at al (1983) found that propranolol affected both α- and β-adrenoceptors, causing hypotension and tachycardia when administered intravascularly to exercised dogfish (*Squalus acanthias*).

Hexamethonium, a nicotinic ganglionic blocker, has a high specificity for interfering with stimulus transmission across pre- and postganglionic synapses by competing with acethylcholine at nicotinic receptors on the postsynaptic membranes. Hexamethonium administration decreased the peak epinephrine and norepinephrine levels approximately 65% and 40% respectively, in dogfish (an elasmobranch) after a 1-min exercise (Opdyke et al 1983). As neural control of catecholamine secretion from chromaffin tissues involves both nicotinic and muscarinic cholinergic receptors in teleosts (Al-Kharrat et al 1997; Montpetit and Perry 1999), hexamethonium might reduce the peak of catecholamines in hybrid striped bass after stress but probably not completely (Opdyke et al 1983). Blocking nicotinic cholinergic receptors in the chromaffin tissue and thus reducing the epinephrine levels might explain why hexamethonium reduced the severity of epidermal ulceration in our study.

While we were able to reproducibly induce AUR under our standardized experimental conditions, we did observe some variability in the response of fish in various experiments. Possible reasons for this variability include the small number of fish in the samples, the pharmacokinetics of adrenergic agonists and antagonists (i.e., individual differences in absorption, distribution, metabolism and excretion), and the affinity and density of α-and β-adrenergic receptors in various tissues. This is one possible reason for the different degrees of AUR on different fins, ventral skin and cornea after treatment with the antagonists. Only certain doses of the adrenergic blockers were effective in reducing the severity of AUR. For many drugs, only a narrow dosage range can elicit a positive effect, with adverse effects on either side (i.e., too low = no effect, too high = toxic/antagonistic).

In summary, our data suggest that certain physical environmental manipulations might be used to reduce the risk of fish developing AUR when fish are acutely stressed. However, pharmacological intervention using catecholamine antagonists to reduce the severity of AUR requires further investigation.

Table 3.1. Summary of the effects of environmental manipulations on development of AUR lesions in hybrid striped bass.

Experiment	Caudal fin	Dorsal fin	Pelvic fin	Pectoral fin	Anal fin	Ventrum skin	Cornea
Effect of acclimation to different sized culture systems:							
Epidermal degeneration	x	x	x	x	x	x	-
Epidermal erosion	/ [a]	x	x	x	x	x	-
Epidermal ulceration	/ [a]	x	x	x	x	/ [a]	x
Leucocyte infiltration	/ [a]	/ [a]	/ [a]	/ [a]	/ [a]	-	-
Effect of temperature during acute confinement:							
Epidermal degeneration	x	x	x	x	x	-	-
Epidermal erosion	x	x	x	x	x	x	-
Epidermal ulceration	/ [b]	/ [b]	x	/ [b]	x	x	x
Leucocyte infiltration	x	x	x	x	x	-	-
Effect of temperature prior to acute confinement (acclimation temperature):							
Epidermal degeneration	x	x	x	x	x	-	-
Epidermal erosion	x	x	x	x	x	x	-
Epidermal ulceration	/ [b]	/ [b]	/ [b]	/ [b]	x	/ [b]	x
Leucocyte infiltration	/ [b]	/ [b]	/ [b]	x	x	-	-

/ [a] significantly different in the severity of pathological change between fish acclimated in small and in large culture systems, using ANOVA ($p < 0.05$)

/ [b] linear association between the severity of the lesion (epidermal ulceration or leucocyte infiltration) and temperature using the Cochran-Mantel-Haenszel statistical analysis ($p < 0.05$)

x not significantly different or no linear association

- no lesion

Table 3.2. Summary of the effects of adrenergic stimulation or inhibition on the expression of AUR lesions in hybrid striped bass

Experiment	Caudal fin	Dorsal fin	Pelvic fin	Pectoral fin	Anal fin	Ventrum skin	Cornea
Effect of adrenergic stimulation:							
Epinephrine							
Epidermal degeneration	x	x	x	x	x	-	-
Epidermal erosion	x	x	x	x	x	x	-
Epidermal ulceration	/	/	x	/	x	x	x
Leucocyte infiltration	/	x	x	x	x	-	-
Effect of adrenergic inhibition:							
Phentolamine (α adrenergic antagonist)							
Epidermal degeneration	x	x	x	x	x	-	-
Epidermal erosion	/	x	x	x	x	x	-
Epidermal ulceration	x	/	x	/	x	x	x
Leucocyte infiltration	x	x	x	/	x	-	-
Phentolamine (β adrenergic antagonist)							
Epidermal degeneration	x	x	x	x	x	-	-
Epidermal erosion	x	x	x	x	x	-	-
Epidermal ulceration	x	/	/	x	x	x	x
Leucocyte infiltration	/	x	/	x	/	-	-
Hexamethonium (nicotinic antagonist)							
Epidermal degeneration	x	x	x	x	x	-	-
Epidermal erosion	x	x	x	x	x	x	-
Epidermal ulceration	x	/	/	x	x	x	x
Leucocyte infiltration	x	x	x	x	x	-	-

/ linear association between the concentration of epinephrine or blockers and the severity of epidermal change using the Cochran-Mantel-Haenszel statistical analysis ($p < 0.05$)

x no linear association

- no data

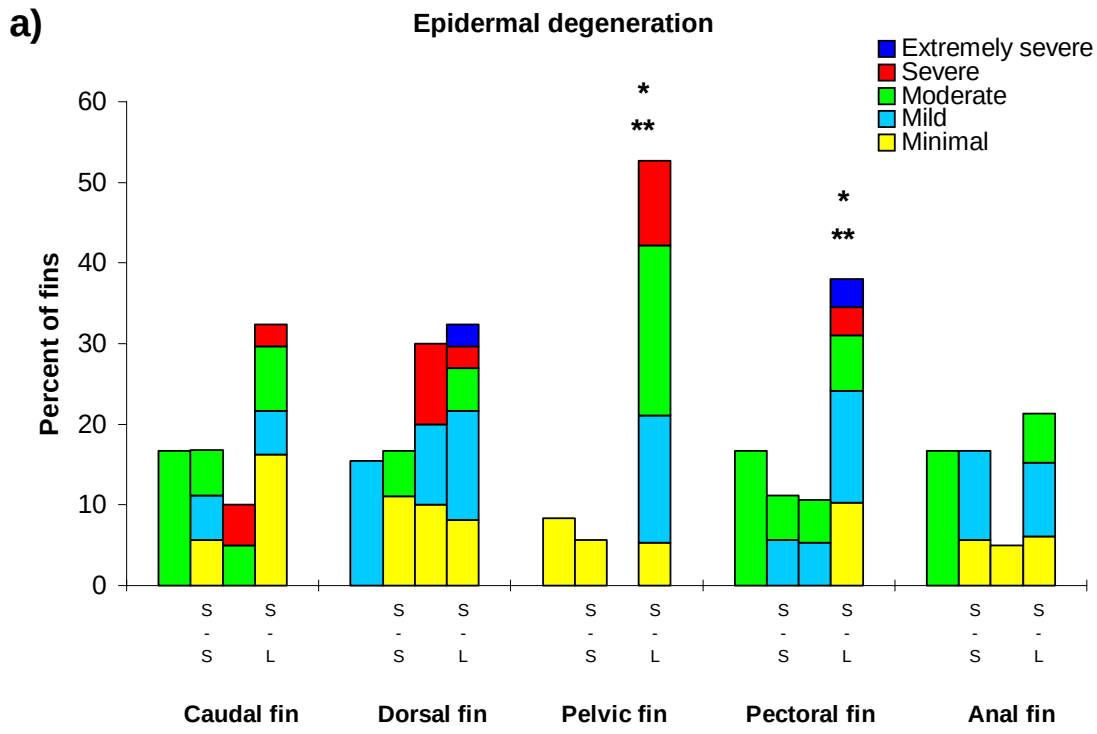
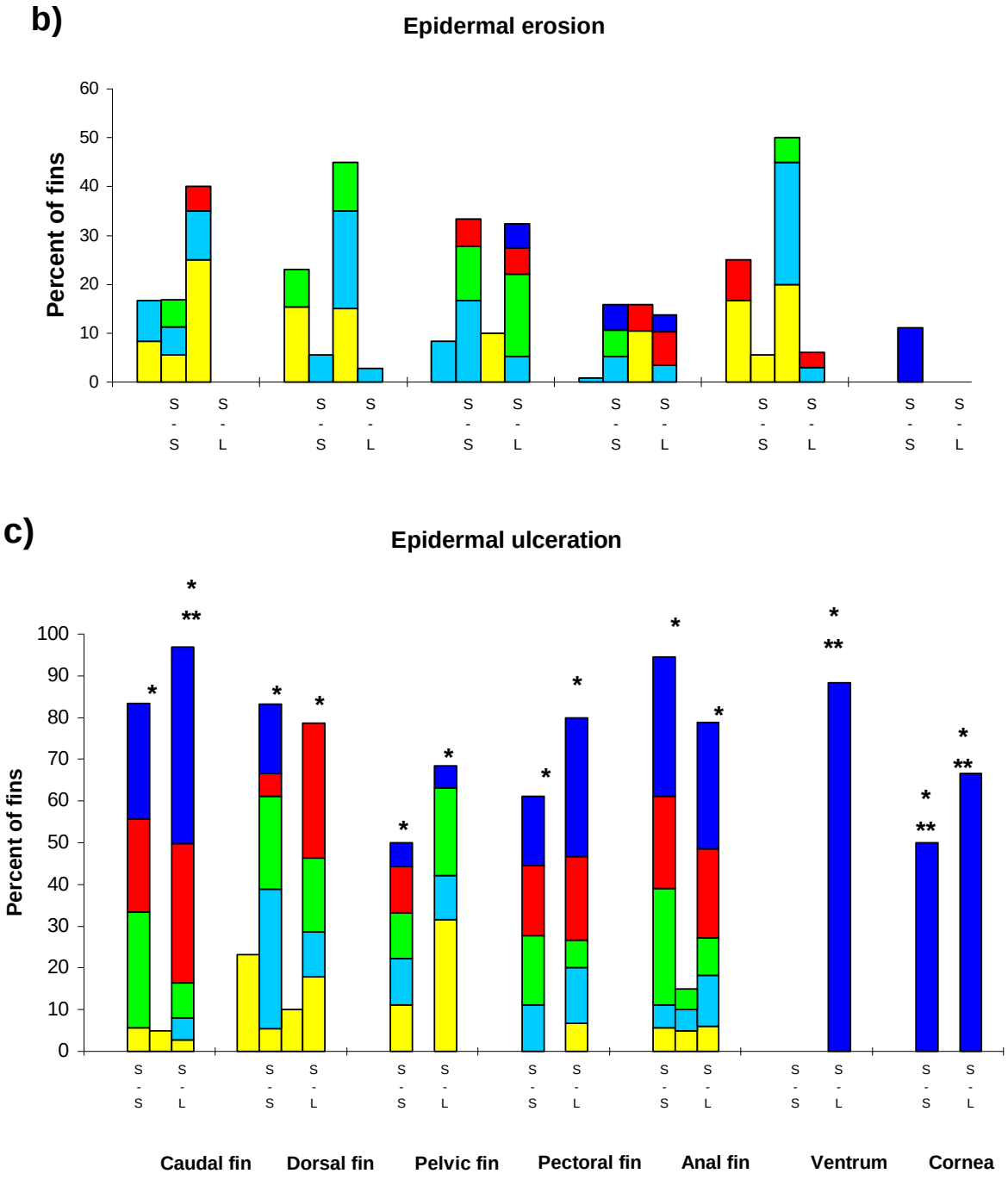


Figure 3.1. Incidence of various degrees of severity of epidermal degeneration, epidermal erosion, epidermal ulceration, and lymphocyte infiltration on each fin type of unstressed (US) and stressed (S) fish acclimated to a small cage (US-S, S-S) or to a large aquarium (US-L, S-L), respectively. There were 3 replications and a total of 12 individuals for both control and stressed fish in each tank size. Data for all 3 experiments was combined for the analysis. * indicates that epidermal damage was significantly greater ($p < 0.05$) in stressed fish than in unstressed fish in the same size acclimation tank by the Fisher test. ** indicates that epidermal damage was significantly greater ($p < 0.05$) in stressed fish from the large tank than in stressed fish from small tank by ANOVA.



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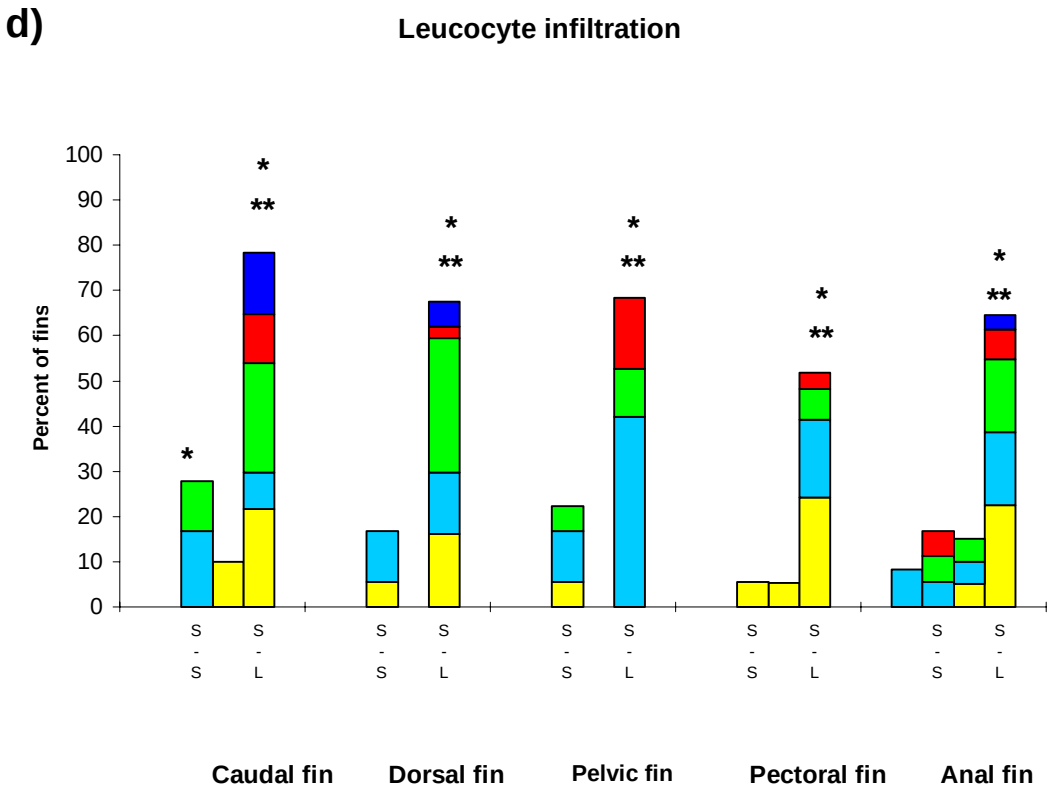


Figure 3.1. Continued.

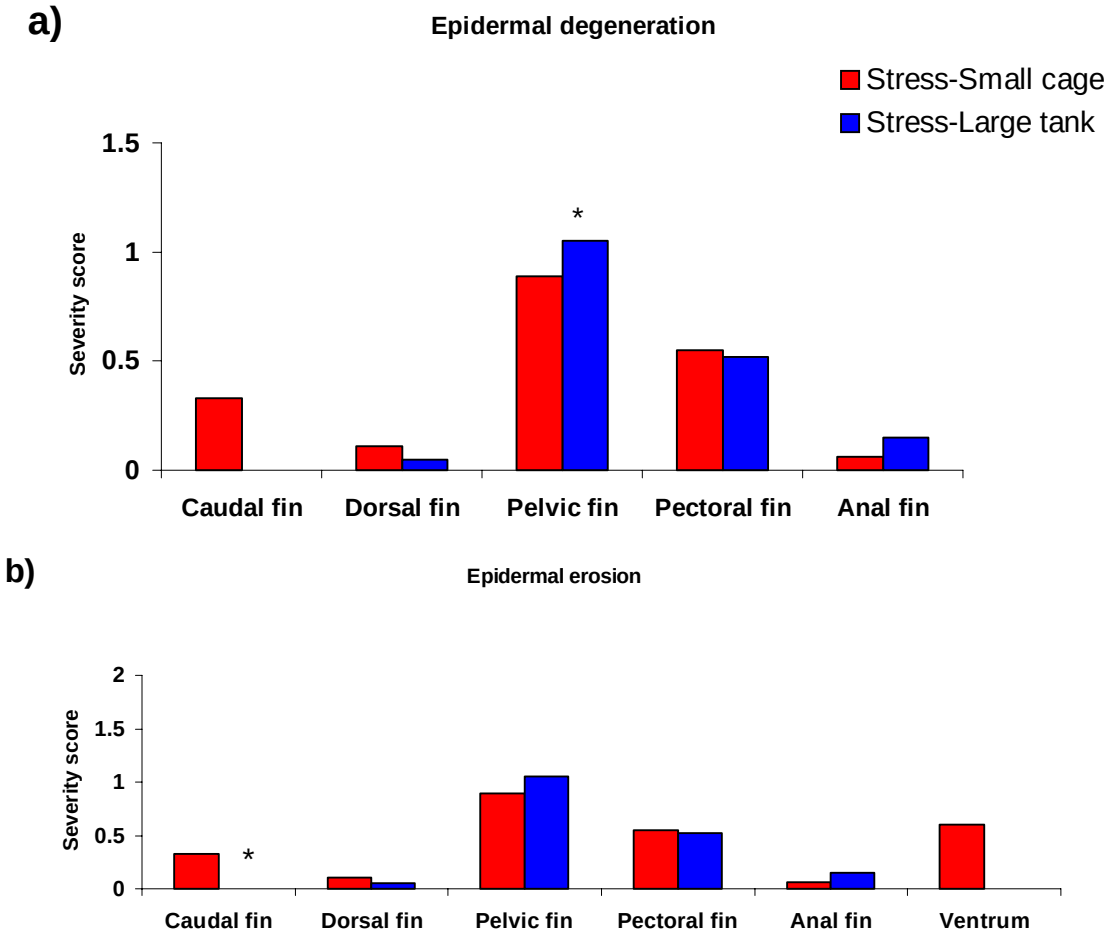
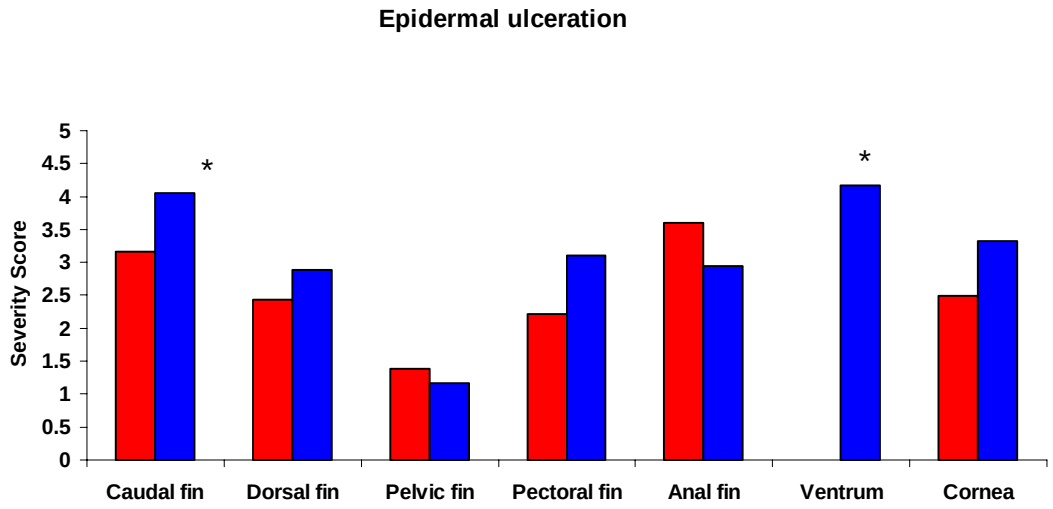


Figure 3.2. Comparison of mean severity scores of pathological changes (epidermal degeneration, epidermal erosion, epidermal ulceration and leukocyte infiltration) between stressed fish acclimated in small cages versus the large aquarium. There were 3 replications and a total of 12 individuals for both control and stressed fish in each tank size. Data for all 3 experiments was combined for the analysis. * indicates that the severity of AUR between stressed fish from small cages versus the large tank was significantly different ($p < 0.05$) by ANOVA.

c)



d)

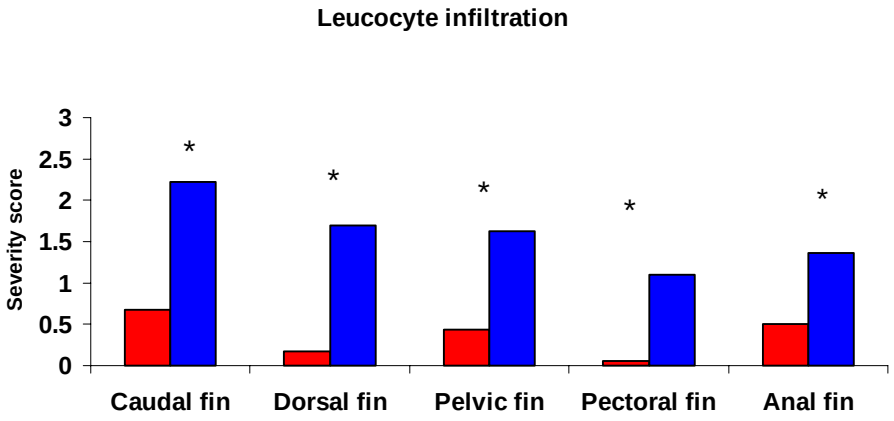


Figure 3.2. Continued.

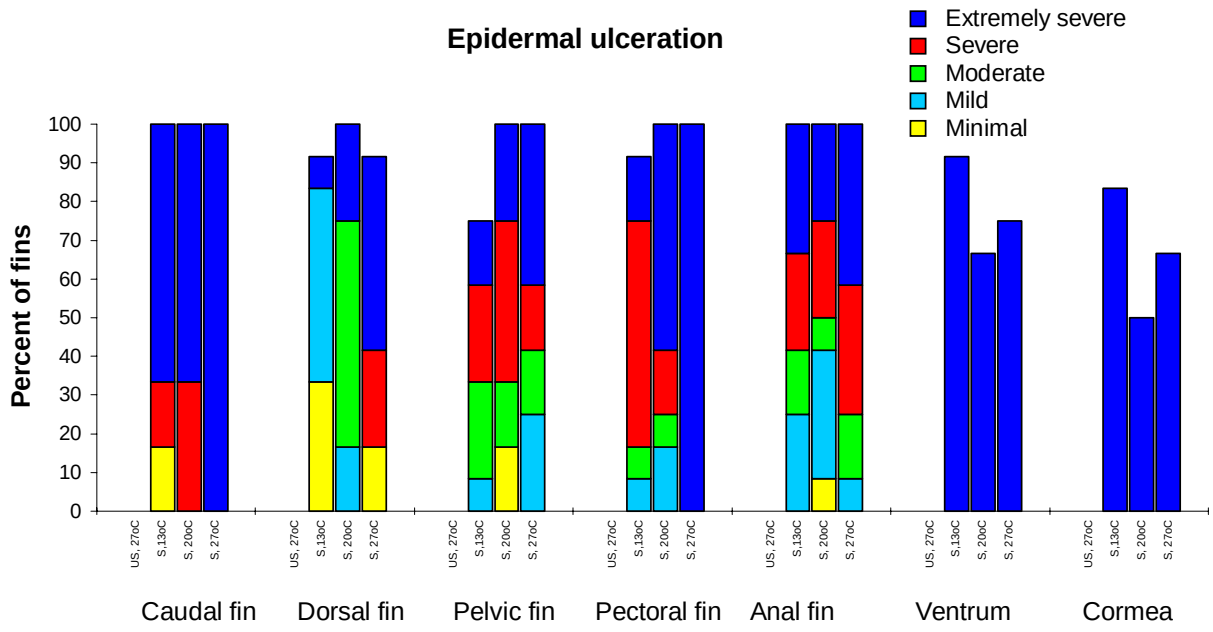


Figure 3.3. Incidence of various degrees of severity of ulceration on each tissue of unstressed (US) and stressed (S) fish during stress at 13, 20, or 27ºC. There were 2 replications and total of 12 fish for each group. Data for both replications were combined. All fish were acclimated to 27°C prior to stressing. Note that none of the unstressed fish had ulceration.

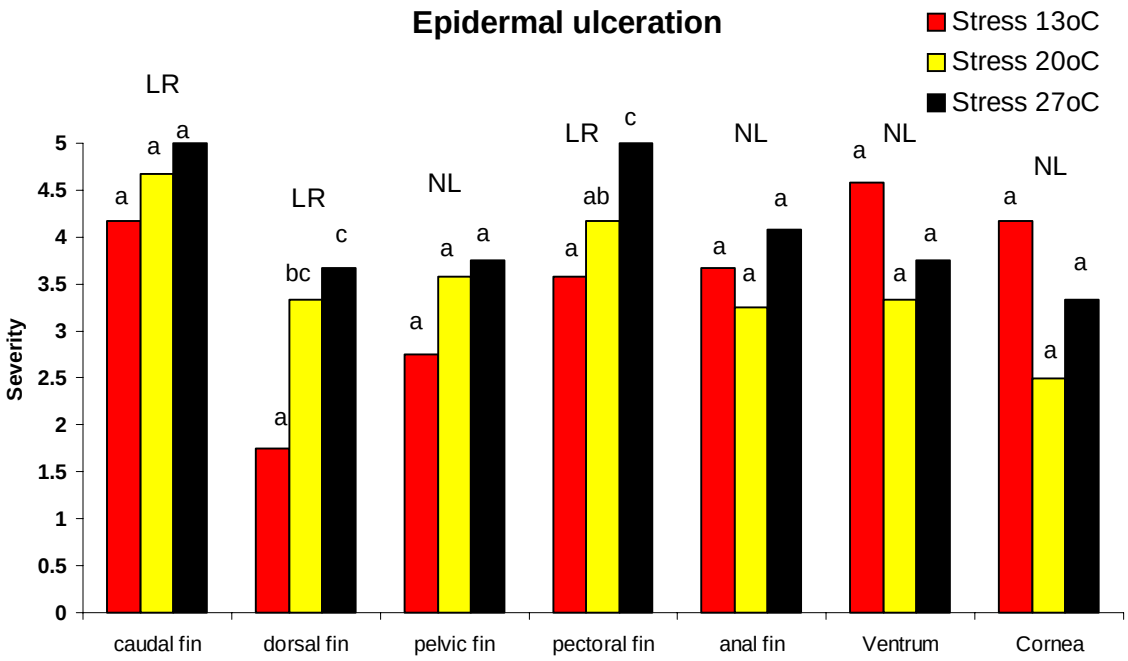


Figure 3.4. The mean severity score of ulceration on each tissue of fish stressed at 13º, 20º, or 27ºC. All fish were acclimated to 27ºC prior to stressing. There were 2 replications and a total 12 fish for each group. Data for both experiments were combined for the statistical analysis. LR indicates that there was a linear association between the mean severity score and temperature using the Cochran-Mantel-Haenszel statistical analysis ($p<0.05$). NL indicates that there was no linear association. Treatments with different letters were significantly different ($p<0.05$) by ANOVA.

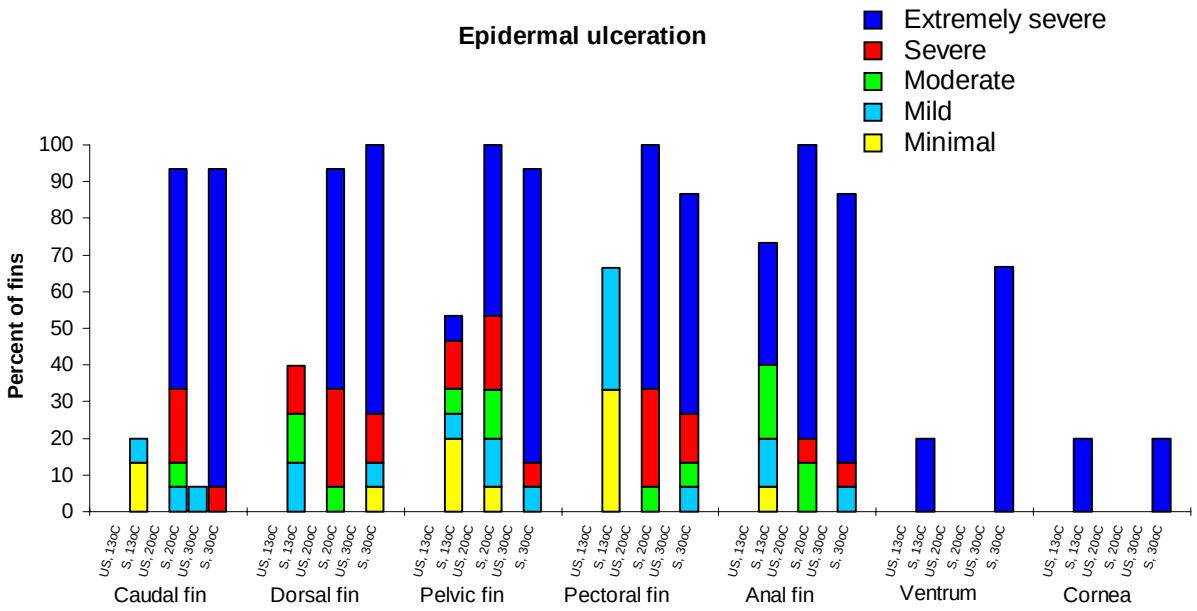


Figure 3.5. Incidence of various degrees of severity of ulceration on each tissue of unstressed (US) and stressed (S) fish at 13º, 20º, or 30ºC. There were 3 replications and a total of 15 fish for each group. Data for all 3 experiments were combined for the analysis. All fish were acclimated to 13º, 20º, or 30ºC prior to stressing.

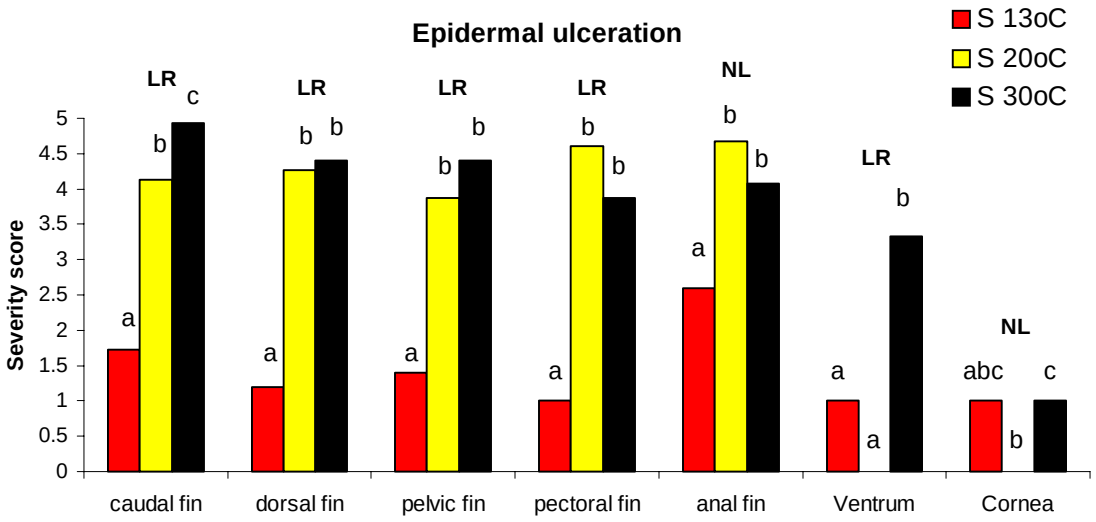


Figure 3.6. The mean severity scores of ulceration on each tissue of fish stressed at 13º, 20º, or 30ºC. All fish were acclimated to 13º, 20º, or 30ºC, respectively, prior to stressing. There were 3 replications and a total 15 fish for each group. Data for all 3 experiments were combined for the analysis. LR indicates that there was a linear association between severity and temperature using the Cochran-Mantel-Haenszel statistical analysis ($p<0.05$). NL indicates that there was no linear association. Treatments with different letters were significantly different ($p < 0.05$) by ANOVA.

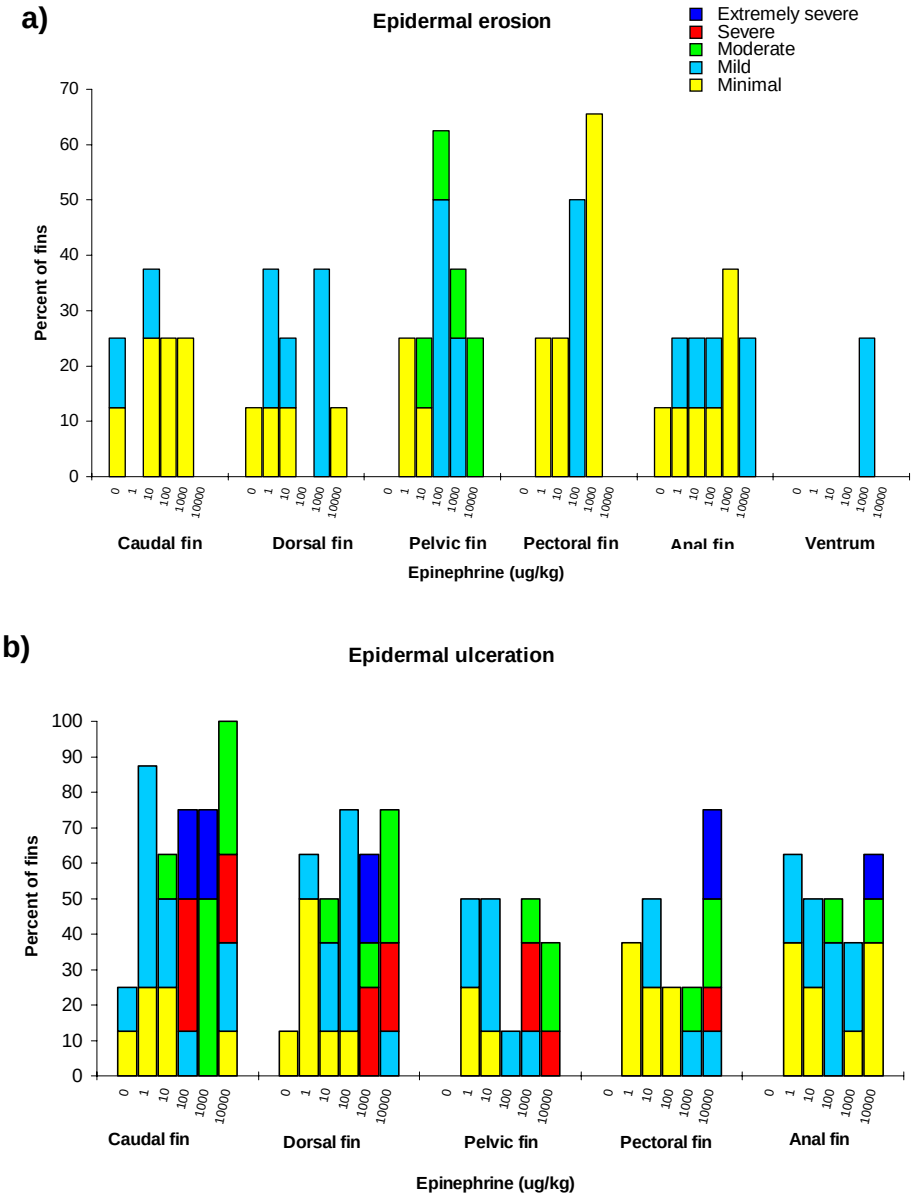


Figure 3.7. Incidence of various degrees of severity of epidermal erosion and ulceration on each fin type of hybrid striped bass administered epinephrine intraperitoneally at a dose of 1, 10, 100, 1,000 or 10,000 µg/kg body weight; control fish were treated with normal saline. The experiment was performed at 27ºC. There were 4 replications and a total of 8 fish for each group. Data for all 4 experiments were combined

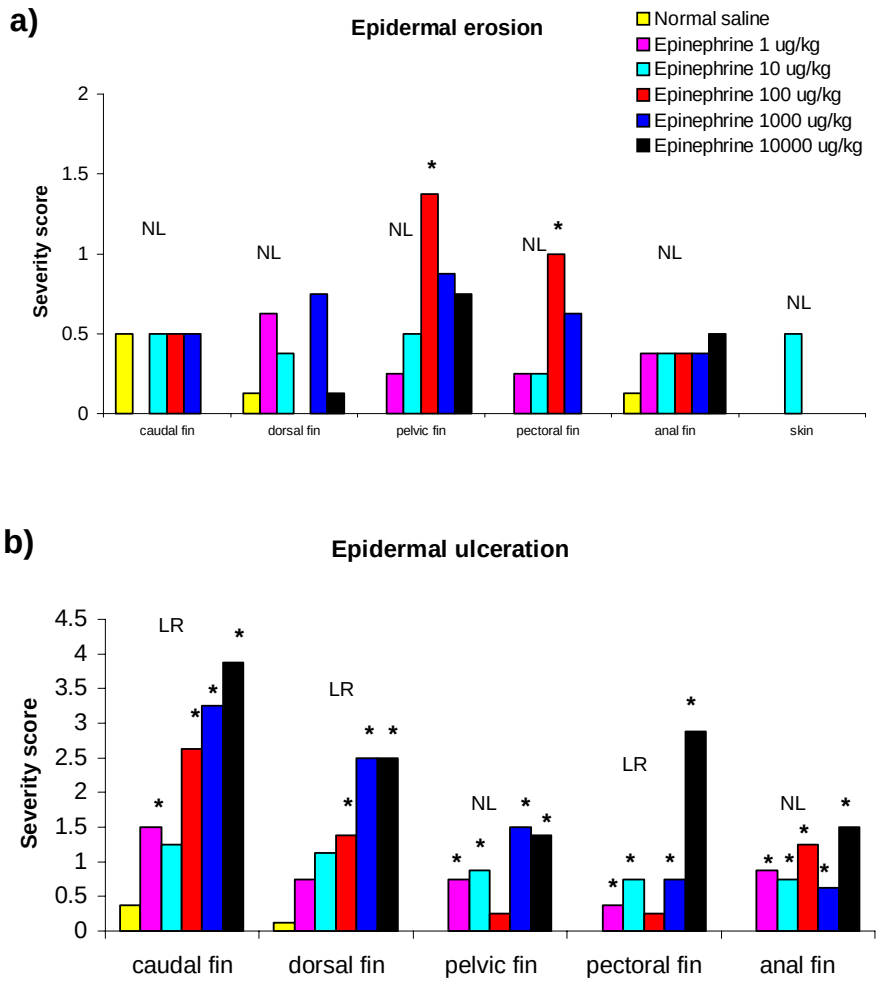


Figure 3.8. The mean severity scores for epidermal erosion and ulceration on each fin type and ventrum skin of hybrid striped bass treated with either normal saline or epinephrine at 1, 10, 100, 1,000 or 10,000 µg/kg body weight. There were 4 replications and a total 8 fish for each group. Data for all 4 experiments were combined for the analysis. LR indicates that there was a linear association between the mean severity score and temperature using the Cochran-Mantel-Haenszel statistical analysis ($p<0.05$). NL indicates that there was no linear association.

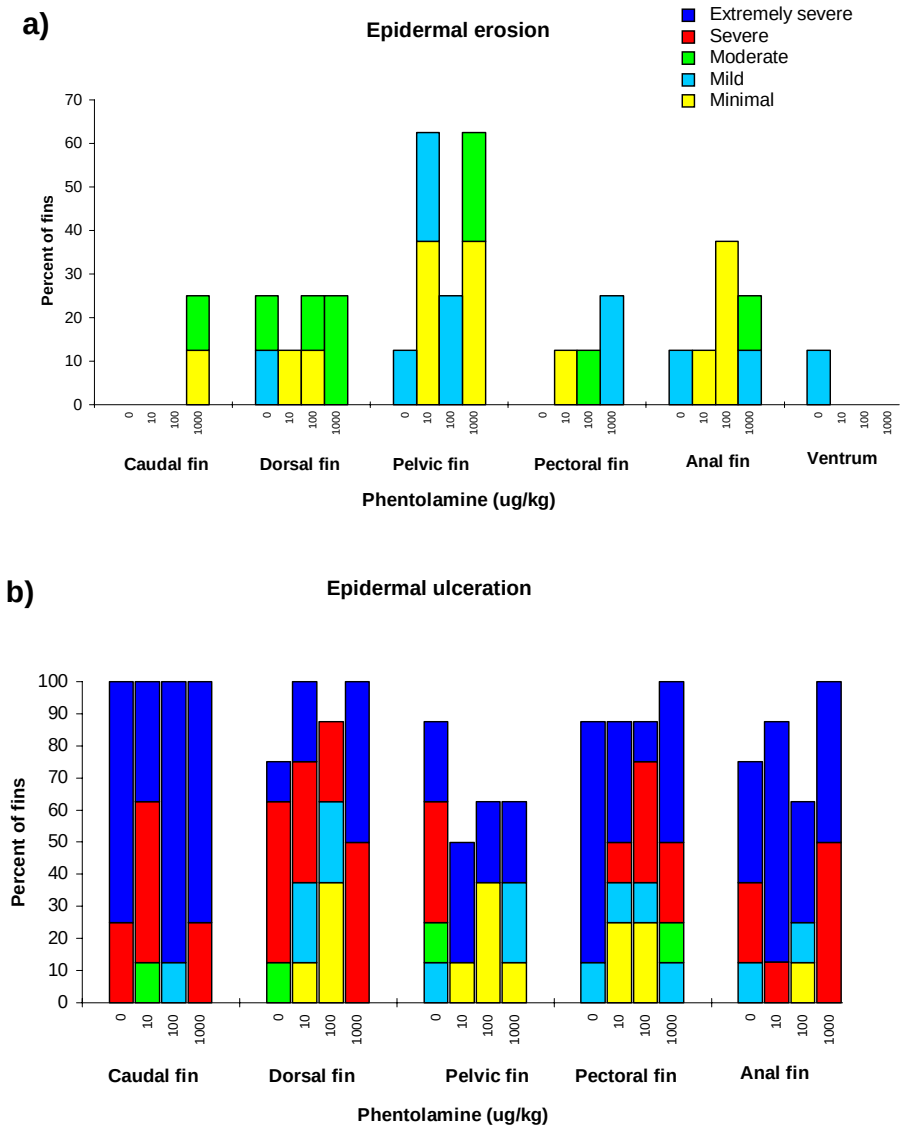


Figure 3.9. Incidence of various degrees of severity of epidermal erosion and ulceration on each fin type and ventrum skin of hybrid striped bass administered phentolamine (α-adrenergic antagonist) at 0, 10, 100 or 1000 µg/kg body weight prior to confinement stress at 27ºC; control fish were treated with normal saline. There were 4 replications and total 8 fish for each group. Data for all 4 experiments were combined for the analysis.

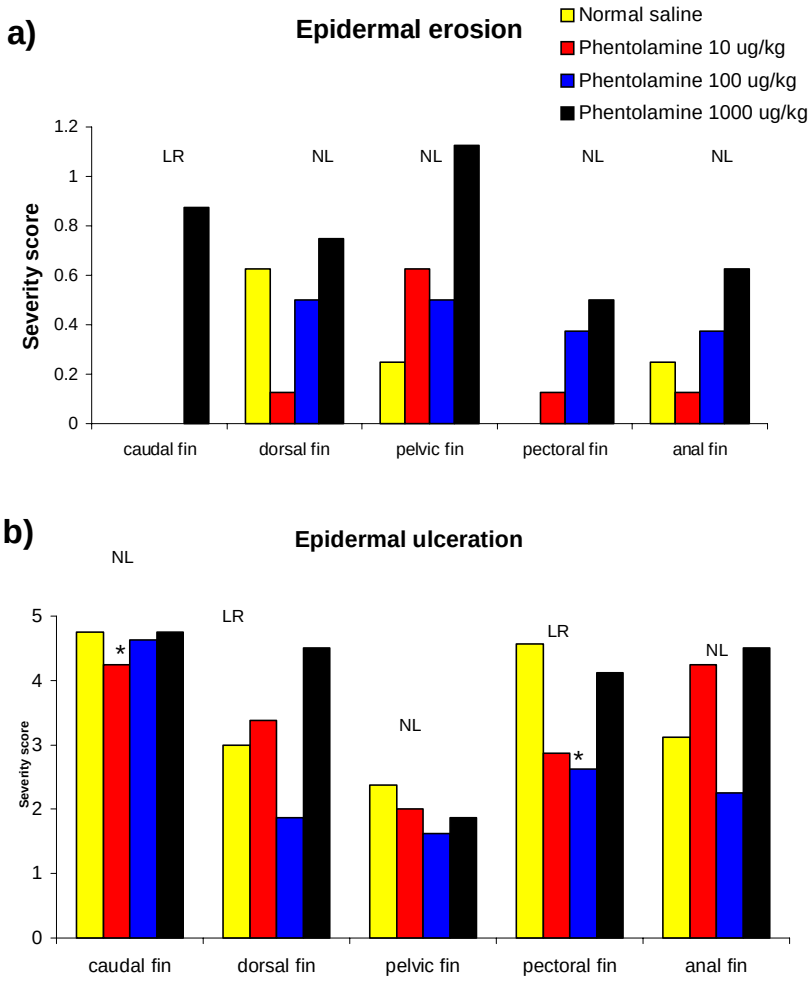
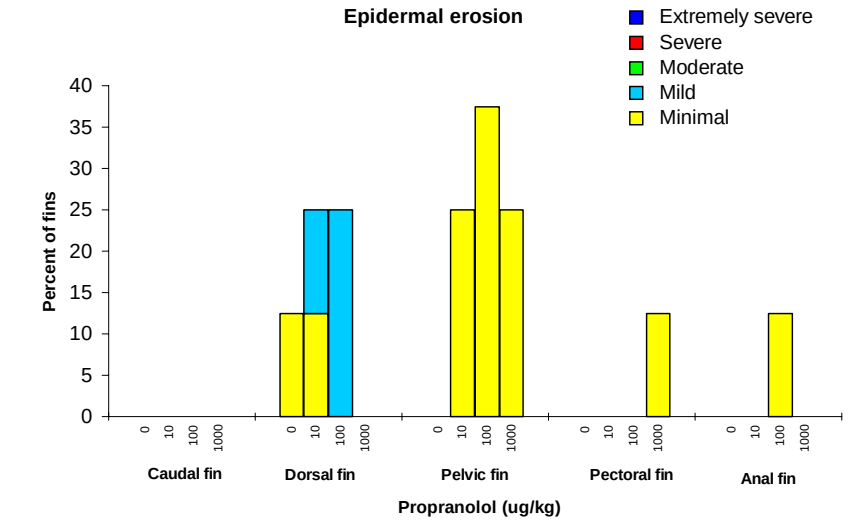


Figure 3.10. The mean severity scores of epidermal erosion and ulceration on each fin type of hybrid striped bass treated with phentolamine at 0, 10, 100 or 1000 µg/kg body weight before acute confinement at 27ºC. LR indicates that there was a linear association between mean severity score and phentolamine concentration using the Cochran-Mantel-Haenszel statistical analysis ($p<0.05$). NL indicates that there was no linear association. There were 4 replications and total 8 fish for each group. Data for all 4 experiments were combined for the analysis.



b)

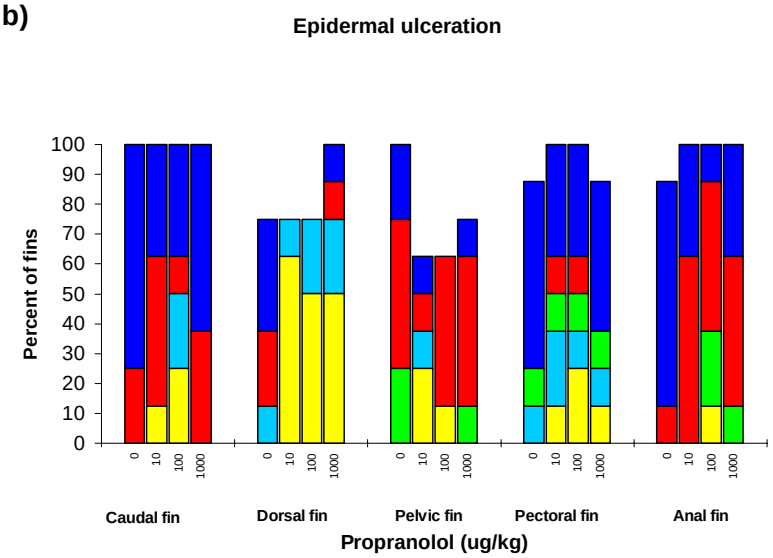


Figure 3.11. Incidence of various degrees of severity of epidermal erosion and ulceration on each fin type of hybrid striped bass intraperitoneally administered normal saline or propranolol (β-adrenergic antagonist) at 0.01, 0.1, or 1.0 µg/kg body weight before confinement stress at 27ºC. There were 4 replications and total 8 fish for each group. Data for all 4 experiments were combined

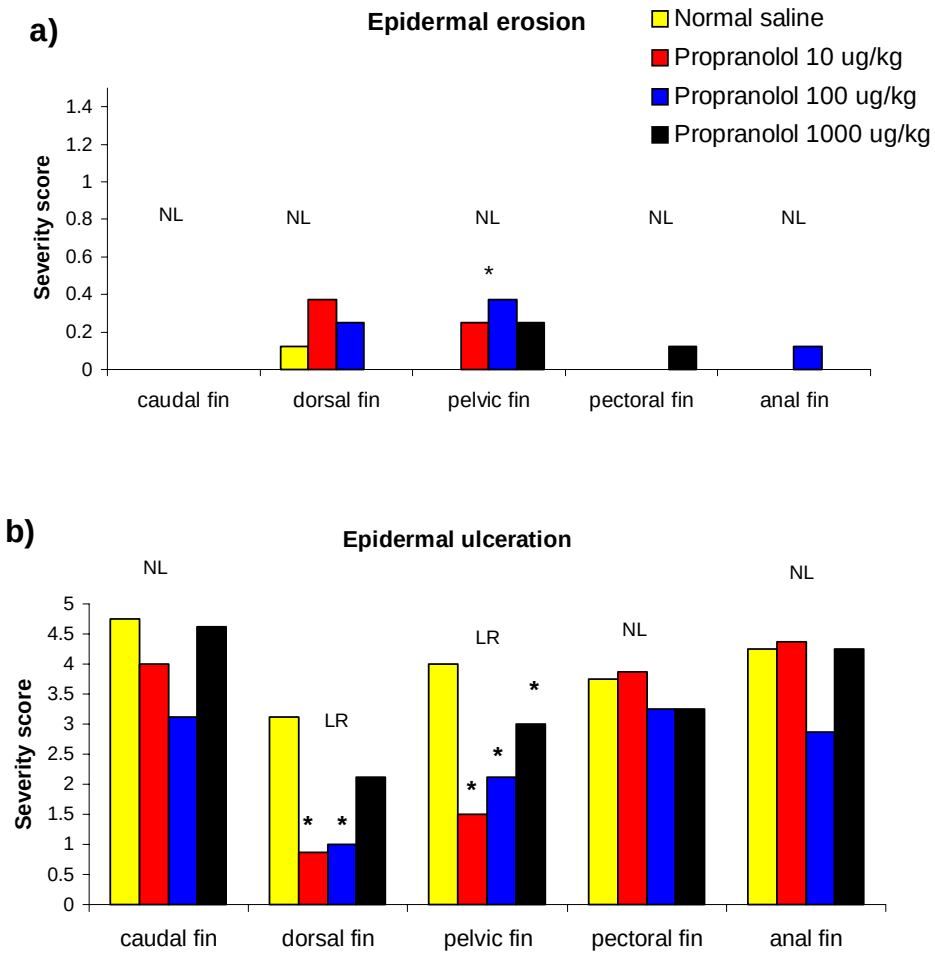


Figure 3.12. The mean severity scores of epidermal erosion and ulceration on each fin type of hybrid striped bass treated with normal saline or propranolol at 0.01, 0.1, or 1.0 µg/kg body weight. LR indicates that there was a linear association between mean severity score and propranolol concentration using the Cochran-Mantel-Haenszel statistical analysis ($p<0.05$). NL indicates that there was no linear association. There were 4 replications and total 8 fish for each group. Data for all 4 experiments were combined for the analysis.

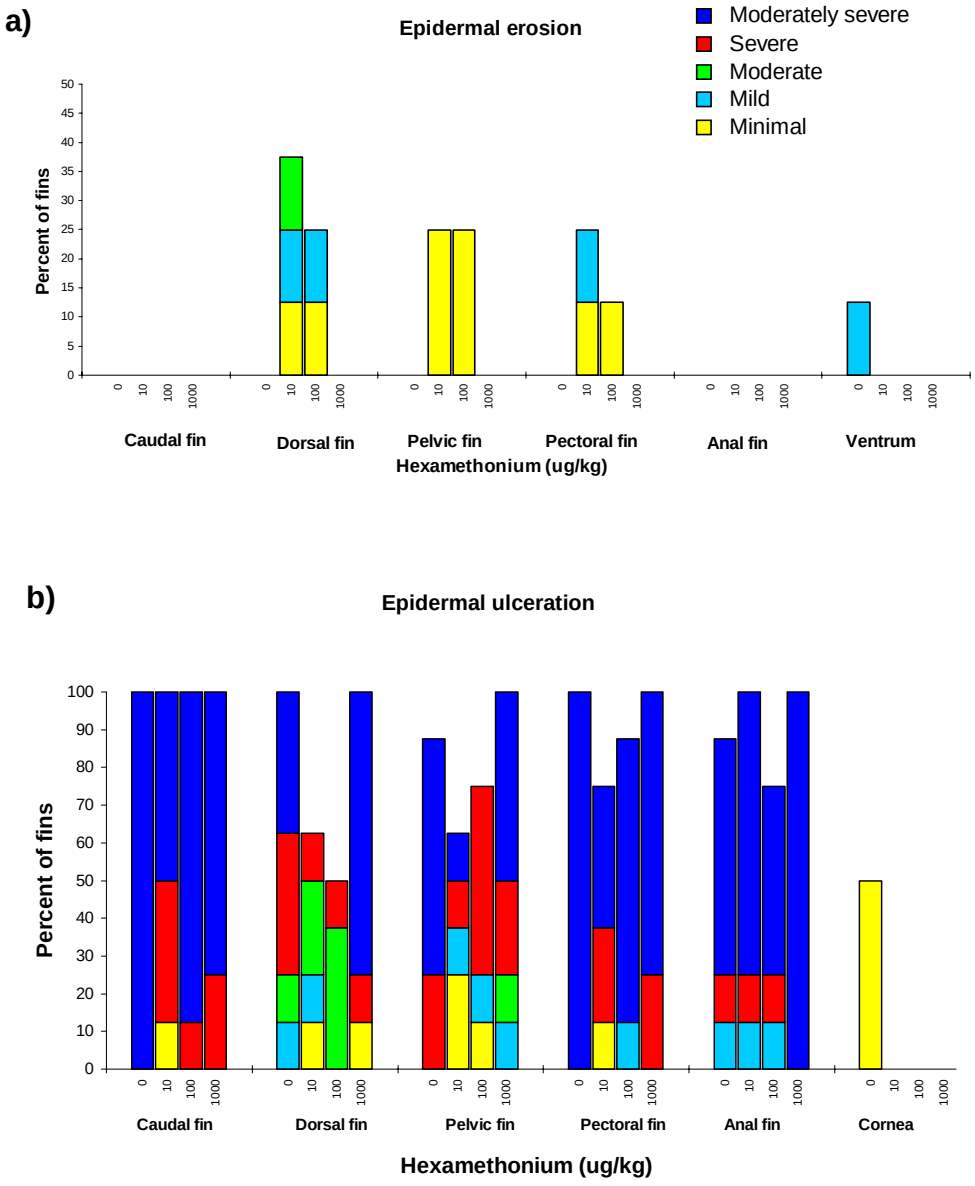


Figure 3.13. Incidence of various degrees of severity of epidermal erosion and ulceration on each fin type and ventrum skin of hybrid striped bass intraperitoneally administered saline or hexamethonium (nicotinic ganglionic blocker) at 10, 100 or 1000 µg/kg body weight before confinement stress at 27ºC. There were 4 replications and a total of 8 fish for each group. Data for all 4 experiments were combined for the analysis.

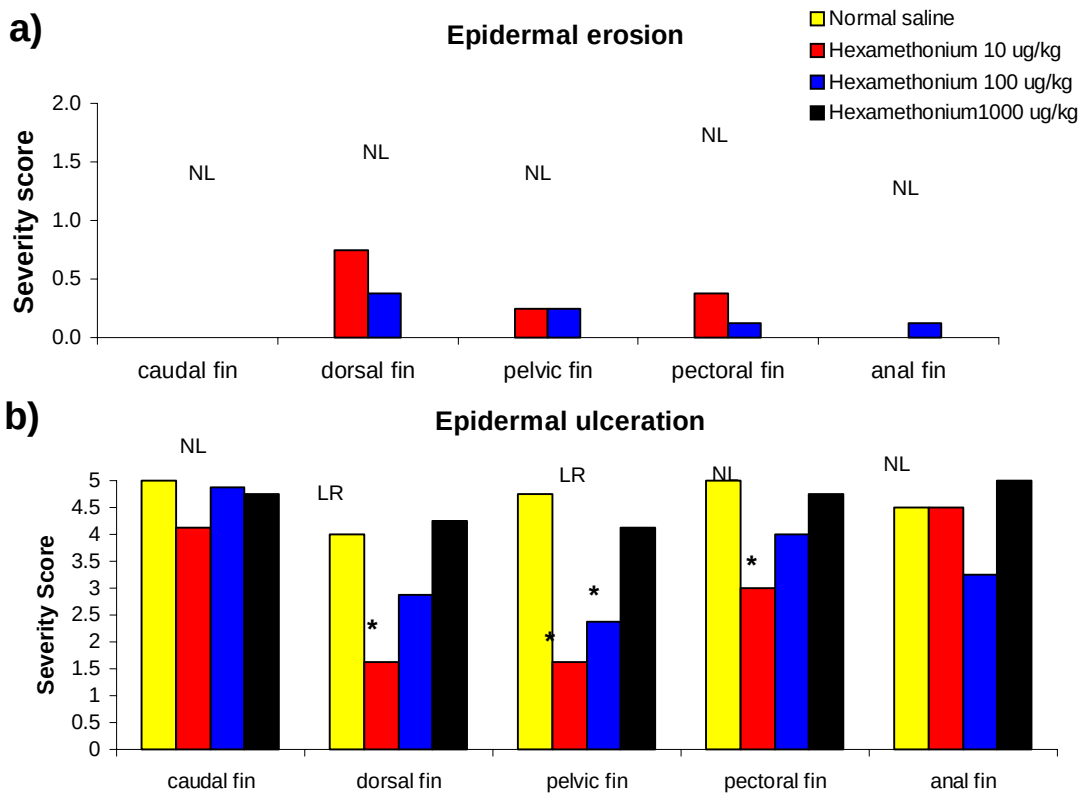


Figure 3.14. The mean severity scores of epidermal erosion and ulceration on each fin type of hybrid striped bass treated with normal saline or hexamethonium at 10, 100 or 1000 µg/kg body weight. There were 4 replications and total 8 fish for each group. Data for all 4 experiments were combined for the analysis. LR indicates that there was a linear association between mean severity score and temperature using the Cochran-Mantel-Haenszel statistical analysis ($p<0.05$). NL indicates that there was no linear association.

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VI.

THE ACUTE ULCERATION RESPONSE (AUR): A WIDESPREAD AND POTENTIALLY SERIOUS CAUSE OF SKIN INFECTIONS IN FISH.

ABSTRACT

In previous studies, we found that rapidly confined hybrid striped bass (*Morone saxatilis* male x *M. chrysops* female) developed a syndrome characterized by the immediate and dramatic loss of their skin. We have named this phenomenon the Acute Ulceration Response (AUR). AUR is characterized by the rapid onset of severe epidermal erosion, ulceration and degeneration on the body skin and fins, as well as corneal ulceration, in stressed hybrid striped bass. In the present study, we have shown that acute confinement stress can also cause AUR in a taxonomically wide array of fish species, including guppy (*Poecilia reticulata*), freshwater angelfish (*Pterophyllum scalare*) and channel catfish (*Ictalurus punctatus*) after a 2-hr stress. However, we could not induce AUR in rainbow trout (*Oncorhynchus mykiss*). The AUR lesions were similar to those seen previously in hybrid striped bass. As AUR might be expected to predispose fish to secondary microbial infections, we examined the skin of hybrid striped bass for bacterial infection after experimental induction of AUR. These experiments showed that even fish with severe skin damage could rapidly heal their wounds without obvious consequences within several days. Bacterial numbers in AUR lesions remained low, ~10^4 cfu/g of fin tissue, throughout the recovery period. However, if hybrid striped bass with AUR were exposed to even low doses (1 zoospore/ml) of the water mold *Saprolegnia*, a relatively weak, opportunistic pathogen, the fish developed severe saprolegniosis as soon as 48 hr after challenge, with 87.5% infected and 87.5 % dead within 4 d post-challenge. In contrast, none of the control fish (no AUR, but exposed to *Saprolegnia*) developed saprolegniosis. These data provide strong evidence that AUR might play a critical role in skin ulcer epidemics of many fish species that are

preceded by an acute stress. Furthermore, our data suggest that environmental pathogen load plays a critical role in determining if AUR lesions will heal spontaneously or instead will lead to devastating disease losses.

INTRODUCTION

Fish diseases are one of the most important problems in the aquaculture industry. Fish diseases are not caused by a single factor but rather are the end result of the interactions of the pathogen(s), the fish (host) and the environment (Sniezko 1974). Fish-microorganism interactions are usually harmless if the fish's immune system is not compromised by a stressor. However, fish diseases frequently occur after fish are subjected to stressful conditions (Pickering 1998; Plumb 1999a; Sniezko 1974).

Fish in intensive culture are continuously affected by various stressors, including environmental fluctuations (e.g., water temperature, pH, dissolved oxygen concentration) and management practices (e.g., crowding from handling or transporting) (reviewed in Wedemeyer 1996). All of these factors can affect fish homeostasis, rendering them susceptible to a wide variety of pathogens. However, despite the general acknowledgement that various stressors can lead to an infectious disease outbreak, the specific mechanisms responsible for these outbreaks is poorly understood.

By far the most common diseases of fish are those that affect the skin. Epidermal damage, especially skin ulceration, is a well-recognized biomarker of polluted or stressful environments (Bernet et al 1999; Sindermann 1990). Epidermal damage may occur via

direct contact (e.g., toxicants, physical trauma); however, skin damage might also occur as an indirect response to largely unknown physiological changes induced by toxins, hormones, or other chemical mediators (Noga 2000).

Previously, we discovered that striped bass (*Morone saxatilis*) and hybrid striped bass (*Morone saxatilis* male X *M. chrysops* female) subjected to acute confinement rapidly (in as little as 15 min) developed skin ulceration, which we have named the Acute Ulceration Response (AUR) (Udomkusonsri, et al, Unpublished Data). With AUR, the fins of stressed fish become ragged and blanched at the distal edges. Epidermal erosion and degeneration eventually lead to severe skin ulceration on the fins; the skin on the body and the corneal epithelium also become ulcerated. Whether other fish are susceptible to AUR has not previously been determined.

Besides the severe osmotic stress that can be imposed by acute skin loss, skin ulceration can also greatly increase the risk of contracting an infectious disease. The skin of fish is a primary defense mechanism that contains many specific and nonspecific defense factors, including immunoglobulin, lectin, complement-like proteins, lysozyme and antimicrobial polypeptides (Robinette and Noga, 2001; Yano 1996). When AUR occurs, fish lose this protective barrier, and opportunistic bacteria (e.g., *Aeromonas*, *Pseudomonas*), parasites, viruses or water molds (e.g., *Saprolegnia* sp.) might be more readily able to invade the skin and cause infection (Kerby 1993; Plumb 1997, 1999b; Wedemeyer 1996).

While many of these pathogens are relatively easily treatable, some are not and cause widespread mortality in both hatchery and wild fish. For the latter pathogens, prevention of

infection is critical for management. One of the most classical of the stress-related pathogens is the water molds (oomycetes) (Neish 1977; Pickering and Willoughby 1982). Water molds are also one of the most important microbial pathogens of fish, as well as one of the most difficult to treat (Plumb 1997; Roberts 2001). Most clinically important members are in the Family *Saprolegniaceae*, with 3 genera commonly affecting aquacultured fish: *Saprolegnia*, *Aphanomyces*, and *Achlya*. They cause a disease commonly called saprolegniosis, which affects virtually all species and ages of freshwater fish (Pickering and Willoughby 1982). Because saprolegniosis is so difficult to treat, understanding means of preventing its occurrence are critical to its successful management.

Thus, the objectives of our study were to determine whether we could reproduce AUR in various commercially important fish species after confinement stress, and whether fish recovering from AUR were more susceptible to microbial infections, especially saprolegniosis.

MATERIALS AND METHODS

Fish used for experiments

Hybrid striped bass (*Morone saxatilis* male X *M. chrysops* female), 13 months old and 95-130 mm total length, were maintained in a 760-liter holding aquarium at 14ºC. Fish were fed a commercial feed at approximately 2% of their body weight daily and maintained on a 12-hour light:12-hour dark photoperiod. The low temperature and restricted feed provided a constant supply of small, similar-size fish for all experiments. Water quality during all

experiments was: dissolved oxygen 6.8-7.5 mg/l, temperature 27ºC, pH 6.65-6.87, unionized ammonia <0.001 mg/l and nitrite <0.10 mg/l.

Bacterial concentrations in fish recovering from AUR

Twenty-one hybrid striped bass were acclimated in a 1500-liter aquarium at 27ºC for 2 weeks. After acclimation, 3 fish were sacrificed with anesthetic overdose (200 mg/l tricaine buffered with 400 mg/l sodium bicarbonate) as control, unstressed fish; the other 18 fish were then subjected to acute stress by confining fish individually in plastic mesh boxes (size 3.5 x 14 x 12 cm) for 2 hr at 27ºC. Boxes were agitated every 15 sec per min during confinement. Confinement caused AUR on the fins and skin of stressed fish (Udomkusonsri et al, in preparation). After the 2-hr stress, 3 fish were sacrificed with anesthetic overdose and fixed in 10% neutral buffered formalin to confirm induction of AUR by histology. The remaining 15 fish were placed in triplicate, 75-liter aquaria at 27ºC (5 fish per aquarium). One fish from each aquarium (3 fish total) was sacrificed at various times after the acute stress: 16 hr, 24 hr, 2, 4 and 7 days.

Caudal fins were aseptically collected from the most distal part of the caudal fin (since AUR progresses from the tip toward the base of the caudal fin). Tissues were weighed and minced into fine pieces (~0.5 x 0.5 mm^2 or less) with a sterile scalpel, and placed into sterile microcentrifuge tubes having 1.0 ml of buffered peptone water (BPW) (0.1% peptone, Sigma Chemical Co., St. Louis, MO, in 10 mM phosphate buffer). After the tissue was vortexed well in BPW, the supernatant was transferred to another sterile tube. The supernatant was serially diluted 10-fold in BPW and cultured on cytophaga agar (CA - 0.5 g

tryptone, Difco, Detroit, MI; 0.5 g yeast extract, Difco; 0.2 g sodium acetate, Sigma; 0.2 g beef extract, Sigma; 11.0 g agar, Difco; in 1 liter water, at pH 7.2) and brain heart infusion agar (BHIA - 26 g BHI agar, Difco, in 500 ml water). CA and BHIA plates were incubated at 25ºC. Bacterial colonies were counted after incubating duplicate plates for 3 days or until all colonies appeared. Cytophaga agar is a low-nutrient, non-selective medium for the isolation of fish pathogenic cytophagas, flavobacteria and flexibacters; BHIA is an enrichment, non-selective medium for general bacterial culture (Austin and Austin 1993; Frerichs 1993). The dominant colonies in the CA and BHIA were identified as gram-positive or gram-negative by gram staining.

Susceptibility of fish with AUR to saprolegniosis

Water mold culture

Saprolegnia sp. (Isolate # 97-2005A1) was isolated from skin lesions of striped bass (*Morone saxatilis*) held at North Carolina State University College of Veterinary Medicine by S Wada in 1997. After several passages at that time, the water mold had been cryopreserved in liquid nitrogen until use in the present studies. The isolate was maintained on corn meal agar (Difco) at 20ºC. For experiments, it was cultured on glucose-yeast extract (GY) agar (1 g glucose, Sigma; 1.25 g yeast extract; 1.5 g agar; in 100 ml deionized water) at 19ºC for 2 days. Agar with mycelia was then cut aseptically into 8 by 8 mm. squares and placed into a petri-dish having 30 ml GY broth. After 2 days, the agar remnants were removed and the mycelia were washed three times with sterile sporulation medium [MSM (modified Griffin's sporulation medium), 0.25 mM $CaCl_2$, Sigma; 0.25 mM KCl, Sigma; 20 mM Hepes, Sigma; 0.01 g/l phenol

red, Sigma; pH 7.4 (Griffin 1978)]. The mycelia were then incubated in MSM at 19ºC for 24 hr to induce zoospore formation. Zoospores were collected by filtering the entire culture through sterilized Whatman 541 filter paper. The zoospore suspension was counted under an inverted phase-contrast microscope and then added to experimental tanks at a concentration of 1 zoospore/ml. In preliminary experiments, stressed hybrid striped bass were challenged with the *Saprolegnia sp.* Isolate at a concentration of 10 or 50 zoospores/ml; all fish developed saprolegniosis 2 days after challenge.

Experimental fish

Forty hybrid striped bass were acclimated to a 1500-liter aquarium with freshwater at 19ºC. After 2 weeks, AUR was induced in 20 fish by confining fish individually for 1 hr in plastic mesh boxes as described above. Four stressed fish were sacrificed with anesthetic overdose and fixed in 10% neutral buffered formalin to confirm induction of AUR by histology. The other 16 fish were placed into four 75-liter aquaria (4 fish/aquarium) filled with freshwater at 19ºC. For the *Saprolegnia*-challenged group, zoospores (final concentration of 1 zoospore/ml) were added to duplicate aquaria immediately after the fish were placed into the aquaria. Before zoospores were added, the biological filters were disconnected and air stones were left running to provide oxygen to the water. Fish were exposed to zoospores for 24 hr and then moved to new aquaria at 19ºC. For the sham-challenged group, no zoospores were added to the other duplicate aquaria.

The remaining twenty fish were taken from the acclimating aquarium and four fish were sacrificed immediately with anesthetic overdose as control, unstressed fish to confirm the

absence of AUR via histology. The other 16 fish were randomly distributed into four 75-liter aquaria (4 fish/aquarium) filled with freshwater water at 19ºC. For the challenged, unstressed group, zoospores were added to replicate aquaria to give a final concentration of 1 zoospore/ml. Fish were challenged with zoospores for 24 hr and then moved to clean aquaria. No zoospores were added to the other two duplicate aquaria (negative control = no AUR, no zoospores). All aquaria were covered during the tests to reduce possible cross-contamination of water mold zoospores. Fish were observed daily for 14 days for signs of saprolegniosis, grossly evident as a cottony mass on the body. When fish appeared moribund with typical saprolegniosis lesions, they were biopsied for wet mounts or culture, and sampled for histopathological evaluation. All of these procedures were done to all zoospore- and sham-challenge fish.

Confirmation of water mold identification

Water mold infection was confirmed by observation of broad, aseptate, hyphae in wet mounts of skin lesions. The water mold was identified as *Saprolegnia* by induction of asexual zoosporangia. Briefly, infected tissues were inoculated onto GY agar. The GY plates were incubated at room temperature and visible growth was usually seen within 48 hr. The agar with mycelia were cut into 8 x 8 mm squares, and processed the same as the methods used to produce zoospores. Asexual sporangia were observed after incubation in MSM for 24-48 hr at 19ºC.

Induction of AUR in other fish species

Guppies (*Poecilia reticulata*) (2.8-4.0 cm total length) and freshwater angelfish (*Pterophyllum scalare*) (3.5-4.5 cm total length) were acclimated to 150-liter aquaria at 25ºC

and channel catfish (*Ictalurus punctatus*) (9.0-11.0 cm total length) were acclimated to an 1100-liter aquarium at 30ºC. Rainbow trout (*Oncorhynchus mykiss*) (4.5–5.0 cm total length) were acclimated in a 150-liter aquarium at 15ºC. After 2 weeks of acclimation, fish were confined individually in confining boxes for 2 hr at 25ºC for guppies and angelfish, 30ºC for channel catfish and 15ºC for rainbow trout. Confinement boxes were 3.5 x 7 x 12 cm for guppies and angelfish and 3.5 x 14 x 12 cm for catfish and rainbow trout. Boxes were agitated every 15 sec per min during the 2-hr confinement. Fish were sacrificed with anesthetic overdose after the acute stress. Fin tissues (caudal fin of guppies; caudal, dorsal and anal fins of angelfish; caudal, dorsal, pelvic, pectoral and anal fins of catfish and rainbow trout) and flank skin of catfish and rainbow trout were fixed in 10% NBF and processed for light microscopy. Fin tissues were evaluated for pathological changes as described below.

Histopathology

Tissues of all fish to be examined histologically were fixed in 10% NBF; calcified tissues were also decalcified in 100 g/l ethylenediaminetetraacetic acid (EDTA) in 0.1M phosphate buffer (pH 7.2). Tissues were then embedded in paraffin, and processed routinely for light microscopy. All sections were stained with hematoxylin and eosin (H&E). For quantitative analysis of damage due to AUR, all tissues were evaluated blindly as described in Noga et al (1998). Briefly, lesions were scored on a scale of 1-5, with (1) being minimal damage (1-20% of area affected by the lesion), (2) being mild damage (21-40% of area affected by the lesion), (3) being moderate damage (41-60% of area affected by the lesion), (4) being severe damage (61-80% of area affected by the lesion) and (5) being extremely

severe/high damage (81-100% of area affected by the lesion). All fin tissues were oriented in the longitudinal plane, and then evaluated for pathological changes: epidermal erosion, epidermal ulceration, and leucocyte infiltration at the affected fins. Flank skin and cornea were evaluated for epidermal erosion and ulceration. In *Saprolegnia* challenge experiments, sections were also stained with Gomori methenamine silver stain (GMS) to demonstrate water mold hyphae.

Statistical analysis

For comparing the severity of pathological change between control and stressed fish, one-way analysis of variance (ANOVA) was performed with the Statistical Analysis System (SAS Version 8, SAS Institute, Cary, NC) and a p-value <0.05 was set as the limit for statistical difference.

RESULTS

Bacterial concentrations in fish recovering from AUR

After 2-hr stress, all hybrid striped bass developed gross evidence of AUR (blanched and ragged fins). Advanced AUR was confirmed via histopathology in the 3 fish sampled at time 0. The number of bacteria from the caudal fins of stressed fish ranged from 0.3-9.7 x 10^4 bacteria/g of tissue on CA and 0.6-7.4 x 10^4 bacteria/g of tissue on BHIA. There were consistently low numbers of bacteria at all sampling times for up to 7 days post-stress (Figure 4.1a, b). One of three fish sampled on day 7 had reddening on the caudal fin, but the

amount of bacteria was similar to the other fish sampled at the same time (data not shown). All bacteria examined were gram-negative bacilli.

Susceptibility of fish with AUR to saprolegniosis

After the 1-hr confinement, the stressed fish displayed gross evidence of AUR that was confirmed by the subsample of fish that was examined histologically prior to the *Saprolegnia* challenge. These fish displayed skin and fin lesions diagnostic for AUR: epidermal degeneration, epidermal erosion, epidermal ulceration, and corneal ulceration. No fish died after the 1-hr confinement, but *Saprolegnia*-challenged fish began to develop signs of water mold infection (i.e., a cottony mass growing on the body skin and fins) within 2 days post-challenge. Fish were lethargic and lost equilibrium as the hyphal mass became more extensive. Mortality began within 2 days after challenge (Table 4.1). All dead fish showed signs of saprolegniosis grossly prior to death. Water mold-infected fish had white cottony masses on the skin of the head, operculum, dorsum, ventrum, peduncle, and/or fins (Figure 4.2a). Infected fish typically died within 12 hr after the water mold infection was grossly evident. One of the stressed fish challenged with zoospores did not develop any signs of water mold infection after 14 days, while one of the stressed fish from the sham-challenged group did develop saprolegniosis (Table 4.1).

The positive and negative controls (unstressed fish), with and without zoospore challenge, respectively, showed no water mold infection during the 14-day experimental period. Nor did these control fish have any visible damage on the skin, fins or cornea.

Histopathologically, water mold-infected fish displayed mycelial mats on the fins, head, operculum and ventrum, as well as sloughing of epidermis, and edema and degenerative changes in the dermis (Figure 4.2b, c). Biopsies of lesions displayed abundant, branching, aseptate, hyphae; some lesions also had immature zoosporangia that were identified as *Saprolegnia* sp. (Figure 4.2d). This was further confirmed by the identification of *Saprolegnia* zoosporangia in all lesions from zoospore-challenged fish (n=7) and one sham-challenged fish which cultured on GY agar.

Induction of AUR in other fish species

Guppies, freshwater angelfish and channel catfish, but not rainbow trout, developed gross and histopathological lesions typical of AUR after the 2-hr stress. The fin damage in stressed guppies, freshwater angelfish and channel catfish was very evident (Figure 4.3, 4.4, 4.5) and the histopathological changes on the affected fins showed epidermal degeneration, erosion, and ulceration (Figures 4.6a-f). The stressed guppies developed epidermal ulceration (severity score = 1.8) on their caudal fins significantly more severe than lesions observed in unstressed fish which had no epidermal damage ($p < 0.05$) (Figure 4.7a). A similar response was seen in stressed freshwater angelfish, where the severity of epidermal ulceration in caudal, dorsal and anal fins (average severity score = 3.6, 3.1 and 1.7, respectively) were significantly greater than in that in control, unstressed fish which did not have any epidermal damage ($p < 0.05$) (Figure 4.7b).

Stressed channel catfish also developed epidermal damage on the fins, with epidermal ulceration affecting the caudal, pectoral and anal fins (average severity score of epidermal

ulceration = 2.0, 0.4 and 3.3 respectively); however, there was no evidence of epidermal ulceration on dorsal or pelvic fins of stressed catfish. The severity of epidermal ulceration and lymphocyte infiltration on caudal and anal fins was significantly greater in stressed catfish than in controls ($p < 0.05$) (Figure 4.7c). The ventral skin of stressed catfish did not developed ulceration.

DISCUSSION

Many fish are susceptible to AUR

We successfully induced AUR in guppies, freshwater angelfish and channel catfish after a 2-hr confinement stress. The most severely affected fish species was the freshwater angelfish, followed by the channel catfish, and then the guppies, in which the average severity scores of caudal fin ulceration were 3.6, 2.0 and 1.8, respectively. However, none of the lesions were as severe as what we have consistently reproduced in hybrid striped bass, which the average severity of caudal fin ulceration were 4.1 (Udomkusonsri et al, Unpublished Data). We were not able to induce AUR in rainbow trout. Epidermal erosion and ulceration of caudal fins occurred prominently in both the angelfish and guppies. Channel catfish developed AUR on the caudal, pectoral and anal fins, but not on the dorsal and pelvic fins. The reason for the different susceptibilities of various fish species to AUR is unknown. We have evidence that the adrenergic response to stress may be at least partly responsible for the development of AUR in hybrid striped bass (Noga et al 1998). Epinephrine can cause peripheral vascular shutdown, which might be at least partly responsible for the tissue damage. The increase in blood catecholamines after acute stress varies greatly among different fish species. For example, the

plasma epinephrine (E) and norepinephrine (NE) levels of channel catfish after an acute stress (holding in a net for <1 min) were 2.17±0.26 and 8.07±2.44 pmol/ml (Finkenbine et al 2002). These plasma levels of E and NE in stressed channel catfish were equivalent to the resting levels in other fish. The resting levels of E and NE in rainbow trout are even lower: 2.6 and 3.3 pmol/ml, respectively, compared to 179.7 and 51.1 pmol/ml in stressed trout in fresh water (Tang and Boutilier 1988). The E and NE levels in acute (30 min) hypoxia stressed eel (*Anguilla anguilla*) were 4.5 and 2.8 pmol/ml compared to 0.84 and 0.53 pmol/ml in resting levels (Perry and Reid 1992).

Many other factors might influence the development of AUR and further studies are needed to understand the molecular mechanisms affecting its expression. However, it is interesting to note that the more phylogenetically advanced teleosts (e.g., *Poecilia*, *Pterophyllum*, *Morone*, Superorder Acanthopterygii) were the most susceptible to AUR, compared to more primitive species (*Oncorhynchus*, Superorder Protacanthopterygii; *Ictalurus*, Superorder Ostariophysi). Although not confirmed histologically, we have also observed gross lesions that appear to be AUR in other higher teleosts, including largemouth bass (*Micropterus salmoides*), spot (*Leiostomus xanthurus*) and croaker (*Micropogonias undulatus*) (all Superorder Acanthopterygii) (E Noga, Unpublished Data). It is also important to realize that the experimental conditions that we used might not have been optimal in inducing AUR in a particular species. Thus, even rainbow trout might develop AUR under different circumstances.

The procedure that we used to induce AUR in these species was similar to the protocol that we used to induce AUR in hybrid striped bass. To induce AUR, we used temperatures near the upper physiological tolerance range of these species (25-30ºC) because in previous studies with hybrid striped bass, we found that AUR lesions were most severe in hybrid striped bass stressed at high temperature (27ºC) and least severe at low temperature (13ºC) (Udomkusonsri et al, In Preparation). However, high temperature is not necessarily required to induce AUR in fish, since we also were able to elicit severe AUR lesions in hybrid striped bass at 19ºC (see below).

Fish with AUR do not always develop secondary infections

Many bacterial diseases affect striped bass and their hybrids. Most of these bacteria are saprophytic, facultative and opportunistic organisms (Plumb 1999a) and include *Flexibacter columnaris, Vibrio, Aeromonas, Pseudomonas, Pasteurella piscicida, Edwardsiella tarda, Enterococcus., Streptococcus., Mycobacterium and Nocardia* (Nedoluha and Westhoff 1997; Plumb 1991, 1999a).

We found that skin of healthy, unstressed, hybrid striped bass had low numbers of bacteria (~10^4 bacteria/g of caudal fin tissue) when assayed using either BHIA or CA. After induction of AUR, bacterial numbers remained low (less than 10^5 bacteria/g of tissue). The number of commensal bacteria on healthy skin of teleost fish typically ranges from 10^3-10^8 organisms per g of tissue (Horsley 1977). Thus, the bacterial concentrations in fish with AUR lesions remained well within the normal range present on healthy skin for one week after the insult. At this time, all AUR lesions appeared completely healed when examined via

steromicroscopy (data not shown). We had expected that fish with advanced AUR lesions to readily develop secondary bacterial infections. Open skin wounds are commonly considered a major portal of entry for many pathogens (Darwish et al 2000; Madetoja et al 2000). However, these fish were clearly able to remain healthy while their lesions healed. The temperature during acclimation, stress and recovery (27ºC) was within the optimal range for juvenile hybrid striped bass growth (Woiwode and Adelman 1991) and is also within the optimal range for their immune function (Hrubec et al 1996; Wang et al 1997). Fish were also not crowded (5 fish per 75 liter aquarium), which probably facilitated their recovery. Another possible reason for the lack of infections is the presumably low pathogen load present in the recovery aquaria. Dechlorinated tapwater was used in the recovery aquaria and the chorination process destroys most pathogens. Certainly, there are a number of microbes present in any aquarium, but the presence of certain pathogens can tremendously increase the risk of developing an infection (see **AUR greatly increases the susceptibility of fish to Saprolegnia infection**).

Thus, our data suggest that quality of the recovery environment (such as optimal water temperature, low number of bacteria) probably played an important role in decreasing the risk of infectious disease after the fish developed AUR.

AUR greatly increases the susceptibility of fish to Saprolegnia infection

Hybrid striped bass exposed to acute confinement for 1 hr at 19ºC developed AUR lesions that were similar to the lesions that occurred when fish were stressed at 27ºC for 2 hr

(Udumkusonsri, *et al*, Unpublished Data). The epidermis was severely ulcerated on the fins, ventrum skin and cornea.

The great majority of fish having AUR that were challenged with *Saprolegnia* zoospores developed saprolegniosis, while fish without AUR did not develop saprolegniosis after challenge with zoospores. Many studies have demonstrated that skin damage promotes water mold infection (Howe et al 1998; Howe and Stehly 1998; Singhal et al 1987; Xu and Rogers 1991). In those studies, the skin was injured experimentally by scraping, descaling or physical abrasion prior to exposing fish to the water mold zoospores. Howe et al (1998) and Howe and Stehly (1998) demonstrated that water mold challenge failed to induce the infection in healthy (unstressed) channel catfish and rainbow trout, respectively, when challenged with *Saprolegnia* zoospore at 5 zoospores/ml. And in our study, water mold infection appeared in areas associated with damage from AUR, especially the fins and ventrum. However, skin on the operculum and head, which appeared normal by histopathological examination in control stressed fish, also became infected. This suggests that histopathology probably cannot accurately detect all damage present since it can only evaluate a small area of the tissue. We have recently discovered that fluorescein staining of skin is a much more sensitive method for identifying skin ulcers (Noga and Udomkusonsri 2002) and would be useful in future studies of site susceptibility.

Saprolegniosis is highly common in fish under intensive aquaculture (Copland and Willoughby 1982; Noga 1993). Fish stressed by net handling or social interaction are susceptible to *Saprolegnia.* (Whisler 1996) and striped bass and their hybrids can often

develop *Saprolegnia* infection after they are injured or stressed (Kerby 1993; Plumb 1991, 1997). Increased susceptibility to saprolegniosis from epidermal damage has been experimentally demonstrated in many fish including rainbow trout, channel catfish, common carp (*Cyprinus carpio*), roho (*Labeo rohita*), and mrigal (*Cirrhina mrigala*)(Howe et al 1998; Howe and Stehly 1998; Singhal et al 1987; Xu and Rogers 1991).

In virtually all studies of experimental saprolegniosis, fish have been challenged with *Saprolegnia* zoospores at much higher concentrations than we used [e.g., 200 zoospores/ml (Hatai and Hoshiai 1993), 50-100 zoospores/ml (Wood et al 1988), and ≥5 zoospores/ml (Bly et al 1992, 1993; Howe et al 1998; Howe and Stehly 1998). Furthermore, fish in most studies were stressed by rapidly lowering the temperature or abrading the skin prior to challenge. The concentrations of zoospores in natural waters is normally low (e.g., 25-1,500 zoospores/l in Windermere lake, in UK (Willoughby 1962); however, the hatchery pond water had higher background zoospore number (e.g., 50-4,600 zoospores/l in Wraymires hatchery [Willoughby 1962], 200-22,200 zoospores/l [Willoughby and Pickering 1977]). Bly and colleagues reported that channel catfish ponds typically have 5-10 zoospores/ml in the winter and 1-6 zoospore/ml during summer (Bly et al 1992, 1993). Bly et al (1993) reported that catfish exhibited saprolegniosis when zoospore concentrations exceeded 5 zoospores/ml in the winter. Thus, the very low zoospore concentration (1 zoospore/ml) that we used is well within the concentration that might be encountered by fish in aquaculture or even natural waters.

Water molds are classical opportunistic pathogens (Neish 1977; Pickering and Willoughby 1982) and outbreaks of saprolegniosis among farmed fish are associated with many

kinds of stress, such as adverse water temperature, poor water quality, handling or crowding (Bailey 1984). Stress is believed to cause immunosuppression that increases the susceptibility of the fish to water mold infection (Bly et al 1992; Pickering and Christie 1981; Pickering and Duston 1983). And certain hormonal changes, especially hypercortisolemia, are well-known to increase the susceptibility of some fish to saprolegniosis (Pickering and Pottinger 1985; Pottinger and Day 1999). However, the specific mechanism(s) that allows a water mold to invade and infect the skin has remained elusive. There is evidence that the dinoflagellate *Pfiesteria* can cause skin damage and water mold infection by direct feeding on the skin or via some toxin-mediated process that leads to skin sloughing (Noga et al 1996; Vogelbein et al 2002). AUR also appears to be a major means by which stress can directly lead to a water mold outbreak. Several host defense mechanisms against water molds have been proposed (Noga 1993; Robinette et al 1998; Willoughby 1989) and most of these are severely compromised by AUR. First, attached zoospores might be physically removed by the constant renewal of mucus. Channel catfish developing saprolegniosis after an acute drop in water temperature displayed a decreased number of epidermal mucous cells after the temperature drop (Quiniou et al 1998). Second, an unidentified "morphogen" (growth-influencing substance) in the mucus secretion of brown trout (*Salmo trutta*) was found to inhibit the growth of mycelia, but not kill them (Wood et al 1988). Third, a cell-mediated response can be directed against mycelia that invade into deeper tissues beyond the epithelium (Noga et al 1989). These inflammatory cells, called epithelioid cells, have epithelial features (desmosomes, tonofilaments), and appear in chronic granulomatous lesions such as ulcerative mycosis in Atlantic menhaden (*Brevoortia tyrannus*). Finally, the most direct evidence for chemical protection against water mold infection are the

studies of Robinette et al (1998), who showed that histone-like proteins (HLPs), broad-spectrum polypeptide antibiotics naturally produced by hybrid striped bass and many other fish, were lethal to *Saprolegnia* zoospores at low concentrations. HLPs reside in the skin mucus and epidermis (Noga et al 2002) and thus loss of the skin completely removes this defense. Thus, the dramatic and severe skin loss that occurs with AUR would certainly compromise these epithelial defenses.

We chose to perform the *Saprolegnia* challenge experiments at 19ºC rather than a higher temperature because this was within the optimal temperature range for *Saprolegnia*'s growth and infectivity. For example, high temperature (>28ºC) decreases the severity of saprolegniosis, while a lower temperature (22-25ºC) causes more severe disease (Khulbe et al 1995). However, 19ºC might suppress the immunity of hybrid striped bass and increase their susceptibility to saprolegniosis. Hrubec et al (1996) reported that a temperature of 18ºC or less caused immunosuppression, in which there was delayed and decreased antibody response to *Aremonas salminicida*, in hybrid striped bass (*M saxatilis* male X *Morone chrysops* female). Also, the response of hybrid striped bass (*M saxatilis* female X *Morone chrysops* male) T lymphocytes to mitogens was reduced or inhibited when incubated at 17ºC (Wang et al 1997). These findings support the contention that lower temperature is immunosuppressive to hybrid striped bass. However, 19ºC was not suppressive enough to allow infection of zoospore-challenged fish not having AUR (Table 4.1).

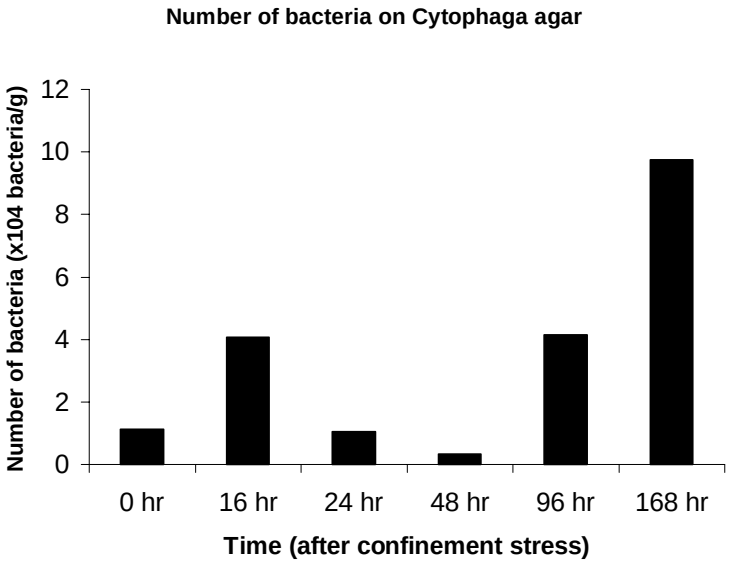
Interestingly, one of the eight sham-challenged fish also developed saprolegniosis (Table 4.1). While we covered all aquaria during the experiment to avoid cross-contamination

by zoospores, it is possible that our quarantine methods were inadequate to prevent the transfer of some zoospores to this aquarium. However, we feel it is more likely that this fish might have been infected by a water mold that might have been inadvertently introduced into the aquarium with the water or some other fomite (e.g., filters, etc.) that was used to establish the aquaria. Water molds are ubiquitous saprophytes and are virtually impossible to eliminate from water sources and thus it is highly possible that some might be latently present in the conditioned filter medium used for the aquaria or the water used to hold the fish prior to their transfer to the experimental aquaria. In either case, it suggests that even extremely low levels of zoospores were sometimes sufficient to infect hybrid striped bass having AUR.

CONCLUSION

AUR is a widespread phenomenon that can affect at least several diverse and economically important fish species. The broad range of species that are susceptible suggest that AUR might be a major cause of skin infections in most aquacultured fish. While fish can spontaneously recover from AUR if maintained in a healthy environment, exposure to even low numbers of relatively weak pathogens can lead to serious, highly lethal epidemics. Further studies to determine ways of preventing the development of AUR are clearly warranted.

a)



b)

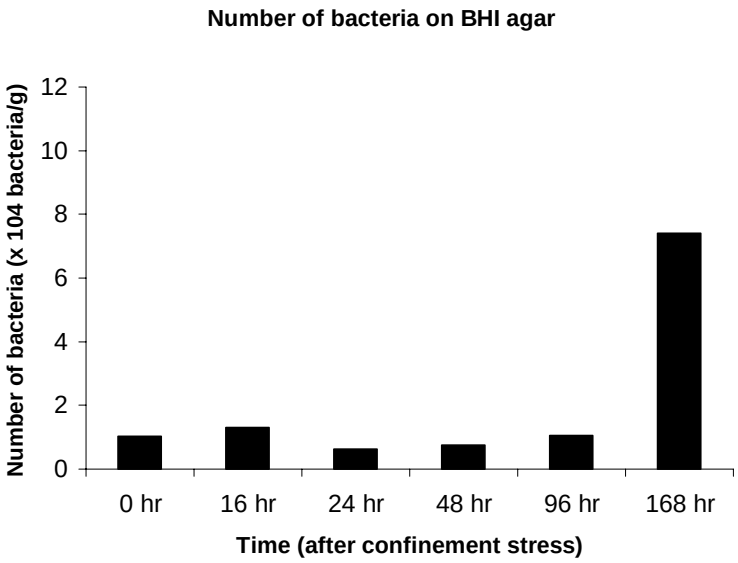


Figure 4.1. Bacterial concentration (bacteria/g of tissue) isolated on cytophaga agar (a) and BHI agar (b) incubated at 25°C. There were 3 fish at each period of time.

Table 4.1. Infectivity of unstressed and stressed hybrid striped bass after challenge with *Saprolegnia* zoospores (1 zoospore/ml for 24 hr). All fish with saprolegniosis died.

Treatment	Number of fish with Saprolegniosis				Cumulative % with Saprolegniosis
	Day 2	Day 3	Day 4	Day 14	
Positive control[a] (n=8)	0	0	0	0	0
Negative control[b] (n=8)	0	0	0	0	0
Challenged[c] (n=8)	2	4	1	0	87.50
Sham-challenged[d] (n=8)	1	0	0	0	12.50

[a] Unstressed hybrid striped bass were exposed to *Saprolegnia* zoospores

[b] Unstressed hybrid striped bass were not exposed to *Saprolegnia* zoospores

[c] Hybrid striped bass were subjected to 1 hr confinement stress and then exposed to *Saprolegnia* zoospores

[d] Hybrid striped bass were subjected to 1 hr confinement stress, but not exposed to *Saprolegnia* zoospores

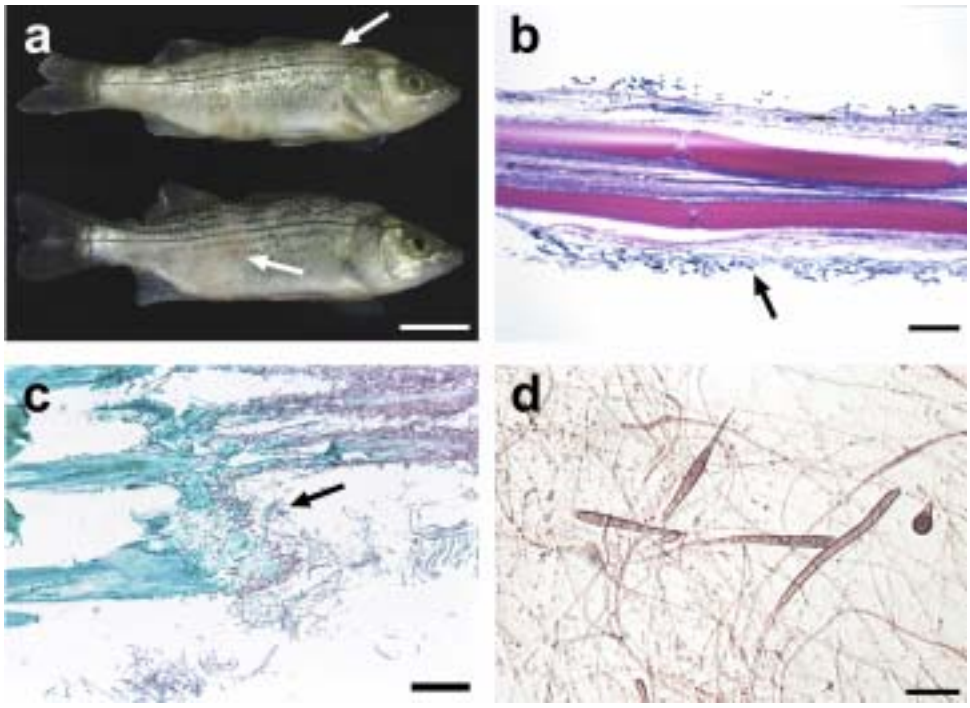


Figure 4.2. Skin lesions in hybrid striped bass with AUR that were challenged with *Saprolegnia* zoospores. a) Fish from the challenged group with saprolegniosis. Hyphal patches cover the head, operculum, dorsum, ventrum, peduncle, and fins (arrows). Bar = 1 cm. b) Histological section of caudal fin of a fish from the challenged group showing epidermal ulceration and hyphae (arrow) on the surface of the lesion. H&E. Bar = 100 μm. c) Histological section of a fish from the challenged group showing severely ulcerated fin epithelium at the distal edge and hyphae (arrow) on the degenerated dermal surface of the lesion. Lymphocytes are also infiltrating into the dermis. GMS. Bar = 300 μm. d) Microscopic identification of *Saprolegnia* in infected fin tissue as shown by numerous branching, aseptate, hyphae and typical zoosporangia containing zoospores. Bar = 150 μm.

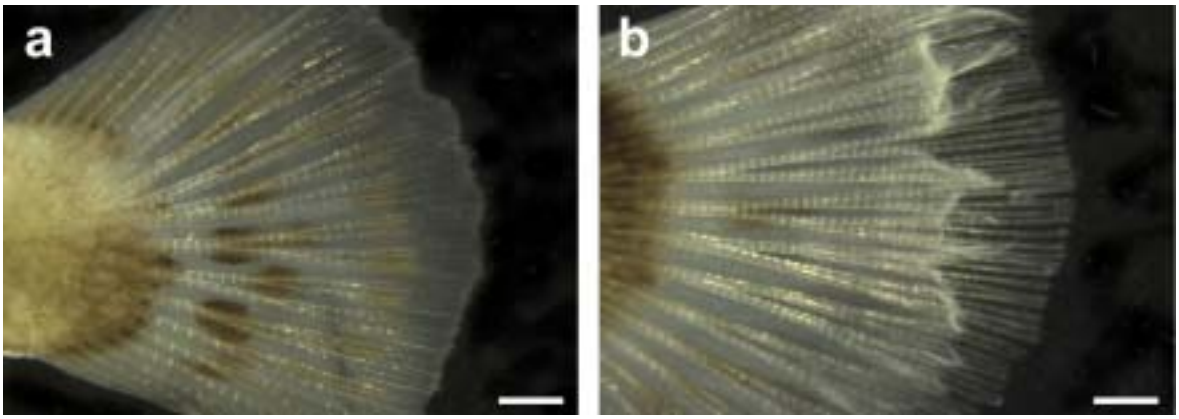


Figure 4.3. Caudal fins of control (a) and 2-hr stressed (b) guppies under stereomicroscopy. Note that stressed fins are ragged and split at the distal edges. Bars = 11 mm.

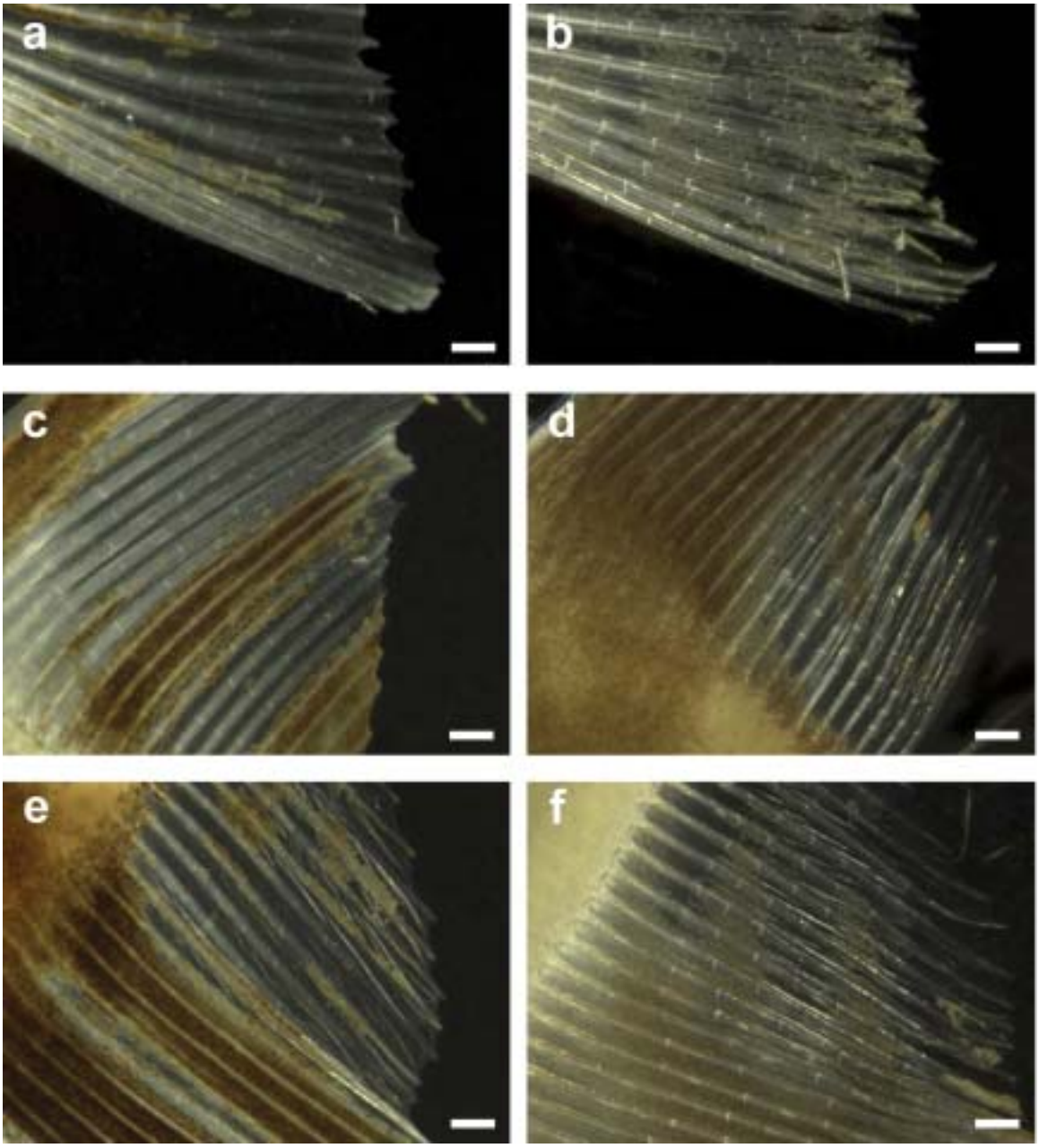


Figure 4.4. Caudal, dorsal and anal fins of control and 2-hr stressed angelfish under stereomicroscopy. Note that stressed fins are ragged and split at the distal edges. Control caudal fin. a) Stressed caudal fin. b) Control dorsal fin. c) Stressed dorsal fin. d) Control anal fin. e) Stressed anal fin. Bars = 0.05 cm.

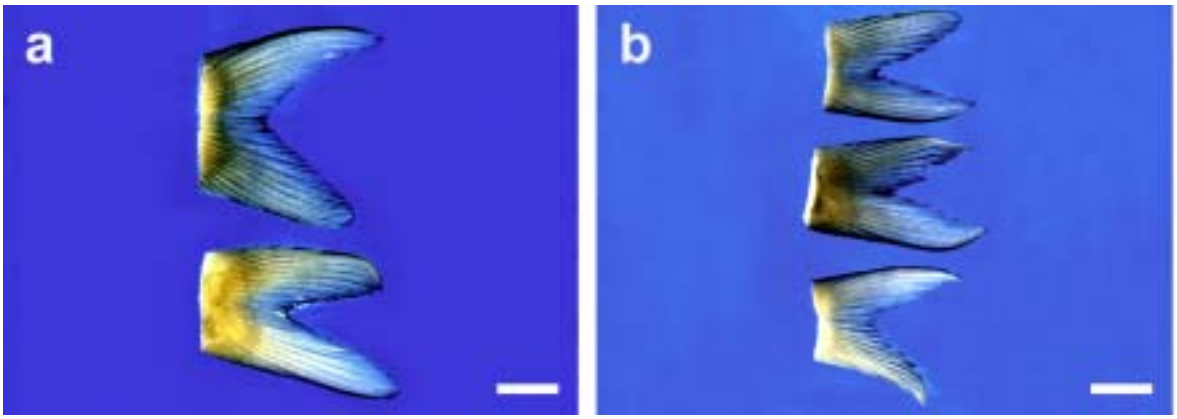


Figure 4.5. Caudal fins of control (a) and 2-hr stressed (b) channel catfish. Note that stressed fins are ragged and split at the distal edges. Bars = 1 cm.

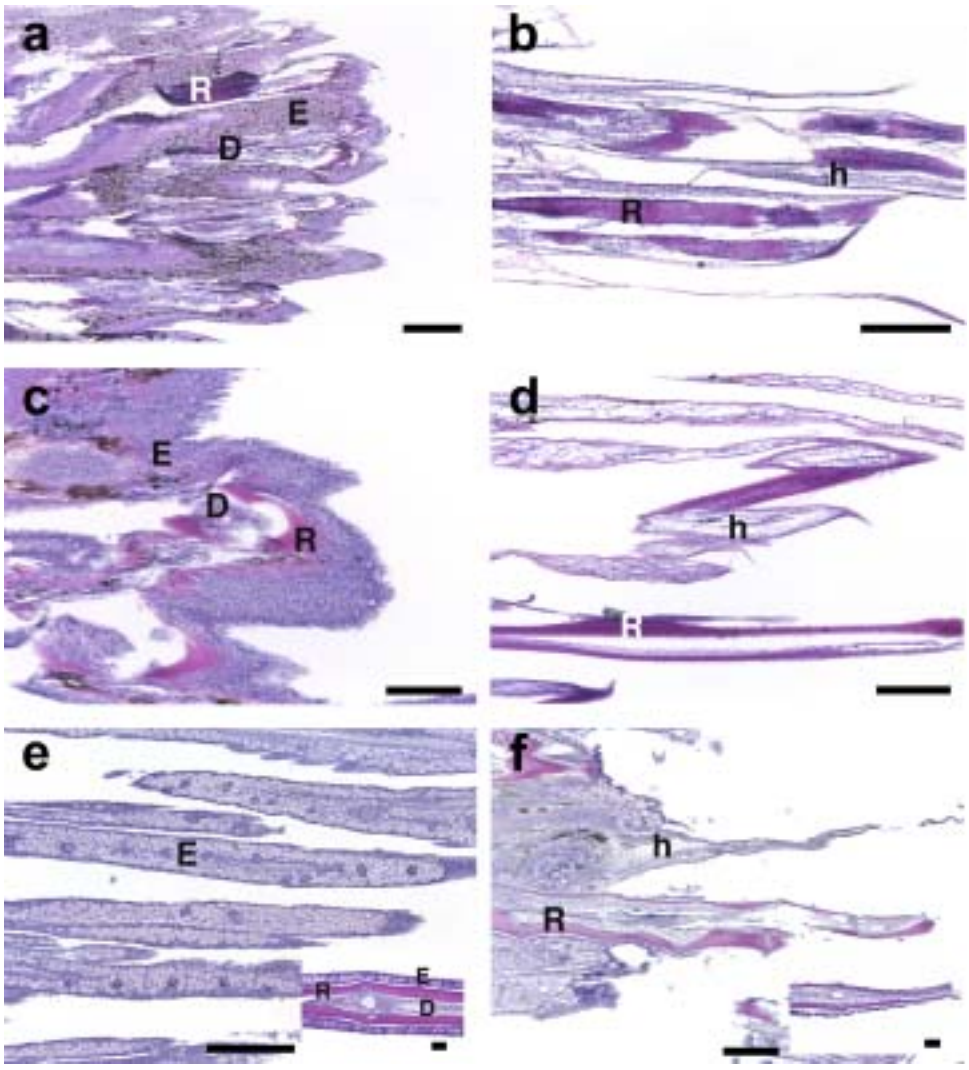
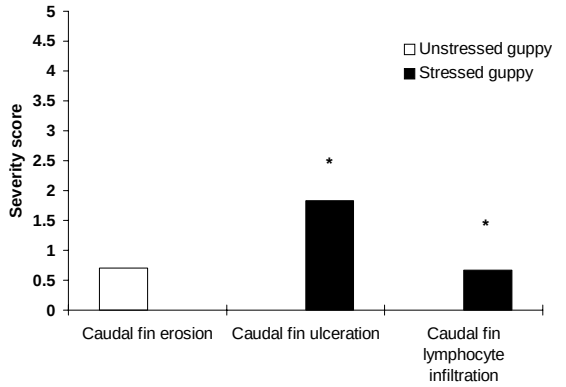


Figure 4.6. Histological sections of caudal fins of control (a) and 2-hr stressed (b) guppies, control (c) and 2-hr stressed (d) freshwater angelfish, and control (e) and 2-hr stressed (f) channel catfish. E epidermis; D dermis; R fin ray; h hypodermal edema. H&E. a) Bar = 300 μm. b) Bar = 150 μm. c, d) Bar = 150 μm. e) Bar = 300 μm; Inset: Bar = 100 μm. f) Bar = 300 μm; Inset: Bar = 50 μm.

a)



b)

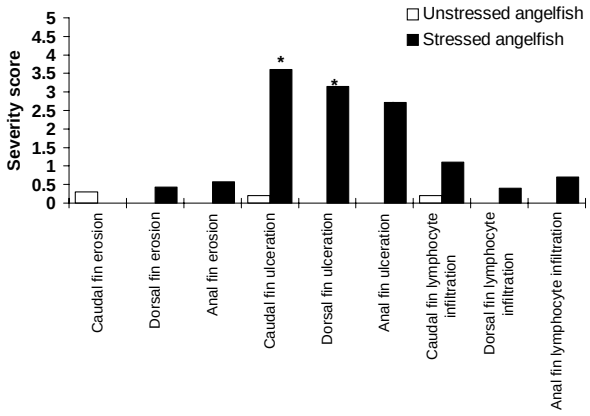


Figure 4.7. Comparison of the severity of pathological changes (epidermal erosion, epidermal ulceration and lymphocyte infiltration) between control versus stressed guppies (a), freshwater angelfish (b) and channel catfish (c). There were 2 replications for each fish species: control (n=6) and stressed (n=6) guppies, control (n=6) and stressed (n=7) angelfish, and control (n=3) and stressed (n=8) channel catfish. * indicates that the severity of AUR between control and stressed fish was significantly different ($p < 0.05$) by ANOVA.

c)

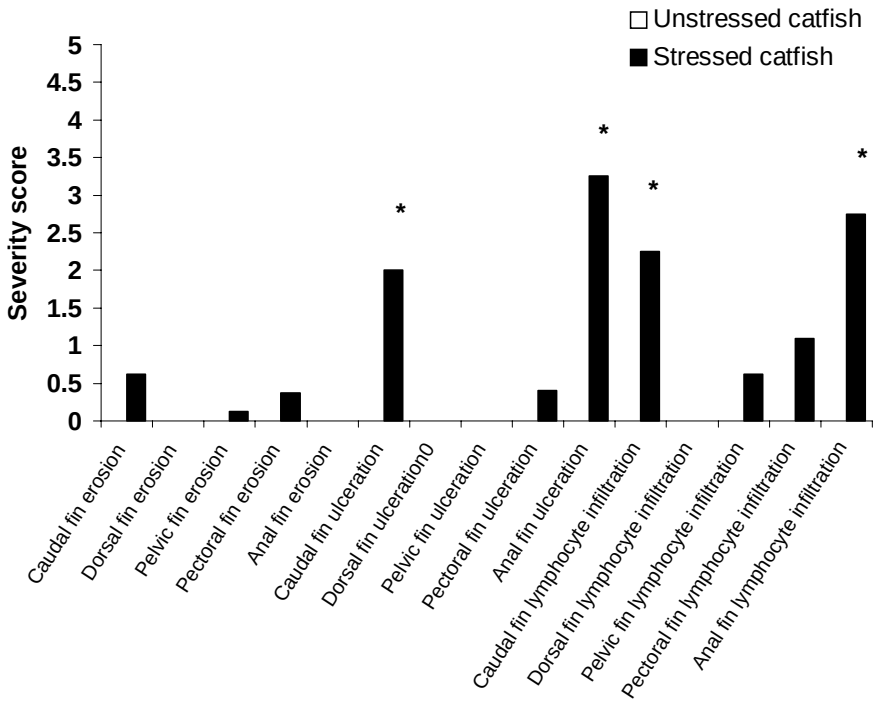


Figure 4.8. continued

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V

CONCLUSION, PERSPECTIVE OF AUR STUDIES AND TECHNOLOGY TRANSFER

CONCLUSIONS

The Acute Ulceration Response (AUR) is a rapid response to 2-hr confinement stress in hybrid striped bass (*Morone chrysops* female X *Morone saxatilis* male). AUR is also developed in other fish species, including guppy (*Poecilia reticulata*), freshwater angelfish (*Pterophyllum scalare*) and channel catfish (*Ictalurus punctatus*) after a 2-hr stress. Thus, AUR is a unique response to acute stress in many teleosts. The histopathological AUR is characterized with epidermal erosion, ulceration and degeneration and leucocytes infiltration on the fins, body skin, including corneal ulceration. The earliest detectable changes of AUR occurred rapidly within 15-min stress with epidermal swelling and erosion, and the AUR severity is time-dependent response. The environmental factors (acclimation space (aquarium size), temperature during acclimation, temperature during confinement) had an influence in the AUR severity. The acclimation fish in small space, or lower temperature and lower the temperature during stress can decrease the severity of AUR. In addition, exogenous adrenergic modulators influenced the risk of developing AUR. Administration of epinephrine caused AUR-like epidermal damage in dose-dependent response, however, the epidermal damage was less severe than AUR which induced by confinement stress. Thus, epinephrine may play a role in AUR development. Adrenergic antagonist and ganglionic blockers (phentolamine, propranolol and hexamethonium) had reduced the severity of AUR when administered before stress. AUR fish were highly susceptible to saprolegniosis when challenged with *Saprolegnia* zoospore even at the low concentration (1 zoospore/ml). However, AUR fish recovered in a healthy environment (low microorganism, optimal temperature) did not develop the skin infection. These data present here suggest that AUR

might play a critical role in skin ulcer epidemics of many fish species that are preceded by an acute stress and environmental plays a critical role in determining if AUR lesions will heal spontaneously or instead will lead to devastating disease losses.

PERSPECTIVE OF AUR STUDIES AND TECHNOLOGY TRANSFER

The studies lead to a better understanding the mechanisms responsible for AUR, including the important fish disease. It showed that an acute stress causes ulcer on the fins of food fish (e.g. hybrid striped bass, channel catfish) and pet fish (e.g. guppy and angelfish). This data provides possible explanation as to why a number important infectious disease can occur after stress. After fish lose their protective skin barrier, opportunistic pathogens (e.g., *Aeromonas, Pseudomonas,* and water molds) can invade the skin and cause infection. Skin loss also causes a serious osmotic stress due to loss of the skin barrier. However, environmental microorganism loads is important for an occurrence of fish diseases.

AUR is easily diagnosed by observing blanching and ragged fins or simple wet mounts of fin tissues. We can use the fluorescein stain technique, which is rapid, accurate and non-toxic method, to identify the skin ulcer in the living fish (Noga and Udomkusonsri, 2002). This provides an early warning of potentially serious problems since the skin is lost. Thus, farmers or pet fish owners might monitor their fish closely and/or provide treatment as early as possible.

An understanding in the pathogenesis of the AUR after acute stress may allow the use of stress blockers (environmental or physiological) to prevent the development of AUR. This

study provides useful information on fish health in that fish exposed to acute stress are more likely to develop AUR. Since many types of aquaculture practices may cause acute stress, AUR may be a very common initiating cause of disease. Knowing that AUR exists may warn farmers that their stocks might be prone to develop opportunistic fin infection (e.g. fin rot) and they should be aware of this potential problem.