NITRATE AS AN ENDOCRINE DISRUPTING CONTAMINANT IN CAPTIVE SIBERIAN STURGEON, *Acipenser baeri*

By

HEATHER J. HAMLIN

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2007

To my family

ACKNOWLEDGMENTS

First and foremost I thank Lou Guillette for his tremendous mentorship, for sharing with me his wealth of knowledge and experience and for having more faith in me than I did at times. I am a better person for having worked with him. I thank my advisor and other members of my committee for their encouragement and support: Ruth Francis-Floyd was graciously supportive and gave me the freedom to do what I love; Kevan Main gave me the opportunity and encouragement to fulfill my dream; Daryl Parkyn read my manuscripts and gave me valuable comments; Roy Yanong gave me valuable opinions and comments on my manuscripts and I'll always appreciate his enthusiasm for disease. I'd also like to thank Jim Michaels for allowing me access to my research animals and supporting my research.

This journey would not have been nearly as fulfilling were it not for the comradery of my fellow lab mates: Thea Edwards, who taught me EIAs and introduced me to the lab experience. I'll always treasure our late night conversations about everything from egg cups to egg development; Brandon Moore, who took the time to mentor me and with whom I'll always enjoy scientific discussions; Satomi Kohno, whose patience in teaching lab techniques deserves an award; Iske Larkin, for teaching me the wonders of RIAs. Lori Albergotti, Ashley Boggs and Nicole Botteri, who made me wish I could spend more time in the lab. I'd also like to thank all the undergraduate students who assisted me with collections and lab analyses.

Finally, I'd like to thank my friends and family, without whom the journey wouldn't be worth it: my Mother, Holly Paulsen, who let me have every creature known to man as a child, and spent countless hours with me as an adult collecting data and analyzing samples in this work. It wouldn't have been the same without her help; my Father, Greg Hamlin, who bought me my first aquarium; my best friend Maria Piccioni, who supported me tremendously in this journey;

I’d love to be half the person she thinks I am; Dave Jenkins, who supported me more than he knows; and Amy Leighton, who made my childhood something to treasure.

TABLE OF CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

NITRATE AS AN ENDOCRINE DISRUPTING CONTAMINANT IN CAPTIVE SIBERIAN STURGEON, *Acipenser baeri*

By

Heather J. Hamlin

May 2007

Chair: Ruth Francis-Floyd
Major: Fisheries and Aquatic Sciences

Numerous environmental contaminants have been shown to alter reproductive endocrine function. Such compounds have been termed endocrine-disrupting contaminants (EDCs). EDCs exert their effects through numerous physiological mechanisms, including alterations in steroidogenesis. Although a global pollutant of most aquatic systems, nitrate has only recently begun to receive attention for its ability to alter endocrine function in wildlife. We examined nitrate-induced endocrine disruption using the Siberian sturgeon (*Acipenser baeri*) as a model species. Comparisons of captive populations of sturgeon cultured in nitrate concentrations of 1.5, 11.5 and 57.5 mg/L nitrate-N revealed nitrate induced elevations in plasma concentrations of sex steroids including testosterone, 11-ketotestosterone and 17β-estradiol. Alterations in circulating concentrations of sex steroids can be a response to several physiological mechanisms, including an up-regulation of gonadal steroid synthesis, altered biotransformation and clearance by the liver or alterations in plasma storage by steroid binding proteins.

To gain a better mechanistic understanding of the observed sex steroid elevations we examined mRNA expression patterns of steroidogenic enzymes ($P450_{SCC}$) and receptor proteins (ERβ and GR). We found no significant differences in mRNA expression patterns, indicating

that the observed sex steroid increases were not likely due to an up-regulation of gonadal synthesis.

Cortisol and glucose, commonly examined as indicators of perceived stress, were not found to vary among groups exposed to any of the nitrate concentrations. Because responses to stress can be cumulative, endocrine responses to stress events in fish residing in the various nitrate concentrations were also investigated. Results showed that nitrate does alter the associated stress response, defined by plasma glucose concentrations.

These data suggest that long-term exposure to nitrate is associated with altered endocrine parameters (e.g., plasma hormone concentrations) in Siberian sturgeon. Future work must begin to examine the underlying causes of these changes. Although the data of gene expression suggest that mRNA concentrations of at least one steroidogenic enzyme were not altered, other enzymes in the pathway need to be examined. These data indicated that nitrate concentrations must now be considered in the effective management of sturgeon populations in both natural and captive environments.

CHAPTER 1
INTRODUCTION

Background

Overview of Reproductive Endocrinology in Fishes

The production of circulating hormones is the result of numerous physiological reactions spanning many levels of biological organization. The regulation of hormone production is controlled by mechanisms that both create and destroy these chemical messengers, and is fine-tuned by various stimulatory and feedback mechanisms (Norris, 1997). Tropic hormones regulate many of the activities of the thyroid gland, adrenal gland and the gonads (Norris, 1997). The endocrine regulation of reproduction is initiated in response to environmental cues, which stimulate the release of gonadotropin-releasing hormone (GnRH) from the hypothalamus (Detlaff et al., 1993; Norris, 1997) (Figure 1-1). In response to GnRH, the anterior pituitary releases gonadotropins, which circulate throughout the body, targeting various organs, such as the gonads.

Two chemically distinct gonadotropins have been characterized in fish, GTH-I and GTH-II, which are purportedly analogous to follicle stimulating hormone (FSH) and luteinizing hormone (LH), respectively, in terrestrial animals (Norris, 1997). Because few fish species have defined chemical hormone structures to date, much of the research literature employs heterologous hormones (Van Der Kraak et al., 1998). FSH stimulates oogenesis and spermatogenesis, and LH stimulates final gamete maturation and release. Like FSH and LH, GTH-I and GTH-II consist of an α and β subunit; the α subunit is the same for both gonadotropins, with only the β subunit conferring biological specificity (Norris, 1997; Vasudevan et al., 2002). The β subunits of both gonadotropins have been cloned in Siberian sturgeon (*A. baeri*) and Russian sturgeon (*A. gueldenstaedti*), and based

on their function and position in the phylogenetic tree, it was suggested these compounds be termed FSH and LH, respectively (Querat et al., 2000; Hurvitz et al., 2005).

FSH and LH stimulate gonadal steroidogenesis, and the three steroid hormones relevant to this study are estradiol-17β (E_2), testosterone (T) and 11-ketotestosterone (11-KT). In females, E_2 stimulates gonadal growth, sexual maturation, vitellogenesis by the liver and oogenesis (Knobil and Neill, 1994; Norris, 1997; Denslow et al., 2001). In males, T stimulates sexual maturation, spermatogenesis and spawning, and is implicated in sexual behavior for both males and females (Norris, 1997; Toft et al., 2003). In addition to inducing spermatogonial proliferation, 11-KT likely also participates in the former processes (Schultz and Miura, 2002).

Circulating hormones can be detected by receptors at the periphery of the cell, and through a cAMP mediated process ultimately leads to increased levels of intracellular cholesterol (Stocco, 1999). This cholesterol is mobilized to the outer mitochondrial membrane and is the precursor for steroid biosynthesis. A protein inserted in the mitochondrial membrane, steroidogenic acute regulatory (StAR) protein, functions to transport cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane, and this process is now thought to be the rate limiting step in steroidogenesis (Stocco, 1999). Its function has received considerable attention in recent studies of vertebrates (Stocco, 2001), including fish (Goetz et al., 2004). The inner mitochondrial membrane is the site of activity for the P450 side chain cleavage enzyme ($P450_{SCC}$) that cleaves cholesterol to form the first steroid in the pathway, pregnenolone. Pregnenolone is then converted to progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD). Both P450scc and 3β-HSD are often evaluated in studies of steroidogenesis and related

physiological mechanisms (Takase et al., 1999; Pozzi et al., 2002; Inai et al., 2003). Further pathways of steroid production are shown in Figures 1-2 and 1-3. Quantifying compounds in the biosynthetic pathways will assist in developing a mechanistic understanding of which pathways can be disrupted.

Stress in Fish and Its Effects on Reproduction

Stress has been defined in the literature in a number of ways, encompassing such definitions as diversions of metabolic energy, adaptive changes resulting in modifications to normal physiological states, and any change that impacts long term survival (Selye, 1956; Esch and Hazen, 1978; Wedemeyer and McLeay, 1991; Bayne, 1985; Barton and Schreck, 1987). Ultimately our interest in stress is attendant upon the causative factors mitigating the deleterious response. Once these causative factors are determined, we can then begin the process of remediation. In this sense, understanding stress is a means to an end and becomes a useful tool to predict if negative outcomes are likely to ensue. We can use this diagnostic tool to understand environmental impact and determine at what point action is necessary to effectuate relief.

As in other vertebrates, concentrations of coricosteroid hormones are sensitive indicators of acute stress in fish, and circulating concentrations generally reflect synthesis rates since little hormone is stored in the adrenal (mammals) or interrenal tissue (fish) (Norris, 1997). The production of corticosteroids is initiated by perceived stress events, triggering the release of corticotropin releasing hormone (CRH) from the hypothalamus, which then triggers the release of ACTH from the pituitary (Figure 1-2) (Flik et al., 2006). Circulating ACTH triggers the release of corticosteroids from the interrenal cells of the head kidney in most fish species; in sturgeon cortisol releasing adrenocortical cells are present in small clusters throughout the kidney (Norris, 1997).

The principal corticosteroid in most fish species is cortisol (Kime, 1997; Barton, 2002; Overli et al., 2005), which has been implicated as a causal factor in many of the deleterious effects of stress (Barton and Iwama, 1991; Harris and Bird, 2000; Schreck, 2001; Bernier et al., 2004). Cortisol shows two primary actions in fish, regulation of water and mineral balance and energy metabolism (Wendelaar Bonga, 1997). The effects of corticosteroid hormones are mediated through intracellular receptors, which act as ligand binding transcription factors (Norris, 1997). Fish possess both a glucocorticoid receptor (GR) and a mineralcorticoid receptor (MR) with GR possessing various isoforms (Bury et al., 2003). While cortisol is the predominant physiological ligand for GR, it is still unclear what is the primary ligand for MR in fish, which shows a high affinity for both deoxycorticosterone and aldosterone (Prunet et al., 2006). This is particularly interesting since there is no reliable evidence for the presence of aldosterone in teleosts, and it is becoming accepted that aldosterone is likely absent in most or potentially all fish groups (Prunet et al., 2006).

The molecular characterization of corticosteroid receptors (CR) in the last 10 years has modified the initial consensus of a unique high affinity binding site for cortisol, and now depicts a multiple CR family with two classes of receptors (GR and MR) with splicing isoforms and duplicated genes (GR1 and GR2) (Prunet et al., 2006). Functional analyses in trout show that GR2 has a higher sensitivity to cortisol when compared to GR1, and that these isoforms show different patterns of expression sensitivity depending on the tissues targeted (Greenwood et al., 2003). It has also been shown that GR can be less sensitive to corticosteroids than MR, suggesting that the latter could serve as a high affinity cortisol receptor in fishes, a condition already described in humans (Hellal-Levy et al., 2000).

The significance of cortisol in assessments of stress may be limited when examining chronic stressors, due in part to the acclimation of the interrenal tissues during chronic stress, which is mitigated by negative feedback mechanisms on the hypothalamo-pituitary-interrenal (HPI) axis (Rotllant et al., 2000). Other bio-markers, such as expression levels of GR, have been shown to be more sensitive indicators of chronic stress. Quantification of GR in seabass (*Dicentrarchus labrax*) showed significantly reduced GR concentrations after a 3-month exposure to elevated stocking densities (Terova et al, 2005).

Environmental contaminants have been shown to alter the stress response by altering GR activation. Organotins, compounds used as industrial stabilizers in paints now present in aquatic environments, have been shown to block GR activation (Odermatt et al., 2006). Other ubiquitous pollutants such as PCBs and arsenic have also been shown to alter GR receptor functioning (Johansson et al., 1998; Bodwell et al., 2004).

The effects of stress can be manifest at multiple levels of the reproductive endocrine axis (Guillette et al., 1995; Pankhurst and Van Der Kraak, 1997). Although there is limited information on the effects of stress on the release of GnRH on aquatic inhabitants, several studies have been conducted identifying stress impacts on circulating concentrations of GTH-I and GTH-II. For some species of fish such as brown trout (*Salmo trutta*), confinement stress results in an increase in circulating concentrations of GTHs (Pickering et al., 1987; Sumpter et al., 1987). For other species, such as the white sucker (*Catostomus commersoni*), capture and transport stress results in depression of GTHs to undetectable concentrations within 24 h of capture (Stacey et al., 1984).

The effects of stress on concentrations of gonadal steroids in both terrestrial and aquatic animals is well documented, resulting in a depression in plasma concentrations of

both androgens and estrogens in most species studied to date (Francis, 1981; Pickering et al., 1987; Carragher and Pankhurst, 1991). These reductions can be attributed to altered secretion of gonadotropins (Gray et al., 1978) as well as by direct inhibition of gonadal steroid synthesis (Saez et al., 1977; Sapolsky, 1985).

Cortisol has also been implicated in altering endocrine function. Cortisol's negative effects on reproduction includes depressed plasma concentrations of sex steroids (Pankhurst and Dedual, 1994; Pottinger et al, 1999). However, this response is dependent upon the hormones involved and the species investigated. Elevated plasma concentrations of cortisol in Stellate sturgeon (*A. stellatus*) females have been shown to result in correspondingly lower concentrations of circulating plasma T and 11-KT, however, E_2 and progesterone (P) remain constant (Semenkova et al., 2002). Similarly, Bayunova (2002) observed an inverse relationship between cortisol and T after a 9-h period of confinement stress for both male and female stellate sturgeon. Consten et al. (2002) investigated whether the decrease in plasma 11-KT of male carp was caused by a direct effect of cortisol, or by an indirect effect (such as a decrease in plasma LH). Experimental animals were fed cortisol-containing food pellets over a prolonged period, and the results indicated that cortisol had a direct inhibitory effect on testicular androgen secretion that was independent of LH secretion. Reductions in reproductive hormones can lead to a myriad of deleterious reproductive effects such as decreased gamete quality, embryo mortality, and behavioral changes (Pankhurst and Van Der Kraak, 1997; Pankhurst, et al., 1995).

Endocrine Disruption in Aquatic Vertebrates

Xenobiotics, or man-made chemicals, have been shown to disrupt normal hormone function, and have received considerable attention over the last decade (Colborn and Clement, 1992; Guillette, 2000; McLachlan 2001). Compounds evaluated as endocrine

disrupting contaminants have generally included common environmental pollutants which have demonstrated abilities to mimic hormones, alter hormone production, or act as anti-hormones (Guillette, 2000). Molecularly, xenobiotics have the ability to bind directly to steroid hormone receptors or other proteins that initiate or facilitate the transcription of genes (Thomas, 1990; Rooney and Guillette, 2000). Compounds such as polychlorinated hydrocarbon pesticides (e.g., DDT derivatives), polychlorinated biphenyls (PCBs) and others have been shown to bind to estrogen receptors manifesting estrogenic or anti-estrogenic actions in mammals and birds (Bulger and Kupfer, 1985; Rooney and Guillette, 2000). Extensive work has been conducted in fishes, and evidence indicates similar mechanisms occur (Thomas, 1990; White et al., 1994; Tyler et al., 1998a; 1998b; 1999; Jobling et al., 1995; 1996; 1998; 2002).

Numerous studies document a vast array of endocrine disruptive effects in fish located in polluted aquatic systems and areas downstream of sewage or other industrial treatment plants (Jobling et al., 2003; Toft et al., 2004). Male walleye (*Stizostedion vitreum*) collected near a metropolitan sewage treatment plant exhibited depressed serum T concentrations and elevated serum E_2 concentrations compared to reference males (Folmar et al., 2001). Reduced plasma concentrations of T have also been documented in lake whitefish (*Coregonus clupeaformis*) and white sucker (*Catostomus commersonii*) exposed to bleached Kraft mill and pulp mill effluent respectively (Munkittrick et al., 1992; 1994). Female mosquitofish downstream from Kraft paper-mill effluent in Florida demonstrated masculinization of the anal fins, which is an androgen-dependent trait (Parks, et al., 2001). Male mosquitofish from a Florida lake contaminated with known endocrine disruptors displayed shorter gonopodium, significantly reduced whole body T concentrations, reduced

liver weights and had reduced sperm counts versus those of a reference population (Toft et al., 2003).

Compounds such as the natural steroid E_2 have been measured in both industrial and municipal sewage treatment effluents, which represent the principle sources of natural estrogens in the aquatic environment (Lai et al., 2002). Exposure to E_2 caused disruptions in sexual differentiation in young zebrafish and altered egg production patterns in adults (Brion et al., 2004). Exposure of the riverine species the roach (*Rutilus rutilus*) to a host of chemicals persistent in typical British waters, revealed significantly increased incidences of intersexuality and plasma vitellogenin concentrations and attributed these alterations to estrogenic constituents of sewage effluents (Jobling et al., 1998).

Considerable work also has been conducted on abnormalities of the reproductive system of Florida's alligators in relation to environmental contamination, notably in Lake Apopka, located northwest of Orlando. These studies report reductions in circulating concentrations of sex steroids, alterations in gonadal morphology, phallus size, enzyme activity and steroidogenesis (Guillette, et al., 1999; 2000). These modifications were attributed to both embryonic and post-hatching exposure to a complex mixture of chemicals from agricultural activities and stormwater runoff, including PCBs, p,p′-DDE, dieldrin, endrin, mirex, and oxychlordane. Excess nitrate has also been shown to alter steroidogenesis and endocrine function in several aquatic species (Guillette and Edwards, 2005; Barbaeu, 2004). Detailed lists of known endocrine disrupting contaminants and their documented effects are readily available (Edwards, 2006), and will be discussed in further detail in Chapters 3, 4 and 5.

Nitrate in Natural Water Systems

In nature, organic and inorganic nitrogen is cycled through various environmental processes such as nitrification, denitrification, fixation and decay. Nitrification and denitrification processes are essential to the health of aquatic ecosystems. These processes generally begin with ammonia, which is broken down to nitrite by aerobic nitrifying bacteria (usually *Nitrosomonas* sp.), which is then converted by another group of bacteria to nitrate (usually by *Nitrobacter* sp.). Nitrate is often then fixed by plants as a nutrient, or undergoes denitrification (Sharma and Ahlert, 1977). Complete denitrification converts nitrate to either nitrogen gas or organic nitrogen. Incomplete denitrification, resulting from inadequate sources of carbon or environmental conditions, results in nitrate's conversion back to nitrite, or even ammonia, by anaerobic denitrifying bacteria (Van Rijn et al. 2006).

Over the last several decades, concentrations of nitrate in natural water bodies from anthropogenic impact has increased significantly (Pucket, 1995), which has resulted in nitrate concentrations in many water sources far in excess of the EPA drinking standard of 10 mg/L nitrate-N (Kross et al., 1993; U.S. EPA, 1996). In northern Florida, concentrations as high as 38 mg/L nitrate-N were recorded in the Suwannee River (Katz et al., 1999). In addition to its direct effects, nitrate can encourage excessive algal and plant growth, adversely impacting the ecology of the affected area (Attayde and Hansson, 1999; Capriulo et al., 2002).

Nitrate in Aquaculture and Its Implications as an EDC

As discussed previously, elevated concentrations of stress hormones have been shown to result in decreased concentrations of circulating sex steroids. Environmental contaminants have been shown to elicit a stress response, thereby decreasing circulating concentrations of sex steroids. In fact, some of the earliest reports of vertebrate stress

responses were induced by chemical exposure (Selye, 1936). While it is clear many man-made chemicals have considerable impact on hormone function in aquatic animals, it is less clear if naturally occurring compounds could also have the same effect. Contaminated aquatic ecosystems such as Lake Apopka, Florida provide ample opportunity to observe severe abnormalities of the reproductive system, and are decidedly "unhealthy" for aquatic life. In aquaculture, aquatic animals are exposed to xenobiotic and natural compounds often far in excess of those experienced in nature, but resultant abnormalities are often overlooked since aquaculture fish are not necessarily expected to mimic wild fish. After all, they are held at higher densities, eat dramatically different diets, and are often held under unnatural temperature and light regimes. Additionally, definitions of acceptable water quality standards of natural water environments (generally under EPA regulation) versus those of intensive aquaculture systems (under the regulation of the farm manager) are usually dramatically different. Commercial aquaculture operations have limited budgets (if any) for in-depth research into the factors that are contributing to the success or failure of husbandry practices and protocols. Therefore, water quality estimates of "safe" operating levels in aquaculture are often the result of trial and error practices based on growth or mortality events. For species such as sturgeon, which take many years to reach reproductive maturity, and whose economic viability relies heavily on proper egg production, it may be important to investigate more thoroughly the sublethal effects a potential hazard may impose.

Nitrate has been overlooked as a material water quality hazard in both natural and aquaculture settings. Emerging information implicates nitrate as a hazard at concentrations once thought to be innocuous for both reptile and amphibian species (see Guillette and

Edwards, 2005). It has been shown that vertebrate mitochondria are capable of nitric oxide (NO) synthesis via non nitric oxide synthase (NOS) activity (Zweier et al., 1999) using nitrite as a precursor. Nitrate can be converted to nitrite *in-vivo* (Panesar and Chan, 2000), and it is thought other enzymes can generate NO directly from nitrate (Meyer, 1995). Nitric oxide is a gas that plays diverse roles in cellular signaling, vasodilation, immunity and has been documented to inhibit steroid hormone synthesis (DelPunta et al., 1996; Panesar and Chan, 2000; Weitzberg and Lundberg, 1998). As discussed previously in this chapter, StAR and $P450_{SCC}$ are key factors regulating steroidogenesis. NO has been shown to alter the activity of StAR and may also alter $P450_{SCC}$ by binding to the heme group which is present in all enzymes of the P450 family (White et al., 1987). Bulls fed nitrate showed reduced sperm motility and degenerative lesions of the germ layers of the testes (Zraly et al., 1997). Medaka exposed for 2-months to no more than 75 mg/L NO_3-N showed reduced fertilization and hatching rates (Shimura et al., 2002). A study of female mosquitofish (*Gambusia holbrooki*) in Florida showed reduced reproductive activity and embryo number in fish exposed to 5.06 mg/L NO_3-N (Edwards et al., 2006b).

Reproductive hormone concentrations have been shown to be especially vulnerable to chemical and physical strain (Pickering, 1987), which as discussed can cause numerous reproductive complications. Since nitrate has been shown to negatively impact the reproductive physiology of a number of aquatic species (Edwards et al. 2006a; Edwards et al., 2006b) and sturgeon have been shown to be unusually susceptible to environmental impact (Akimova and Ruban; Dwyer et al., 2005), it stands to reason that nitrate could be an endocrine disrupting contaminant for Siberian sturgeon, and is worthy of investigation.

In the United States and elsewhere, water is becoming a valuable and limited commodity, and its use is tightly regulated. New aquaculture operations will not be afforded the vast quantities of water established facilities have been permitted to use, and will therefore need to use recirculating technologies which enable these facilities to reuse a significant portion of their water. In most of these recirculating facilities, the limiting factor for water exchange is nitrate concentration.

Sturgeon as a Model Species

Sturgeons belong to one of the most ancient groups of *Osteichthyes*, and are naturally distributed above the 30th parallel. Although they can be found almost everywhere along the Pacific and Atlantic coasts, the Mediterranean and Black Seas, as well as rivers, lakes and inland seas, most sturgeon populations are sparse and occur in significant numbers in only a few regions (Detlaff et al., 1993). The Caspian Sea represents a unique reservoir, producing the bulk of the world's sturgeon capture fisheries. Sturgeon include anadromous, semi-anadromous and river-resident (freshwater) forms. The Siberian sturgeon have both semi-anadromous and river resident populations (Detlaff et al., 1993).

Sturgeon have preserved primitive structural features relating them to chondrosteans, while at the same time the structure of their eggs is more similar to amphibians than either chondrosteans or teleosts, since the inclusions of yolk are distributed throughout the cytoplasm. Although sturgeon produce great numbers of large eggs, affording them great ecological advantage in hostile environments, ironically this production is at the nexus of their dwindling population. Sturgeon eggs, termed caviar when processed, are a prized delicacy and commands very high prices. This has lead to over fishing on a grand scale (Birstein, 1993; Williot et al., 2002). This over fishing, in

concert with other anthropogenic impacts, such as river damming and pollution, has resulted in the reduction, or in some cases decimation, of sturgeon stocks worldwide (Williot et al., 2002). Aquaculture has been proposed as a mechanism to help save wild populations, either by reducing fishing pressures or by providing animals for stock enhancement. Due to the high value of caviar, sturgeon aquaculture has great promise.

As discussed above, nitrate is the limiting factor for water exchange in recirculating aquaculture systems. The less water a facility uses, the greater the possible concentrations of nitrate, and although research is underway to develop technologies to reduce nitrate concentrations, it is unclear what affects nitrate has on fish residing in these systems. Additionally, environmental nitrate from anthropogenic sources is increasing at an alarming rate worldwide (Rouse et al., 1999), and with pollution implicated in reductions in wild sturgeon populations in the Caspian Sea, the world's largest sturgeon reservoir, the need to understand the affects of nitrate on sturgeon is becoming more and more apparent. That egg production is paramount to the viability of sturgeon as an aquaculture species, and is of obvious ecological importance, necessitates an understanding of the affects of nitrate on the reproductive system in particular.

Research Objectives and Hypotheses

The goal of this study was to gain a better mechanistic understanding of the potential for nitrate-induced disruptions in reproductive function, using Siberian sturgeon as a model. Based on previous studies reviewed in this Chapter, I hypothesize that given nitrate's ability to alter steroidogenic activity, notably through NO induced alterations in P450 enzyme activities, that the fish exposed to elevated nitrate will demonstrate reduced concentrations of plasma sex steroid concentrations, and these reductions will be mirrored in gonadal mRNA expression patterns of $P450_{SCC}$, ERβ and GR. I theorize that these

alterations would not be caused by a generalized stress response, but by disruptions in steroidogenic mechanisms directed at the production of sex steroids, notably T, 11-KT and E_2.

Compensatory mechanisms required to combat physiological challenges consumes energy and physiological resources that could otherwise be used to carry out other essential functions. Therefore, an animal experiencing simultaneous stressors, such as nitrate exposure in combination with an induced stressor such as confinement, may not be as adept at responding to the stress events as an animal experiencing a single stressor. I therefore hypothesize that long-term exposure to elevated nitrate will alter the associated stress response. In addition, given that GR has been shown to parallel chronic stress, I predict GR mRNA expression will be significantly reduced in animals exposed for 30 days to elevated nitrate.

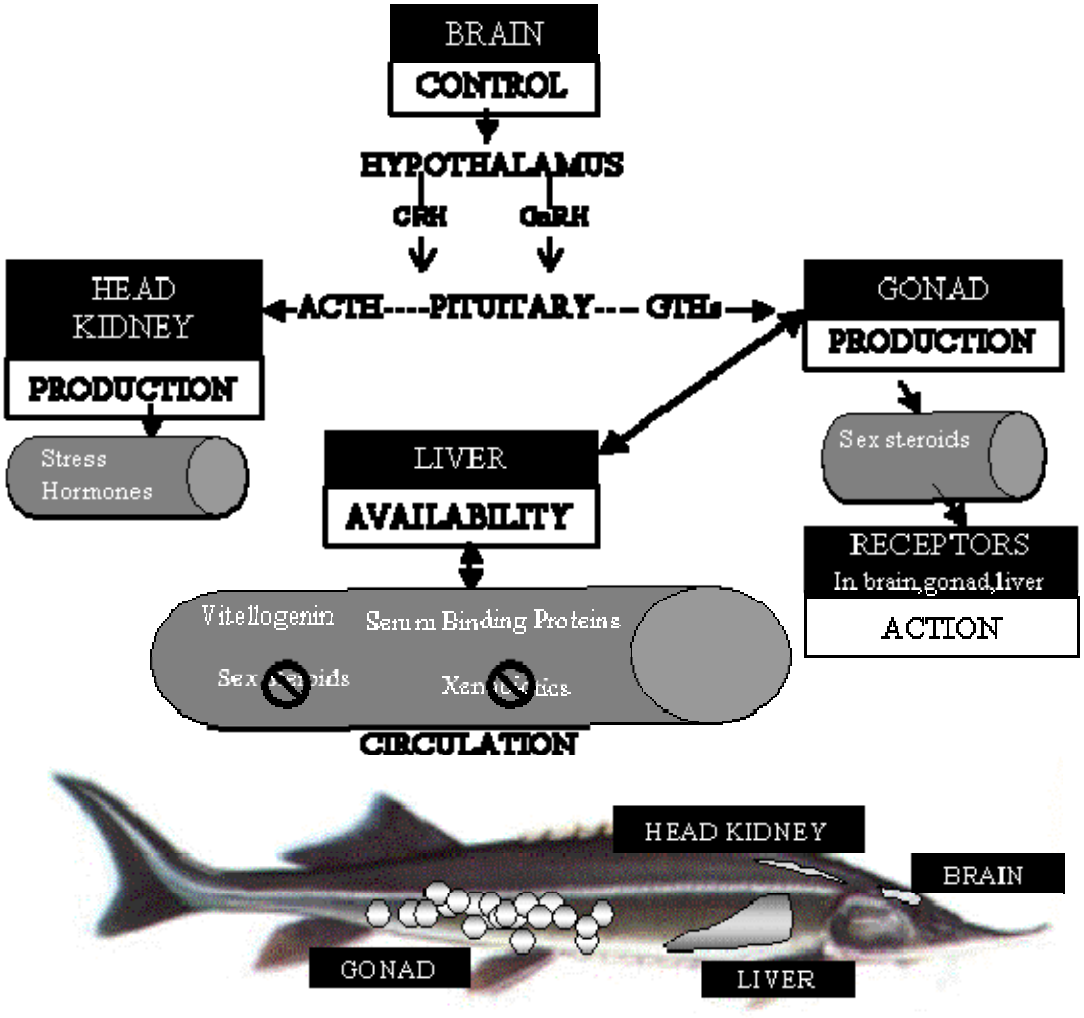


Figure 1-1. Overview of the hypothalamic-pituitary-gonadal axis in sturgeon. The hypothalamic-pituitary-gonadal axis in sturgeon is similar to that of other vertebrates. Gonadotropic releasing hormone (GnRH) from the hypothalamus controls the release of gonadotropins (GTHs) from the pituitary that then enter circulation. The gonad responds by producing various sex steroids including 17β-estradiol, which stimulates hepatic vitellogenin production. These processes are essential for normal ovarian follicle development. Similar to other fish species, the hypothalamic release of corticotropin-releasing hormone (CRH) controls the release of adreno-corticotropin hormone (ACTH) from the pituitary, which controls the release of glucocorticoids from the interrenal cells of the head kidney.

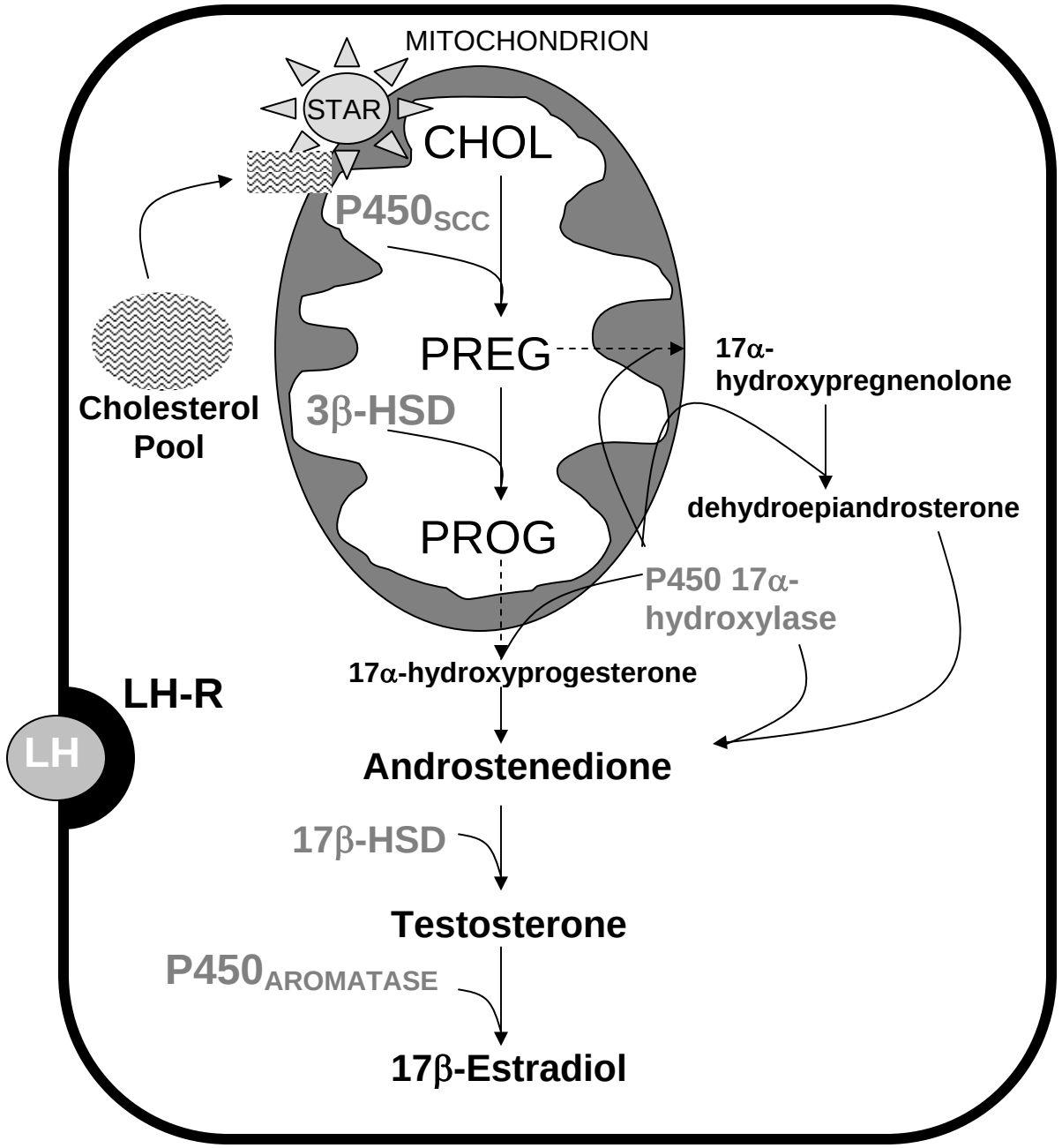


Figure 1-2. Representative steroidogenic pathway of steroid hormones in gonadal cells. In response to ligand binding of the receptor, the transfer of free cholesterol into the mitochondria facilitated by steroidogenic acute regulatory (StAR) protein, is considered the acute rate limiting step in steroidogenesis. The enzymatic conversion of cholesterol to pregeneolone by $P450_{SCC}$ is considered the chronic regulatory step in steroidogenesis. Pregnenolone or progesterone is released into the cytoplasm/smooth endoplasmic reticulum to be converted to androstenedione, which is in turn converted into testosterone and 17β-estradiol by 17β-HSD or aromatase respectively.

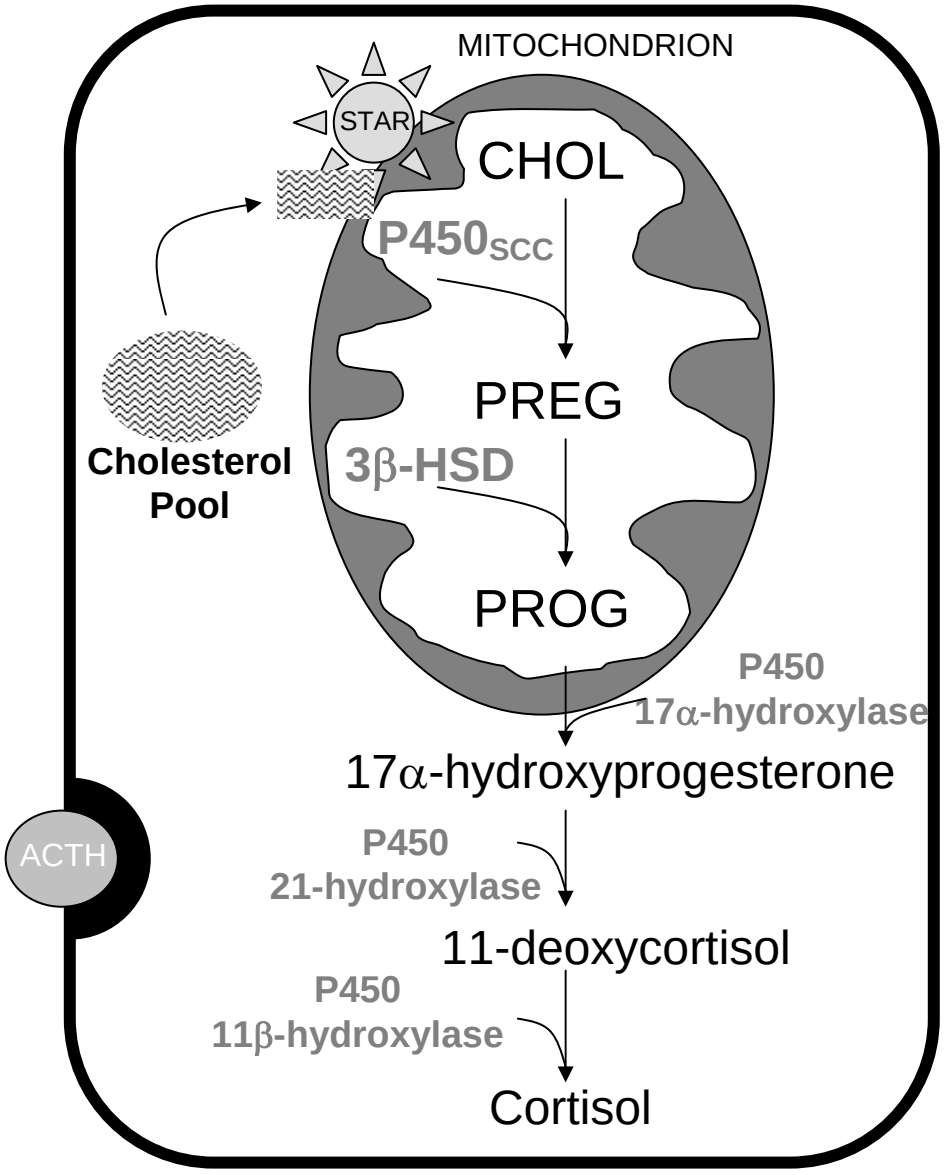


Figure 1-3. Representative steroidogenic pathway of cortisol production in an interrenal cell. In response to ligand binding of the receptor, the transfer of free cholesterol into the mitochondria facilitated by steroidogenic acute regulatory (StAR) protein, is considered the acute rate limiting step in steroidogenesis. The enzymatic conversion of cholesterol to pregeneolone by $P450_{SCC}$ is considered the chronic regulatory step in steroidogenesis. 17α-hydroxyprogesterone is released into the smooth endoplasmic reticulum for further processing and eventual conversion 11-deoxycortisol and cortisol.

CHAPTER 2
NITRATE TOXICITY IN SIBERIAN STURGEON

Introduction

Ammonia is a product of the biological degradation of proteins and nucleic acids. Nitrifying bacteria convert ammonia to nitrite, which is in turn converted to nitrate, the end product of nitrification (Sharma and Ahlert, 1977). Ammonia, and to a less extent nitrite, are ecologically relevant compounds and the toxicity of these compounds, both in terms of tolerable thresholds and physiologic mechanism to aquatic animal health, has been well documented (Rubin and Elmaraghy, 1977; Meade, 1985; Huertas et al., 2002). Nitrate, however, does not normally reach toxic concentrations in natural environments or in recirculating systems with high water exchange, and has therefore received comparatively less attention as a material water quality hazard (Knepp and Arkin, 1973; Russo, 1985; Bromage et al., 1988; Meade and Watts, 1995). The absence of obvious patho-physiological effects in most aquatic species at ecologically relevant concentrations of nitrate, rationalizes the belief that nitrate is relatively non-toxic (Jensen, 1996). While nitrate is indeed much less toxic than ammonia or nitrite on a mg/L basis, nitrate commonly rises to levels far in excess of those of the other compounds in intensive aquaculture environments with limited water exchange (Knepp and Arkin, 1973; Hrubec, 1996), and warrants more detailed investigations into the effects these levels may have.

Excess nitrate in aquaculture has traditionally been reduced by water exchange or the operation of denitrification filters (Timmons et al., 2001). Current trends in environmental regulation are limiting the amount of water which may be consumed or discharged, reducing the feasibility of using large influxes of water to remove excess nitrate.

Denitrification filters can be technically challenging and costly, and as aquaculture operations become water limited, nitrate will become a considerable concern.

The levels of nitrate that are likely to cause concern are unknown for many aquatic species, as are how susceptibilities to nitrate change ontogenetically. For large species such as sturgeon, it is logistically difficult and costly to conduct acute toxicity evaluations on broodstock size animals. However, evaluations using smaller animals may not mimic responses of larger fish. New evidence implicates nitrate as a material water quality hazard at levels much lower than previously suspected for other aquatic species (Guillette and Edwards, 2005) and recommended levels of nitrate for warm-water fishes (90 mg $N0_3$-N) (U.S. E.P.A., 1986) has been shown to be highly toxic to amphibians (Marco et al., 1999).

Although a great deal of research needs to be conducted to elucidate the effects of sublethal exposures, acute testing will assist researchers in understanding how sensitive a particular species is to nitrate, and can be used as a tool to predict if susceptibilities may change over time. The most common analytical method for evaluating acute toxicity in fish is the LC_{50} (Parish, 1985). An LC_{50} describes a lethal concentration (LC) at which 50% of the experimental population dies in a specified period of time. LC_{50} data allows us to determine if a substance is toxic, how toxic it is, and allows for multi-species comparisons of sensitivity. The objectives of this study were to determine the acute toxicity of three ontogenetic size classes of Siberian sturgeon (*Acipenser baeri*) to nitrate, using the LC_{50} criterion, to determine how life stage influences this response.

Methods

Study Animals and Pre-Testing Conditions

Siberian sturgeon were reared from eggs in 250 L troughs in a recirculating system containing well water. Fish were initially fed *Artemia* and a soft moist formulated feed (Silver Cup™, Nelson and Sons Inc., Murray, UT). When the fish reached 1.5 g they were transferred to 1300 L tanks and were fed only formulated feeds by this time. Dissolved oxygen was monitored daily and rarely went below 90% saturation (Oxyguard Handy Beta, Point Four Systems Inc., Richmond, BC, Canada). Temperatures were slowly increased throughout the fish's development, and ranged from 15°C (at hatch) to 23.5°C. Other water quality parameters prior to the toxicity trials were evaluated weekly (ammonia-N and nitrite-N, Lamotte Smart Colorimeter, Chestertown, MD; nitrate, Ion 6 Acorn Series, Oakton Instruments™, Vernon Hills, IL; pH, Acorn 6 Series, Oakton Instruments™, Vernon Hills, IL). In addition to the above parameters, alkalinity, chloride, total hardness and calcium hardness (Hach Company™, Loveland, CO) were tested at the beginning and end of each 96-h toxicity trial.

Range-Finding Studies

Small-scale range finding studies using at least three nitrate concentrations with five fish/concentration were conducted prior to each test until a suitable test range was determined. Suitability was defined by total mortality in the highest concentration and no mortality in the lowest concentration in 96 hours within a narrow test range. Tests generally required 2-3 range finding studies per toxicity trial. Tanks were evaluated for mortalities every 3-4 hours from 08:00 to 20:00, and dead fish were immediately removed and inspected for condition.

Test Procedures

Three partial exchange 96-h toxicity tests were conducted in triplicate using three weight classes of Siberian sturgeon spanning 3 orders of magnitude, with 10 fish per test container. Experiments were conducted over time using fish from the same cohort to eliminate cohort variability. New experimental animals were used for each trial. Water for each of the evaluations consisted of degassed well water (nitrate-N 1.4 ± 0.3 mg/L) from which nitrate solutions were created from food-grade sodium nitrate (JLM Marketing, Tampa, FL). Initial concentrations were confirmed with an Auto Analyzer™, and were periodically spot-checked with an ion specific probe (Ion 6 Acorn Series, Oakton Instruments™, Vernon Hills, IL) throughout the trials to ensure concentrations matched initial target values. Each trial evaluated four geometrically constant concentrations of nitrate, as well as triplicate well water and sodium controls. Sodium controls were achieved with NaCl (Morton Salt™, Chicago, IL) with concentrations adjusted to match the sodium in the highest nitrate concentration in the trial. Tanks were randomly assigned to each treatment. Tanks were evaluated for mortalities every 3-4 hours from 08:00 to 20:00 and dead fish were immediately removed and inspected for condition.

The first trial evaluated concentrations of 555, 888, 1420, and 2273 mg/L nitrate-N using 6.9 ± 0.31g fish. This trial was conducted in glass aquaria filled with 32.4 L of test solution, submersed in a water bath to maintain a temperature of 21°C. A 50% water exchange with the appropriate nitrate concentration was conducted half way through the trial to eliminate collateral effects from elevated ammonia or nitrite. Fish were not fed two days prior to and throughout the trial, and fecal debris was siphoned twice daily.

At least twice daily, observations were made of fish behavior (orientation, gill ventilation rate, swimming speed) and appearance throughout the trial. The second trial evaluated concentrations of 216, 323, 485, and 727 mg/L nitrate-N using 66.9 ± 3.4 g fish. This trial was conducted in fiberglass tanks filled to 670 L. The water was maintained at 23.5°C. The third trial evaluated concentrations of 234, 421, 758 and 1364 mg/L using 673.8 ± 18.6 g fish. This trial was conducted in fiberglass tanks filled to 587 L**,** and the temperature was maintained at 23.5°C.

Statistical Analyses

Data from replicates were pooled prior to calculating the median lethal concentration. Median lethal concentrations and 95% confidence intervals were evaluated by the trimmed Spearman-Karber method for 24, 48, 72, and 96-hr time periods. Testing ranges, determined by range finding studies, were designed to evaluate a 96-hr time period. Therefore, shorter time periods did not always result in enough mortality to compute the LC_{50} values. Normal distribution was evaluated with the Shapiro-Wilk's test. A linear regression of $\log_{10}$ transformed data was conducted to predict susceptibilities of larger sturgeon using StatView® statistical software package (SAS® Institute, Cary, NC).

Results

No animals died in either the well water or sodium controls for any of the size classes tested, and appeared healthy throughout the trial. The 96-h LC_{50} of nitrate to 6.9 ± 0.31 g Siberian sturgeon was 1028 mg/L nitrate-N (Table 2-1). Moribund fish in this size class tended to gill rapidly, but most showed few outward signs of toxicity except a stiffening of the musculature and lethargy (decreased swimming speed, frequent resting periods). The 96-h LC_{50} of nitrate to the 66.9 ± 3.4 g and 673.8 ± 18.6 g sturgeon was 601 mg/L and 397

mg/L nitrate-N respectively. Moribund fish in these treatments tended to exhibit additional evidence of the toxicity such as reddening around the mouth, and red specks and/or patches along the length of the body, most notably at the base of the pectoral fins. Log transformed nitrate vs. log transformed LC_{50} values are shown in Fig. 2-1. Water chemistry parameters were as follows: unionized ammonia-N (NH_3) ≤ 0.04 ± 0.02 mg/L; nitrite-N ≤ 0.01 mg/L; pH 7.9 ± 0.2; alkalinity 208 ± 12 mg/L; chloride 90 ± 5 mg/L (exclusive of the NaCl control); total hardness 260 ± 10 mg/L; calcium hardness 160 ± 10 mg/L. Dissolved oxygen levels were maintained at ≥95% saturation throughout the trials. The Shapiro-Wilk's test indicated normal distribution for all treatments. The 6.9 ± 0.31 g sturgeon were maintained at 21.0°C while the latter two size classes were maintained at 23.5°C, which are typical temperatures for these size stages. Placing all three size classes at the same temperature would not represent a realistic rearing condition, and previous toxicity tests with this species has not demonstrated a significant difference in LC50 values for temperatures ranging from 20°C-25°C for 6.0 g to 1 kg Siberian sturgeon (H. Hamlin, unpublished data).

Discussion

The United States is now recognizing water as a valuable and limited commodity, and its tight regulation is forcing aquaculture technology to shift toward more sustainable and ecologically responsible practices. Therefore, as the land-based aquaculture industry continues to grow, management strategies are shifting to recirculating systems with lower water exchange. This trend is creating new husbandry concerns as less clean water is available to flush out nitrate. In systems with limited water exchange, nitrate can build to levels of 150 mg/L nitrate-N or more (personal observation), and it is unclear the impact

these elevated levels may have. Critical for the design of any aquaculture operation are the water quality standards to be maintained, and it is important to know what levels of substances are likely to cause concern (Bohl, 1977). The etiology and effects of nitrate toxicity are relatively unknown in fishes, leaving open future opportunities for research in this area. This information can then be used to understand toxicity thresholds and physiologic impact, as well as appropriately engineer remediation systems and technologies.

Results of this study demonstrated the 96-h LC_{50} for fish of 7-700 g to range between 397-1028 mg/L nitrate-N. These numbers are appreciably lower than those reported for most aquatic species tested to date. Comparative nitrate data from representative toxicity studies suggests that the majority of test populations can handle nitrate-N levels of 1000 mg/L nitrate-N or more (4426 mg/L total nitrate) without reaching 50% mortality, when sodium nitrate is used as the source of nitrate (Table 2). Some fish, such as the beaugregory (*Stegastes leucostictus*), exhibit LC_{50} values of over 3000 mg/L NO_3-N (13,280 mg/L total nitrate), substantially above the tolerance of most freshwater fish including Siberian sturgeon (Peirce et al., 1993). Although diet may affect the relative toxicity of nitrate (Chow and Hong, 2002), a pervasive theory in the etiology of nitrate toxicity is that it is endogenously converted to nitrite (Hill, 1999), and it is in fact nitrite that is the biotoxic agent. In terrestrial animals this theory has been the source of numerous debates (Hartman, 1982), and the mechanism of nitrate toxicity in fishes is still unclear.

Anecdotal evidence at Mote Marine Laboratory's Aquaculture Park (Sturgeon Commercial Demonstration Project) has shown Siberian sturgeon to be especially sensitive to nitrate, with larger animals exhibiting increased incidence of toxicity and mortality

starting at levels as low as 90 mg/L nitrate-N (398 mg/L total nitrate, see Guillette and Edwards (2005) for an explanation of the reporting of nitrate concentrations) (H. Hamlin, unpublished data). Susceptibilities have been strongly affected by cohort variability, with certain cohorts being more sensitive to elevated nitrate than others. Although the results in this study demonstrate a strong correlation between size and LC_{50} values, caution must be taken in predicting susceptibilities of varying cohorts of fish, or even fish within the same cohort, since LC_{50} values have been shown to be highly variable (Buikema et al., 1982). Regression analysis of the current data yield a predicted LC_{50} of 247 mg/L nitrate-N (1093 mg/L total nitrate) for 6 kg fish (Fig. 2-1). Regardless of the high variability of toxicological responses to nitrate, it is clear from this study that young Siberian sturgeon are far more tolerant to elevated nitrate than their adult counterparts, and this is the first study to demonstrate this finding.

Often, the dose-response relationship is a scaled association between the concentration of chemical tested and the severity of the elicited response (Lloyd, 1979). In general, younger or immature animals tend to be more susceptible to chemical insult or perturbation than are adults (Macek et al., 1978; Sprague, 1985). In fact, a common chronic toxicity test is the early life stage test, because although this test does not provide total life cycle exposure, it is purported to include exposure during the most sensitive life stages (McKim, 1985). This study found an increased tolerance of Siberian sturgeon to nitrate at younger stages. Although this opposes general convention, this phenomenon has been reported for other fish species with other toxic compounds (Rosenberger et al., 1978).

Acute toxicity tests are an effective tool to establish baseline toxicity thresholds in terms of responses to nitrate over time, and to compare the toxicity of nitrate to other

species. Given the increased sensitivity of Siberian sturgeon to nitrate as compared to other species, it is clear much more work is needed to elucidate the sublethal effects of elevated nitrate exposure. The sensitive nature of sturgeon to nitrate renders them suitable candidates for further investigation of the etiology and nature of nitrate exposure and toxicosis.

Table 1-1. LC_{50} results and test conditions for three size classes of Siberian sturgeon exposed to sodium nitrate

Average weight	6.9±0.31 g	66.9±3.4 g	673.8±18.6 g
24-h LC_{50} (mg/L NO_3-N)	1510	n/a *	803
95% confidence interval	(1826-2631)		(720-897)
48-h LC_{50} (mg/L NO_3-N)	1443	n/a *	522
95% confidence interval	(1309-1590)		(486-562)
72-h LC_{50} (mg/L NO_3-N)	1195	n/a *	438
95% confidence interval	(1086-1316)		(394-487)
96-h LC_{50} (mg/L NO_3-N)	1028	601	397
95% confidence interval	(941-1124)	(557-649)	(357-441)

* Not enough partial kill responses to obtain a valid lethal concentration estimate.

Table 1-2. Representative acute toxicity data for nitrate

Species	NO_3 Source	NO_3-N mg/L	LC_{50}	Reference
Cape sole (*H. capensis*)	$NaNO_3$	5081	24-h LC_{50}	Brownell 1980
Common bluegill (*L. macrochirus*)	$NaNO_3$	2909*	24-h LC_{50}	Dowden and Bennett 1965
Goldfish (C. carassius*)*	$NaNO_3$	2761*	24-h LC_{50}	Dowden and Bennett 1965
Tiger shrimp (*P. monodon*)	$NaNO_3$	1575	96-h LC_{50}	Tsai and Chen 2002
Catla (*C. catla*)	$NaNO_3$	1565	96-h LC_{50}	Tilak et al. 2002
Channel catfish (*I. punctatus*)	$NaNO_3$	1409	96-h LC_{50}	Colt and Tchobanoglous 1976
Chinook salmon (*O. tshawtscha*)	$NaNO_3$	1318	96-h LC_{50}	Westin 1974
Fathead Minnows (*P. promelas*)	$NaNO_3$	1349	96-h LC_{50}	Scott and Crunkilton 2000
Guadalupe Bass (*M. treculi*)	$NaNO_3$	1269	96-h LC_{50}	Tomasso and Carmichael 1986
African clawed frog (*X. laevis*)	$NaNO_3$	1236	240-h LC_{50}	Schuytema and Nebeker 1999
Aquatic Snail (*P. antipodarum*)	$NaNO_3$	1042	96-h LC_{50}	Alonso and Camargo 2003
Florida pompano (*T. carolinus*)	$NaNO_3$	1006	96-h LC_{50}	Pierce et al. 1993
Sao Paulo shrimp (*P. paulensis*)	$NaNO_3$	494	96-h LC_{50}	Cavalli et al. 1996
Pacific treefrog (*P. regilla*)	$NaNO_3$	266	240-h LC_{50}	Schuytema and Nebeker 1999
Guppy fry (*P. reticulatus*)	KNO_3	200	72-h LC_{50}	Rubin and Elmarachy 1977
Caddisflies (*C. pettiti*)	$NaNO_3$	114	96-h LC_{50}	Comargo and Ward 1992

* Publication did not specify whether results were values for NO_3 or NO_3-N

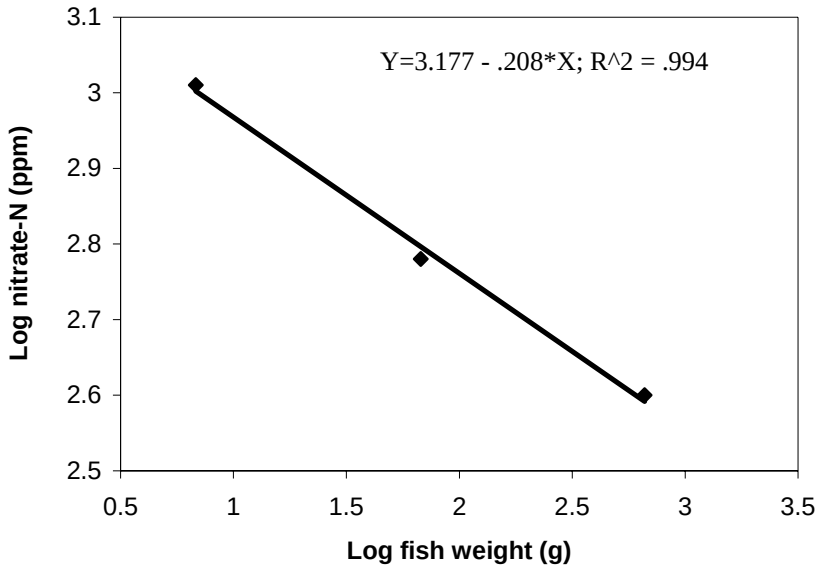


Figure 2-1. Linear regression of $\log_{10}$ transformed nitrate-N (mg/L) lethal concentration values versus log transformed fish weight (g).

CHAPTER 3
STRESS AND ITS RELATION TO ENDOCRINE FUNCTION IN CAPTIVE FEMALE SIBERIAN STURGEON

Introduction

The central focus of comparative physiology and endocrinology involves understanding how various organisms respond to environmental influences. Fish are affected by stress in both their natural and captive environments. It is well recognized that common fishery and aquaculture practices, including crowding, transport and confinement are stressful to fish and can negatively affect reproduction (Pankhurst and Van Der Kraak, 1997). The effects of stress can be manifested at many levels of the reproductive endocrine axis, and measuring the concentration of circulating hormones is a useful endpoint to understand if a stressor affects endocrine function. Numerous environmental stressors, including capture and confinement (Pankhurst and Dedual, 1994), time of day (Lankford et al., 2003), hypoxia (Maxime et al., 1995), and environmental contaminants (Orlando et al., 2002; Guillette and Edwards, 2005) have been shown to induce stress in fish. For most fish, including the Siberian sturgeon and other freshwater chondrosteans, cortisol is the predominant stress hormone (Maxime et al., 1995; Barton et al., 1998; Mommsen et al., 1999). Plasma glucose concentration has also been shown to be an indicator of secondary stress responses (Bayunova et al., 2002).

Sex steroids can have an inverse relationship with plasma concentrations of stress steroids, an effect evident in fish and some other animals (Carragher and Sumpter, 1990; Cooke et al., 2004). Negative effects of stress on reproduction have been attributed to the suppression of LH and FSH secretion from the pituitary gland, disruptions in steroidogenesis pathways, or alteration of hormone degradation by the liver and/or kidney (Krulich et al., 1974). Although plasma concentrations of corticosteroids often parallel acute stress, there is evidence in teleosts that the

estrogenic inhibitory effects of stress are not necessarily mediated by cortisol, and that these effects arise higher in the endocrine pathway than at the level of ovarian steroidogenesis (Pankhurst et al., 1995).

Contradictory evidence has shown that the addition of cortisol to the culture medium reduces the secretions of 17β-estradiol (E_2) and testosterone (T) from cultured ovarian follicles of rainbow trout (*Oncorhynchus mykiss*) (Carragher and Sumpter, 1989). Likewise, carp fed with cortisol-containing food pellets showed reduced androgenic production, independent of LH secretion (Consten et al., 2002). Acute confinement stress in male brown trout (*Salmo trutta* L.) resulted in low concentrations of plasma T and 11-KT in sexually mature animals (Pickering et al., 1987). White sturgeon (*Acipenser transmontanus*) injected with an ACTH analog exhibited a dose-dependent increase in cortisol concentration more than the cortisol concentrations induced by stress events such as transport and handling (Belanger et al., 2001). A few studies, including one examining the effects of stress on serum cortisol concentration in cultured stellate sturgeon, actually demonstrated significantly increased gamete quality in fish with elevated cortisol concentration, speculating that cortisol could be a normal endocrine component of the reproductive system, even though later studies of the same species showed reduced plasma concentrations of sex steroids during stress (Semenkova et al., 1999; Bayunova et al., 2002). It has also been shown that fish require prolonged periods to recover from an acute stress event (Jardine et al., 1996). Other studies have shown that blood removal, a practice often necessary for evaluating endocrine endpoints, can alter blood hemoglobin concentration (Hogasen, 1995).

Stress studies typically focus on the causative factors mitigating the deleterious response, but defining these relationships often requires sampling and research measures that themselves contribute to enhancing the stress response. Understanding the effects of potential stressors is

critical to properly manage wild fisheries or successfully culture endangered or economically important fishes. It is important to know which stressors are naturally present in the fish's environment, which are caused by typical aquaculture practices, and which are induced by the testing procedures themselves (Conte, 2004).

Sturgeons (*Acipenseriformes*) are among the most ancient fishes on earth, originating over 200 million years ago (see review by Birstein, 1993). Twenty-five extant sturgeon species occupy the Northern Hemisphere; however, excessive fishing, loss of spawning grounds and other environmental pressures have contributed to the reduction of sturgeon stocks worldwide, particularly Caspian Sea varieties (Williot et al., 2002). Today, all 25 species of sturgeon are listed as endangered or threatened in some regard (Birstein, 1993). Aquaculture has been proposed as a means to conserve sturgeon, and generating commercial stocks has the dual benefit of providing fish for stock enhancement, as well as for food production, thus conserving wild populations (Beamesderfer and Farr, 1997; Waldman and Wirgin, 1997; Chebanov et al., 2002; Stone, 2002). The Siberian sturgeon is rapidly becoming a species of great economic interest in the United States, and is currently the most widespread sturgeon species utilized for commercial aquaculture in Europe (Gisbert and Williot, 2002). Despite this, very few studies have been conducted to clarify the physiological effects of stress on this species. Understanding the endocrine disruptive effects of induced stress will serve as a baseline for understanding the effects of other environmental stressors, such as contaminants commonly found in both natural and constructed environments. Nitrate, for example, has recently been shown to be highly toxic to Siberian sturgeon in aquaculture environments with limited water exchange (Hamlin, 2006), and is predicted to be of considerable concern for commercial aquaculture operations, which are already being forced to significantly reduce their water usage. Nitrates and other ions have also

been established as ecologically relevant endocrine disruptors in natural environments for numerous other vertebrates (see review by Guillette and Edwards, 2005). For late maturing species such as sturgeons, whose economic viability relies heavily on successful egg production (caviar), it is of particular importance to understand the relationships between stress and reproductive health.

The purpose of this study is to define the relationship between induced stress and circulating concentrations of steroid hormones in cultured Siberian sturgeon, and to identify mitigating stress factors in typical testing procedures, most notably the techniques of blood withdrawal and surgical sexing, to understand what factors contribute significantly to the stress response.

Methods

Fish and Sampling

Three-year-old Siberian sturgeon were collected from two 30,000 L tanks, each from separate commercial recirculating aquaculture systems at Mote Marine Laboratory's Aquaculture Park (Commercial Sturgeon Demonstration Project) in Sarasota, Florida. Experiments were started at approximately 10:30 a.m. in May of 2004. Water chemistry in each of these systems was analyzed weekly for the levels of ammonia-N, nitrite-N, nitrate, and pH prior to the start of experiments. Dissolved oxygen and temperature were monitored continuously using stationary probes, which were spot-checked biweekly for calibration using portable probes. Hardness, alkalinity, and chloride concentration was analyzed the day prior to the start of experiments.

The sturgeon were pulled from the water by hand at the side of the tank and immediately held down on a padded V-shaped surgical table. Pulling the sturgeon from the tank by hand (versus netting) decreased the likelihood of stressing the remaining fish in the tank and allowed immediate access to the fish for blood sampling. Blood was extracted from the caudal vein (5

ml) using a 10 ml syringe (20 gauge needle) within 1 min of capture; most captures took 30 sec for the full blood sample to be drawn. The blood sample was placed into lithium heparin Vacutainer™ tubes, and stored on ice for less than 30 minutes before centrifugation. Plasma was separated via centrifugation (5-10 min at 2000 *g*), placed into cryovials, rapidly frozen in liquid nitrogen and stored at -80 °C for 2-3 weeks prior to analysis.

Surgical Sexing

For surgical sexing, the sturgeon were anesthetized in a 5 °C water bath containing carbon dioxide. Carbon dioxide was used because it is a low regulatory priority anesthetic for fish that are grown for food production and requires no withdrawal period; the sturgeon used in this study were part of a commercial food production program. Pure oxygen gas administered through a fine air stone was used to maintain dissolved oxygen concentrations in the range of 8.0 - 12.0 mg/L in the anesthetic bath, and sodium bicarbonate was added to maintain pH in the range of 6.8 - 7.5 throughout the procedure. The sturgeon generally took 3 - 5 min for full anesthetization. A 2.5 - 3.8 cm incision was made on the ventral side of each fish, approximately 7.5 cm anterior to the vent, along the median axis to allow inspection of the gonads on either side of the fish for sex determination. The incision in each fish was closed by suturing with coated vicryl absorbable suture (Ethicon Inc™., Somerville, New Jersey), and the fish was allowed to recover in a confinement tank. Once anesthetized, the surgical procedure took approximately 1 min/fish, and the fish recovered fully from the anesthesia in 5 - 10 min.

Treatments

Six fish (3 fish/tank) were used for each treatment. All fish were sexed immediately after initial bleedings/sham bleedings; if the fish was male, the sample was discarded, and another fish was extracted until 3 females had been sampled from each tank for each treatment. In this study,

we focused on female sturgeon because they are part of a larger set of studies examining various environmental factors and ovarian development leading to commercial caviar production. The female sturgeon were then weighed and measured just after sexing while they were still under anesthesia. The fish were then placed into a square 0.64 m^3 insulated plastic tote filled with 530 liters of system water for a 4-h period of confinement stress. A numbered cable tie placed around the caudle peduncle identified individual fish. The time at which the fish was removed from the tank for initial bleeding/sham bleeding was considered 0-h.

In all treatments, fish were sexed immediately after initial blood drawing/sham drawing prior to placement in the confinement tank. In treatment 1, fish were bled at 0-h only and placed into an insulated tote as described previously. In treatment 2, fish were bled at 0-h, 1-h and 4-h. In treatment 3, fish were bled at time 1-h and 4-h only, and in treatment 4, fish were bled at 4-h only. For treatments 3 and 4, during the sampling periods when the fish were not bled, the fish were held down on the surgical table momentarily to mimic the bleeding procedure but were not pricked with the needle. Blood sampling times for all treatments during the 4-h period of confinement stress are shown in Fig. 3-1.

Hormone Evaluations

Plasma samples for steroid evaluations were thawed on ice, and the steroid fraction was extracted with diethyl ether. Extraction was repeated twice to enhance extraction efficiency. Plasma cortisol, E_2, T and 11-KT concentrations were analyzed according to the instructions provided with the commercial competitive enzyme immunoassay kits (Cayman Chemical Co., Ann Arbor, MI), specific to each hormone. Each hormone was previously validated for Siberian sturgeon plasma by verifying that serial dilutions were parallel to the standard curve. Samples were run in duplicate and each plate contained duplicate wells for interassay variance and a blank. Individual hormones were all run with plates from the same kit lot # and were completed

in the same testing session to reduce testing variance. Sample plates were analyzed using a microplate reader (BioRad, Hercules, CA). Intra-assay and interassay variances, respectively, were as follows: estradiol, 3.5% and 7.0%; cortisol, 2.0% and 9.1%; testosterone, 3.7% and 12.8%; 11-KT, 4.9% and 11.9%.

Plasma samples for glucose concentration determination were thawed on ice and evaluated according to the instructions provided with the commercial glucose oxidase assay kit (Invitrogen, Amplex® Red, Eugene OR). The sample plate was analyzed using a microplate reader (BioRad, Hercules, CA).

Statistical Analyses

Statistical analyses were performed using StatView for Windows (SAS Institute, Cary, NC, USA). Initial comparisons were made to determine if there was a significant tank effect within treatments. F-tests were conducted to test variances among treatment groups for homogeneity. If variance was heterogenous, data were $\log_{10}$ transformed to achieve homogeneity of variance; however, all reported means ($\pm$ 1 SE) are from nontransformed data. Analyses of variance (ANOVA) of weight, length and hormone concentration was used to compare differences among treatment groups. If significance was determined ($\underline{P} < 0.05$), Fisher's protected least-significant difference was used to determine differences among treatment means.

Results

Morphology and Chemistry

The average fish weights in this experiment ranged from 4.13 to 4.55 kg, and the average fish length ranged from 88.8 to 92.2 cm. Neither weight nor length was significantly different among treatments, and there was no significant tank effect for any tested parameter. Water

chemistry parameters were tested on the day of the experiment and were as follows: un-ionized ammonia (NH_3), ≤ 4.55 µg/l; nitrite, ≤ 0.2 mg/L; pH, 7.5; alkalinity, 200 mg/L; chloride concentration, 85 mg/L; total hardness, 230 mg/L; and calcium hardness, 130 mg/L. Dissolved oxygen concentrations were maintained at ≥ 95% saturation throughout the trial and the temperature was 24°C.

Hormones

The 0-h plasma cortisol concentrations for treatments 1 and 2 averaged 6.65 ± 3.58 and 4.63 ± 1.02 ng/ml, respectively (Fig. 3-2A), and were statistically similar. The 0-h plasma glucose concentrations were statistically similar and averaged 2.13 ± 0.12 and 2.21 ± 0.11 mmol/L for treatments 1 and 2, respectively (Fig. 3-2B). The plasma concentrations of T, 11-KT, and E_2 were statistically similar at 0-h for treatments 1 and 2 and averaged 25.53 ± 2.9, and 10.2 ± 0.8 ng/ml and 672.4 ± 45.9 pg/ml, respectively.

Plasma cortisol concentrations increased significantly ($P \leq 0.05$) in the Siberian sturgeon from 0-h to the 1-h sampling period averaging 70.9 ± 18.7 ng/ml at 1-h, and were not significantly different between treatments 2 and 3 (Fig. 3-2A). Plasma glucose concentrations increased significantly from 0-h to the 1-h sampling period and averaged 4.67 ± 0.40 mmol/L at 1-h, and there were no significant differences among treatments 2 and 3 (Fig. 3-2B). At 4-h, plasma cortisol concentrations were similar for fish in treatments 2 (46.2 ± 15.4 ng/ml) and 3 (36.27 ± 14.0 ng/ml), but were significantly elevated compared with those observed for fish in the treatment 4 group (10.44 ± 2.53 pg/ml) (Fig. 3-2A). Plasma glucose concentrations at the 4-h sampling period were similar for treatment 2 (4.70 ± 0.27 mmol/L) and treatment 4 (4.14 ± 0.38

mmol/L), but were significantly lower than plasma glucose concentration in treatment 3 (5.65 ± 0.41 mmol/L) (Fig. 3-2B).

The evaluation of treatment 2, in which the same group of fish at 0-h, 1-h and 4-h were sampled, demonstrated that plasma T concentrations increased significantly from time 0 to 1-h (20.3 ± 1.76 and 31.45 ± 4.19 ng/ml respectively), with a subsequent decrease at 4-h to a concentration similar to that observed at 0-h (Fig. 3-3A). In the same fish, we observed no differences between bleeding times for E_2 or 11-KT (Fig. 3-3 B,C).

Discussion

The Siberian sturgeon that were exposed to capture and confinement stress exhibited significantly elevated plasma cortisol concentrations 1-h after the initiation of stress, which persisted throughout the 4-h sampling period. This response is similar to the reactions of other fish species exposed to acute stressors (Thomas et. al., 1990). Cortisol and glucose have been shown to be more sensitive to stress than most other plasma constituents except catecholamines, and respond rapidly to a wide range of environmental stressors. Stress in fish and the concomitant increase in cortisol have been implicated in numerous physiological conditions including impaired immune function (Tort et al., 1996), altered feeding behavior (Kentouri et al., 1994), oxygen radical production (Ruane et al., 2002), and reproductive impairment (Pankhurst and Van Der Kraak, 1997). Responses to stress are largely dependent on the severity and type of environmental stressor. Previous studies with Siberian sturgeon exposed to acute and severe hypoxia have shown significantly elevated plasma cortisol concentrations, with a peak concentration of 35,000 pg/ml (Maxime et al., 1995). The basal cortisol concentration in that study was approximately 5000 pg/ml, which is comparable to the basal cortisol concentration obtained in this study. However, the peak concentration of cortisol in our study increased to

nearly 75,000 pg/ml, demonstrating the plasticity of the physiological stress response in this species. In some species, plasma cortisol concentrations can persist for days if the stressor is chronic or severe (Sumpter, 1997).

This study is distinct from other studies in several regards. This is the first study to define the relationship between stress and potential reproductive function, as indicated by the plasma concentrations of various sex steroids, in Caspian Sea sturgeon, habituated to a warm environment and reared under commercial culture conditions from the egg stage. This is also the first study to show the endocrine effects of surgical sexing, a procedure often necessary for sturgeon and other species that do not exhibit sexually dimorphic characteristics. The induced stressors in this study, caused by capture and confinement, bleeding, and surgical sexing are common stressors in a laboratory or fishery environment, and it is important to understand what effects these stressors can have on mitigating experimental responses.

In this experiment, fish underwent capture and confinement stress, with multiple disturbances at 1-h and 4-h. It has been shown that serial stressors evoke cumulative physiological stress responses in other fish species (Waring et al., 1997; Di Marco et al., 1999) and multiple stress events cause fish to be more sensitive to additional acute stress (Ruane et al., 2002). The multiple disturbances in this study likely mitigated the expected decreases in plasma cortisol concentrations after 4-h, because in treatment 4, where fish were captured but not bled until the fourth hour of capture, fish exhibited lower plasma cortisol concentrations than fish in treatment 2 or 3. These lower concentrations could result from a more rapid return toward basal concentrations, due to the lack of repeated stressors, or a reduced stress effect as they were not bled initially, adding additional handling and blood loss to the stress. Our data indicate that serial bleedings intensify the associated stress response, as evidenced by significantly lower

concentrations of F in fish in which a blood sample was not drawn at 0-h or 1-h. This is an important consideration for future studies of this species involving multiple blood samples. Whether elevations in cortisol concentration for the serially bled fish are due to blood volume loss or its associated stressors such as pricking of the fish with a needle, or longer handling times to ensure that a fish is still for actual blood drawing versus sham drawing, is uncertain. It is likely, however, that it is a combination of events, and not solely blood loss that leads to elevated stress in serially bled fish. Note that surgical sexing, an invasive procedure that is often necessary in aquaculture or fishery practice, did not induce a prolonged stress reaction, because fish in treatment 4, which were similarly sexed at 0-h, exhibited plasma cortisol concentrations similar to basal concentrations less than 4-h after the procedure.

The 0-h blood sampling period was started in the morning and the experiment was concluded in the early afternoon. Cortisol concentrations in sturgeon (Belanger et al., 2001; Lankford et al., 2003) and other animals (Young et al., 2004) have been shown to be highly sensitive to diurnal variation, so care was taken in this study to ensure that all samples were collected within a relatively short period to reduce the possibility of daily hormone fluctuations as confounding variables. In addition to the concentrations of sex steroids, it has been shown that plasma cortisol concentration can be altered depending on the reproductive stage in sturgeon (Barannikova et al., 2000) and other species (Pickering and Pottinger, 1985). The female sturgeon in this study were 3 years old, and although all female sturgeon had formed clearly visible ovigerous lamellae or ovarian folds, none of them exhibited vitellogenic oocytes, and they appeared to be in a similar reproductive stage. However, the plasma concentrations of sex steroids in this study were similar to those of fish possessing fully vitellogenic oocytes in subsequent studies.

Interestingly, the concentrations of sex steroids evaluated in this study did not demonstrate an inverse relationship with stress as defined by plasma cortisol concentrations; in fact, plasma T concentration was significantly elevated during periods of peak plasma cortisol concentration (Fig. 3-3A). Although there have been no studies of this kind, in which stress and reproductive function in Siberian sturgeon reared in commercial culture conditions are evaluated, this response is distinct from that in published data with other fish species, including other sturgeon species. Of the reproductive hormones, testosterone has been shown to be highly responsive to stress-induced alterations in sturgeons and other species (Pickering et al., 1987; Bayunova et al., 2002). Plasma E_2 and 11-KT concentrations were not significantly affected by stress within the timeframe of this study. In American alligators, certain environmental toxicants were found to increase plasma T concentrations in juveniles, but did not affect the plasma concentrations of other circulating hormones (Milnes et al., 2004). Our findings do not necessarily indicate, however, that stress is not detrimental to the reproduction of this species. Circulating concentrations of sex steroids are only one endpoint in the reproductive endocrine axis, and stress can manifest itself at many levels of the steroidogenic pathway. For example, sex steroids are generally removed from circulation via clearance by the liver. Reductions in sex steroid production would not necessarily be reflected in circulating concentrations if clearance is concomitantly affected. Other possible mechanisms that would result in the alteration of the reproductive biology of this species include alterations in hypothalamic-pituitary stimulation or alterations in transport mechanics (i.e., transport proteins).

Finally, the elevation in plasma T concentrations described here could be due to a technical problem; that is, although commercial antibodies are screened for cross reactivity and specificity to a wide range of steroids, little is known about the steroid milieu released during stress in

sturgeon. Although unlikely, it is possible that a unique androgen of adrenal origin is released during stress in this species that cross reacts with the antibody used in the testosterone but not in the 11-KT kits. Studies using advanced analytical chemistry could determine the steroids released from stressed Siberian sturgeon. The data presented here indicate that the concentrations of sex steroids in Siberian sturgeon do not show an inverse relationship with elevated plasma cortisol concentration following acute stress, as has been observed for most fish. This altered response needs further study, as this study differed from previous studies of sturgeon in that it coupled sturgeon habituated to warm temperature with a specific stress response. This is the first study to define the relationship between stress and endocrine function in cultured Siberian sturgeon, a threatened and commercially important species. Future studies need to address various aspects of the aquaculture environment (e.g., temperature and water quality), reproductive stage (e.g., juvenile versus adult) and seasonality to determine which variables modify the stress response and thus potentially alter growth and reproductive potential. This work will also serve as a baseline to evaluate the effects of material water quality hazards, such as nitrate, present in both natural and constructed environments.

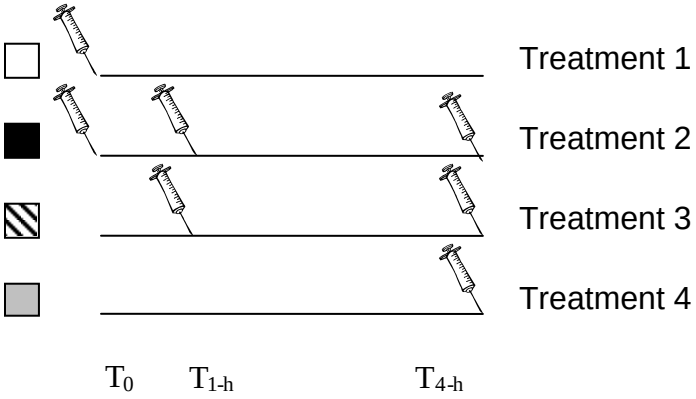


Figure 3-1. Blood sampling times for Treatments 1 to 4 of fish held under confinement stress for 4 hours. Six female Siberian sturgeon were used for each treatment.

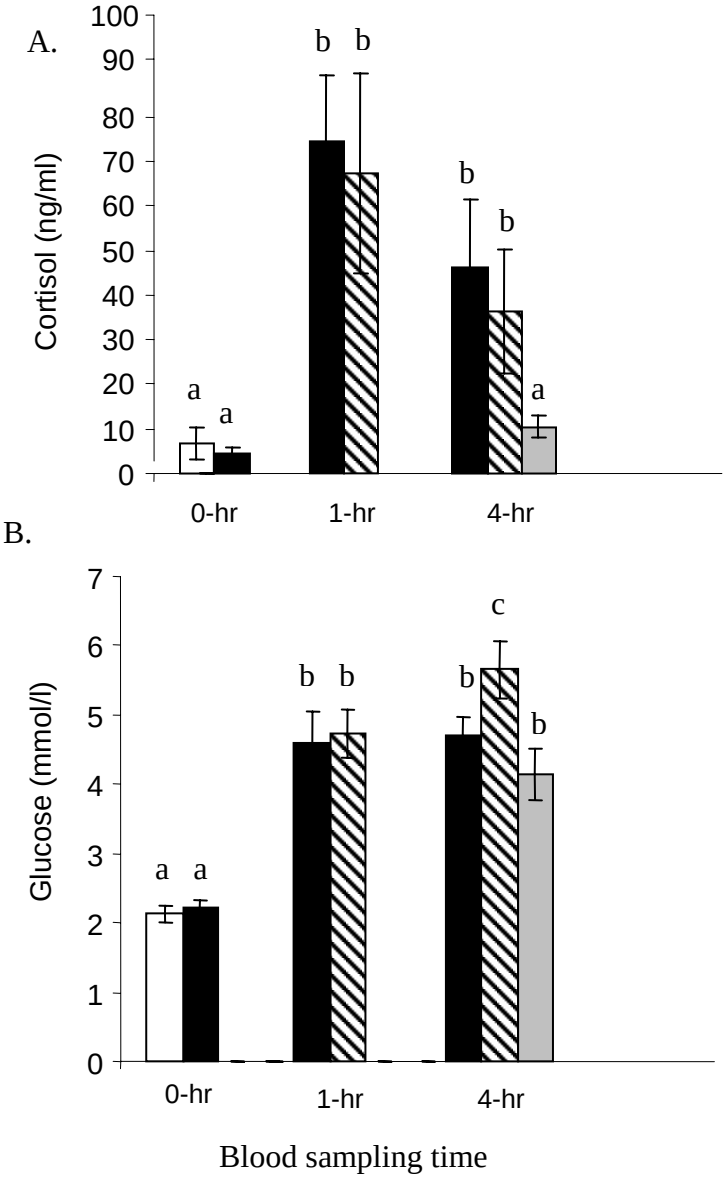


Figure 3-2. Plasma cortisol (A) and plasma glucose (B) concentrations (mean ± S.E.M.) during a 4-h capture and confinement period. Means with the same superscript are not significantly different ($P \geq 0.05$).

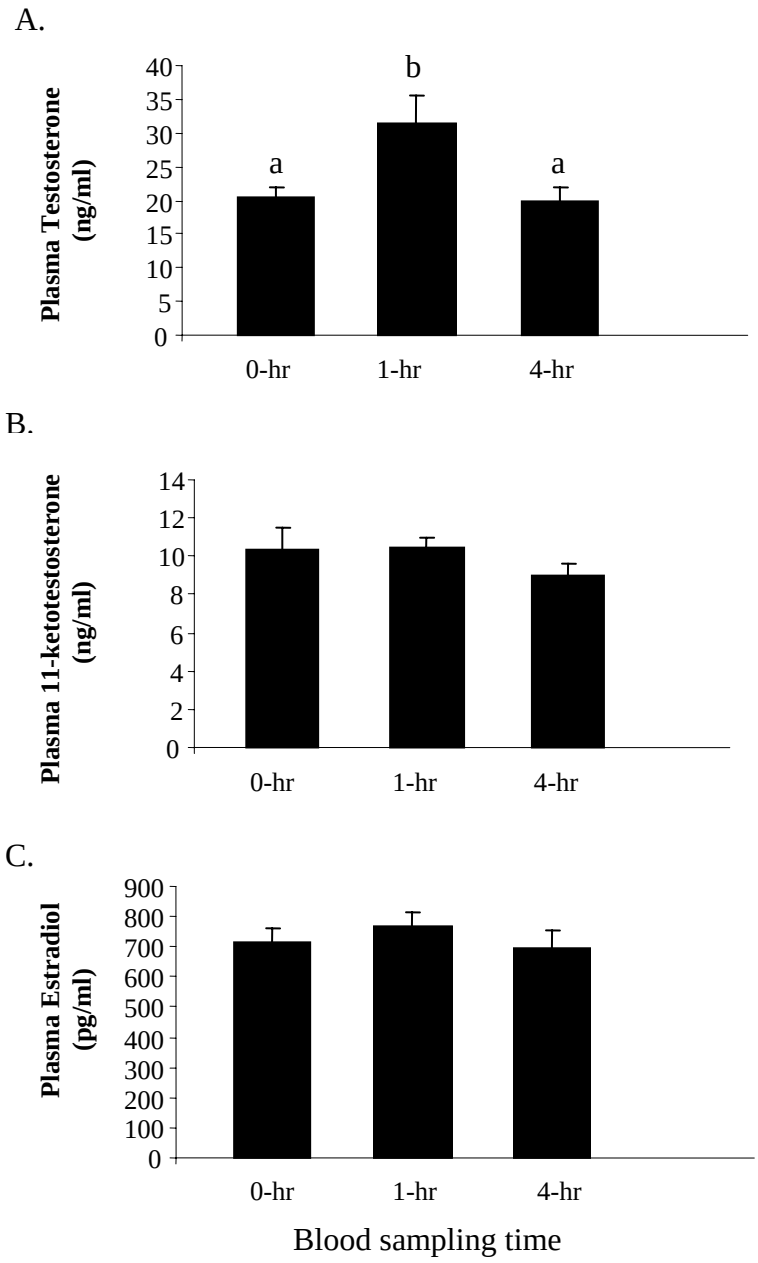


Figure. 3-3. Sex steroid data for treatment 2. Plasma 17β-Estradiol (A), testosterone (B), and 11-ketotestosterone (C) taken from serial bleeds of cultured female Siberian sturgeon throughout the 4-h period of confinement stress (mean ± 1 S.E.M.). Fish were serially bled at 0-h, 1-h and 4-h (see Fig. 3-1 legend for a description of treatment 2 bleeding times). Means with the same superscript or no superscript are not significantly different ($P \geq 0.05$).

CHAPTER 4
NITRATE AS AN ENDOCRINE DISRUPTING CONTAMINANT IN CAPTIVE SIBERIAN STURGEON

Introduction

The endocrine disrupting actions of various chemical contaminants have become a significant concern for comparative endocrinologists (Colborn et al., 1993; Guillette and Crain, 2000). A growing literature describes the effects of endocrine disrupting contaminants (EDCs) for both terrestrial (Iguchi and Sato, 2000) and aquatic (Sumpter, 2005; Milnes et al., 2006) species. These effects include altered reproductive morphology, endocrine physiology and behavior, and involves such endpoints as reduced phallus size, decreased sperm count, depressed reproductive behaviors and altered circulating concentrations of sex steroids (e.g., Guillette et al., 1999; Orlando et al., 2002; Toft and Guillette, 2005). EDCs exert their effects by mimicking hormones, acting as hormone antagonists, altering the function or concentration of serum-binding proteins, or altering the synthesis or degradation of hormones. Aquatic organisms can receive continuous exposure to environmental contaminants throughout their lives, as the aquatic environment receives most of the intentionally released environmental pollutants. Thus, the effects of EDC exposure on aquatic life have received considerable attention (Kime, 1999; McMaster, 2001; Sumpter, 2005; Milnes et al., 2006).

Although nitrate is a ubiquitous component of aquatic environments, and has become a global pollutant in a variety of aquatic systems (Sampat, 2000), it has only recently begun to receive attention for its ability to alter endocrine function (Guillette and Edwards, 2005). The toxicological effects of nitrate have long been known. As early as 1945, nitrate induced methemoglobinemia (Blue Baby Syndrome) in humans was associated with drinking well water contaminated with nitrate (Comly, 1945). Fish are also vulnerable to methemoglobinemia (Brown Blood Disease), and in Siberian sturgeon methemoglobinemia has been associated with a

significant chloride imbalance (Gisbert et al., 2004). Toxicity studies with fish (LC_{50}) have shown lethal concentrations of nitrate to range an order of magnitude or more (Brownell, 1980; Pierce et al., 1993; Hamlin, 2006), demonstrating significant plasticity in response to elevated nitrate among fish species.

Sublethal effects of nitrate include endocrine alterations which have been shown to alter metabolism, reproductive function and development. Frogs (*Rana cascadae*) exposed to 3.5 mg/L nitrate-N metamorphosed more slowly, and emerged from the water in a less developed state than control animals (Marco and Blaustein, 1999). Rodents exposed to nitrate (50 mg/L $NaNO_3$) in their drinking water had significantly lower circulating testosterone (T) concentrations than control animals (Panesar and Chan, 2000). Bulls given oral administration of nitrate (100 – 250 g/day/animal) showed reduced sperm motility, depressed Leydig cell function, and degenerative lesions in the germ layers of the testes (Zraly et al., 1997). Studies in Southern toad tadpoles showed nitrate induced alterations in growth and thyroxine concentrations were mitigated by the source of culture water used, indicating that environmental context plays a significant role in mitigating the effects of nitrate (Edwards et al., 2006a). Mosquitofish (*Gambusia holbrooki*) experienced significant reproductive alterations, such as reduced gonopodium length and fecundity (number of females per unit of female size), in nitrate concentrations as low as 5 mg/L NO_3-N (Toft et al., 2004; Edwards et al., 2006b). Proposed mechanisms for nitrate induced steroidogenic disturbances include mitochondrial conversion to nitric oxide (NO), altered chloride ion concentrations and altered enzymatic action by binding to the heme region of P450 enzymes associated with steroidogenesis (Guillette and Edwards, 2005).

Stress effects on reproduction can be manifest at various levels of the reproductive endocrine axis, and stress has been shown to have inhibitory effects on reproduction for most

aquatic species studied to date (Pickering et al., 1987; Carragher and Sumpter, 1990; Pankhurst and Van Der Kraak, 1997; Consten et al., 2002). For many species of fish, including sturgeon and other chondrosteans, cortisol is the primary stress hormone (Idler and Sangalang, 1970; Barton et al., 1998) and cortisol has been implicated in mediating the inhibitory reproductive effects induced by stress (Pankhurst and Van Der Kraak, 1997; Semenkova et al., 1999; Bayunova et al., 2002). There is evidence in teleosts, however, that the estrogenic inhibitory effects of stress are not mediated by cortisol and that the effects arise higher in the reproductive endocrine pathway (Pankhurst et al., 1995). Tilapia (*Oreochromis mossambicus*) fed pellets containing cortisol to achieve plasma cortisol concentrations typical of acutely stress fish, resulted in decreased plasma concentrations of T and 17β-estradiol (E_2), reduced oocyte diameter and gonad size in females, and reduced plasma T concentrations in males (Foo and Lam, 1993a,b). Female brown trout (*Salmo trutta*) exposed to 2 weeks of confinement stress had significantly reduced plasma T concentrations compared to unstressed fish (Campbell et al., 1994). Plasma glucose concentrations have also been shown to be reliable indicators of secondary stress responses. An animal under chronic stress can demonstrate a reduced capacity to handle subsequent stress events, and studies have shown responses of fish to multiple stressors are cumulative (Barton et al., 1986). Fish residing in laboratories or fish farms are often subjected to chronic stress (sub-optimal water chemistry, crowding, confinement) followed by acute stress events (sampling, netting), which can lead to dramatic and prolonged stress responses (Rotllant and Tort, 1997; Heugens et al., 2001).

Sturgeon are among the most ancient groups of Osteichthyes, and twenty-five extant species occupy the Northern Hemisphere (Birstein, 1993). The dramatic decline in sturgeon populations due to overfishing, pollution, and habitat degradation have led to the necessity of

commercial aquaculture as a means to provide animals for stock enhancement, as well as food production, reducing pressures on wild populations (Beamesderfer and Farr, 1997; Waldman and Wirgin, 1997; Williot et al., 2002; Chebanov et al., 2002). The Siberian sturgeon is one of the leading species of sturgeon adapted to aquaculture (reviewed by Gisbert and Williot, 2002). It was recently discovered that Siberian sturgeon are more sensitive to nitrate toxicosis than most fish species reported to date (Hamlin, 2006). Further, Siberian sturgeon juveniles become less tolerant to nitrate as they grow, a finding of considerable importance for the commercial culture of this species, since adult populations reared in recirculation systems often experience higher nitrate concentrations than their juvenile counterparts. Although understanding what concentrations of nitrate are necessary to avert mortality is generally understood in commercial aquaculture, mortality is not an effective endpoint for producers interested in optimizing growth and reproductive function. Understanding nitrate's effects on reproductive function is especially critical to sturgeon, whose economic viability relies heavily on proper endocrine function, notably the production of eggs (caviar).

The purpose of this study is to begin to determine the potential effects of elevated environmental nitrate on endocrine function, and investigate whether elevated nitrate alters the stress response in captive female Siberian sturgeon.

Methods

Fish and Sampling Procedures

Siberian sturgeon were collected from four 30,000 liter tanks, from separate commercial recirculating aquaculture systems at Mote Marine Laboratory's Aquaculture Park (Commercial Sturgeon Demonstration Project) in Sarasota, FL. Water chemistry in each of these systems was analyzed weekly for ammonia, nitrite, nitrate, and pH prior to commencement of the experiments. Dissolved oxygen and temperature were monitored continuously with stationary

probes, which were spot-checked bi-weekly for calibration with portable probes. Hardness, alkalinity and chloride were analyzed the day prior to commencement of the experiment.

The sturgeon were pulled by hand at the side of the tank and immediately held down on a padded V-shaped surgical table. Pulling the fish from the tank by hand (versus netting) decreased the likelihood of stressing fish remaining in the tank and allowed for more immediate access to the fish for blood sampling. Blood was extracted from the caudal vein (5 ml) with a 10 ml syringe (20 gauge needle) within 1 minute of capture; most captures took 30 seconds for the full sample to be drawn. The blood was placed into lithium heparin Vacutainer™ tubes, and stored on ice for no more than 30 minutes before centrifugation. The plasma was separated via centrifugation (5 - 10 min at 2000 g), transferred to cryovials, flash frozen in liquid nitrogen and stored at -80° C for 1 – 3 weeks prior to analysis.

Surgical Sexing

For surgical sexing, the fish were anesthetized in a 5 - 8° C water bath containing carbon dioxide (CO_2) gas; CO_2 was used because it is a low regulatory priority anesthetic for fish that are grown for food production and requires no withdrawal period; the sturgeon used in this study were part of a commercial food production program. Pure oxygen gas administered through a fine air stone was used to maintain a dissolved oxygen concentration of 9.0 - 13.0 mg/L, and sodium bicarbonate was added to maintain a pH of 6.8 - 7.6 in the bath throughout the procedure. Fish generally took 3 - 5 minutes for full anesthetization. A 2.5 – 3.5 cm incision was made on the ventral side of the fish, approximately 8 cm anterior to the vent, along the median axis to allow inspection of the gonads on either side of the fish for sex determination. The fish was sutured closed with coated vicryl absorbable suture (Ethicon Inc., Somerville, New Jersey).

Experiment 1

Experiment 1 was conducted in July of 2004 and consisted of two treatments, which sampled fish from each of four commercial culture tanks (30,000 l each) located in separate recirculating systems at Mote Marine Laboratory's Aquaculture Park. Two of the culture tanks were held at a nitrate concentration of 11.5 mg/L nitrate-N (50 mg/L total nitrate) for one month, and the other two tanks were held at 57 mg/L nitrate-N (250 mg/L total nitrate) for the same time period (two replicates each). Nitrate concentrations were achieved by adjusting the freshwater input to each system, typical of commercial culture practices. Prior to the 1-month exposure, nitrate concentrations in the four study tanks oscillated between 20 - 60 mg/L nitrate-N routinely. A nitrate concentration of 57 mg/L nitrate-N was chosen as the upper limit in this study, as this is the maximum concentration deemed safe, defined by feeding behavior and mortality, at Mote's Commercial Sturgeon Demonstration Project. The lower concentration of 11.5 mg/L nitrate-N was chosen as this was considered extremely safe, yet realistically achievable under normal aquaculture practices. Although these concentrations may be typical of commercial recirculating aquaculture facilities, these levels are elevated relative to environmental levels or approved drinking water limits of 10 mg/L nitrate-N (U.S EPA, 1996).

Treatment 1 sampled 15 fish from each of the four commercial recirculating culture tanks (two tanks/nitrate concentration; N = 30 per nitrate treatment). Each fish was sampled at time 0 and was surgically sexed immediately after the blood sample was drawn. Only blood samples from female fish were used in the analyses for this study. Each fish was weighed and placed into a holding tank until treatment 2 fish were removed, to avoid stressing fish remaining in the tank.

Treatment 2 sampled 18 fish from each of the four commercial recirculating culture tanks (N = 36 per nitrate treatment). Fish were sampled at time 0, and were then placed into square 0.64 m^3 insulated plastic totes (one tote per nitrate concentration) filled with 530 L of system water for a 6-h period of confinement stress. A numbered tag (Duflex™, St. Paul, MN) was placed on the pectoral fin of each fish for identification. Fish were bled at 1 and 6 – h during the confinement period (Fig. 4-1). After the 6-h sampling period, the fish were surgically sexed as previously described.

Experiment 2

Experiment 2 was conducted in May of 2005 and was procedurally identical to experiment 1 with the following exceptions. Two of the culture tanks were held at a nitrate concentration of 1.5 mg/L nitrate-N (6.5 mg/L total nitrate) for one month, and two tanks were held at 57 mg/L (250 mg/L total nitrate) for the same time period. It should be noted that although the same tanks and population (different individuals) of animals was used in this second experiment, the tanks that previously held the low nitrate concentrations in experiment 1, now held the elevated nitrate concentration and vice versa, to reduce the possibility of tank affect among treatment groups. The exposure in the first experiment should not affect the fish in either nitrate group in the second experiment, since nitrate concentrations typically oscillate in the range of the upper limit (57 mg/L nitrate-N) and the lower limit (11.5 mg/L nitrate-N) routinely in recirculating aquaculture settings, including our facility. Although 11.5 mg/L nitrate-N is considered low in commercial aquaculture, this concentration exceeds that which would occur in unpolluted natural environments. Therefore 1.5 mg/L nitrate-N was chosen in this experiment as it would be more reflective of ecologically relevant exposures. Treatment 1 sampled 15 fish from each of the four commercial recirculating culture tanks (N = 30 per nitrate treatment) and treatment 2 sampled 25 fish from each of the four tanks (N = 50 per nitrate treatment).

Hormone Evaluations

Plasma samples were thawed on ice, and the steroid fraction was extracted twice with diethyl ether. Plasma cortisol (F), E_2 (experiment 1), T and 11-KT were analyzed according to instructions provided with the commercial competitive enzyme immunoassay kits (Cayman Chemical, Ann Arbor, MI), specific to each hormone. Each hormone was previously validated for Siberian sturgeon by verifying that serial dilutions were parallel to the standard curve. Samples were run in duplicate and each plate contained duplicate wells for interassay variance and a blank. Individual hormones were all run with plates from the same kit lot number and were completed in the same testing session to reduce testing variance. Sample plates were analyzed with a plate reader (BioRad™, Hercules CA). Glucose was evaluated with an Amplex™ Red glucose/glucose oxidation kit (Invitrogen™, Carlsbad, CA).

Radioimmunoassays for E_2 (validated for Siberian sturgeon) in experiment 2 were conducted as described previously by this lab (Milnes et al., 2004). Briefly, extracted samples were reconstituted in Borate Buffer (50 ul, 0.05 M, pH 8.0). Antibody (Endocrine Sciences, Tarazana, CA, USA) and radiolabeled steroid (2, 4,6,7,16,17-^{3}H) were added at 12,000 cpm per 100 µl. Interassay variance tubes were similarly prepared from pooled Siberian sturgeon plasma. Standards were prepared in duplicate at 0, 1.56, 3.13, 6.25, 12.5, 25, 50, 100, 200, 400 and 800 pg per tube. Assay tubes were incubated at 4°C overnight. Bound free separation was performed by adding charcoal and centrifuging for 30-min. The supernatant was then drawn off and diluted with scintillation cocktail and counted on a Beckman LS 5801 scintillation counter.

Statistical Analyses

Statistical analyses were performed using StatView for Windows (SAS Institute, Cary, NC, USA). Initial comparisons were made to determine significance within treatments. F-tests were conducted to test variances among treatment groups for homogeneity. If variance was heterogenous, data were $\log_{10}$ transformed to achieve homogeneity of variance, however, all reported mean (± 1 SE) values are from non-transformed data. Analyses of variance (ANOVA) of weights and hormone concentrations were used to compare differences among treatment groups. If significance was determined ($p < 0.05$), Fisher's protected least-significant difference was used to determine differences among treatment means.

Results

Experiment 1

In treatment 1, of the 30 fish sampled and sexed in each nitrate concentration, 19 were females in the 11.5 mg/L nitrate-N group, and 18 were females in the 57 mg/L nitrate-N group. Of the 36 fish sampled and sexed in each nitrate concentration for treatment 2, 16 were females in the low nitrate group, whereas 13 were females in the high nitrate group. The average weight for females in treatment 1 was 4.16 ± 0.53 kg whereas females sampled in treatment 2 was 4.29 ± 0.36 kg. There were no significant differences among the tanks within each nitrate group for any tested parameter.

Water chemistry parameters were tested the day of experimentation and were as follows: unionized ammonia (NH_3) ≤ 4.35 µg/L, nitrite ≤ 0.15 mg/L; pH 7.4, alkalinity 230 mg/L, chloride 94 mg/L, total hardness 240 mg/L and calcium hardness 140 mg/L. Dissolved oxygen concentrations were maintained at ≥ 95% saturation throughout the trial and temperature was 23.3 °C.

Time 0 females in treatment 1 were combined with time 0 females from treatment 2 to evaluate the effects of nitrate exposure for each experiment. Fig. 4-2 and 4-3 illustrates time 0 data for each hormone for experiment 1. Initial concentrations of plasma F or glucose were not different between females in the 11.5 and the 57 mg/L nitrate-N groups, averaging 5.95 ± 1.08 ng/ml and 255.9 ± 6.8 pg/ml respectively. Plasma T, 11-KT and E_2 concentrations were significantly elevated in the 57 mg/L nitrate-N group when compared to concentrations observed in females exposed to 11.5 mg/L nitrate-N ($p \leq 0.05$).

Data for plasma F and glucose concentrations in treatment 2 are shown in Fig. 4-4. There was no significant difference in the stress response, defined by plasma F concentrations, when the females exposed to the two nitrate concentrations were compared. The females in both the 11.5 mg/L and 57 mg/L nitrate-N concentration groups demonstrated a dramatic increase in plasma F concentrations at the 1-h sampling period averaging 42.0 ± 5.7 ng/ml, followed by a significant decrease at the 6-h sampling period. The 6-h plasma F concentrations were still significantly elevated when compared to time 0 concentrations (11.5 ± 1.7 ng/ml). Plasma glucose concentrations were similar for both nitrate groups at time 0 and 1-h, averaging 227.5 ± 12.2 pg/ml at time 0, and rising significantly to an average of 428 ± 17.5 pg/ml by 1-h. The 11.5 mg/L nitrate-N concentration group females demonstrated a significant increase in plasma glucose from time 1-h to 6-h (517.6 ± 19 pg/ml at 6-h), whereas the 57 mg/L nitrate-N concentration group females exhibited no increase in plasma glucose between the 1-h and 6-h sampling period (427.9 ± 25.1 pg/ml). During the six hour captive stress period, we observed no significant changes in plasma T, 11-KT or E_2 concentrations with plasma concentrations within each respective nitrate concentration averaging 10.9 ± 0.8 ng/ml, 4.4 ± 0.4 ng/ml and 784 ± 16.6 pg/ml respectively.

Experiment 2

In treatment 1, of the 30 fish sampled and sexed in each nitrate concentration, 14 were females in the 1.5 mg/L nitrate-N group, and 12 were females in the 57 mg/L nitrate-N group. Of the 50 fish sampled and sexed in each nitrate concentration for treatment 2, 22 were females in the 1.5 mg/L nitrate-N group, and 24 were females in the 57 mg/L nitrate-N group. The average weight for females in treatment 1 was 5.84 ± 0.89 kg and the average weight for females sampled in treatment two was 6.14 ± 1.10 kg. There were no significant differences among the tanks within each nitrate group for any tested water parameter.

Water chemistry parameters were tested the day of experimentation and were as follows: unionized ammonia (NH_3) ≤ 5.35 µg/L, nitrite ≤ 0.20 mg/L; pH 7.6, alkalinity 240 mg/L, chloride 90 mg/L, total hardness 240 mg/L and calcium hardness 135 mg/L. Dissolved oxygen concentrations were maintained at ≥ 95% saturation throughout the trial and temperature was 23.5 °C.

Time 0 females in treatment 1 were combined with time 0 females from treatment 2 to evaluate the effects of nitrate exposure for each experiment. Fig. 4-5 and 4-6 illustrates time 0 data for each hormone for experiment 2. Plasma F concentrations were not significantly different among females when the 1.5 mg/L or the 57 mg/L nitrate-N groups were compared at time 0. Plasma T concentrations were significantly elevated in the 57 mg/L nitrate-N concentration group ($p = 0.010$), with an average of 17.28 ± 4.57 ng/ml for the 1.5 mg/L nitrate-N group, and 31.17 ± 4.57 for the 57 mg/L nitrate-N group. Plasma 11-KT concentrations were not significantly different for either nitrate group at time 0 ($p = 0.091$) with an average of 8.5 ± 2.1 ng/ml for the 1.5 mg/L nitrate-N group, and 13.3 ± 2.9 ng/ml for the 57 mg/L nitrate-N group.

Data for treatment 2 is shown in Fig. 4-7. There was no significant difference in plasma F concentrations between nitrate groups. Initial plasma F concentrations averaged 6.9 ± 1.1 ng/ml, rose to an average of 68.1 ± 6.2 ng/ml at the 1-h sampling period and dropped to an average of 26.8 ± 2.6 ng/ml by 6-h. Plasma F concentrations were significantly different for each sampling period. There was no significant difference in stress response for plasma T or 11-KT for treatment 2 with plasma concentrations averaging 26.4 ± 1.9 ng/ml and 11.7 ± 1.4 ng/ml respectively, across all sampling periods.

Discussion

Absent from most investigations assessing the endocrine disrupting effects of environmental pollutants on aquatic inhabitants, have been studies examining the effects of ions, such as nitrate and nitrite, which are ubiquitous components of most aquatic ecosystems. Anthropogenic activities have dramatically impacted the amount of nitrogenous compounds entering freshwater systems, and recent reports have identified agricultural non-point source pollution, often caused by nitrate laden fertilizers, as the leading cause of water quality deterioration to freshwater systems (Sampat, 2000).

This paper describes the effects of a chronic 30 day exposure of Siberian sturgeon to elevated nitrate on circulating concentrations of plasma glucocorticoids (F and glucose) and sex steroids (T, 11-KT, and E_2). Results of the first experiment, in which animals were exposed to concentrations of 11.5 and 57 mg/L nitrate-N (50 mg/L and 250 mg/L total nitrate respectively), revealed significantly elevated concentrations of plasma T, 11-KT and E_2 in animals exposed to the higher nitrate concentration. Experiment 2, which evaluated the effects of animals exposed to 1.5 and 57 mg/L nitrate-N (6.6 and 250 mg/L total nitrate respectively), also demonstrated an elevated concentration of plasma T and E_2 in animals exposed to the higher nitrate concentration.

Although the results of Experiment 2 did not demonstrate a significant elevation in plasma 11-KT concentration ($p = 0.09$) as shown in Experiment 1 ($p = 0.05$), it should be noted that the second experiment was conducted at a slightly different time of the year, and in animals which were almost 1-yr older. Seasonal variation and stage of reproductive development can have significant impacts on steroid profiles of most fish species (Stacey et al., 1984).

This is the first study to demonstrate a nitrate-induced elevation in concentrations of plasma sex steroids, using a Caspian Sea sturgeon species habituated to a warm environment, typical of commercial culture. Since small-scale trials do not always reflect the scale-up challenges of commercial culture environments, or mimic similar effects on physiologic response, this experiment is unique in that it was conducted at a commercial farm under typical culture conditions. This study is also distinct in that it used naturally occurring nitrate produced by nitrification, to achieve desired nitrate concentrations, versus altering the nitrate environment by chemical addition (e.g. sodium nitrate).

It has been proposed that nitrates and nitrites disrupt endocrine function by entering steroidogenic tissues, where they are metabolized to nitric oxide (NO). NO possesses the ability to bind to the heme moiety of the cytochrome P450 enzymes, which are present at multiple locations along the steroidogenic pathway. The mechanism by which nitrate has led to the elevated concentrations of plasma sex steroids seen in this study is unclear, and more work is necessary to understand the mechanisms involved. Nitrate induced elevations in plasma concentrations of sex steroids does not necessarily imply that nitrate is not detrimental to the reproductive health of this species. Concentrations of circulating plasma sex steroids are only one endpoint in the reproductive-endocrine axis, and disruptions can occur which will not be manifest at the level of circulating steroids. I offer three potential explanations for the elevations

in plasma concentrations of sex steroids seen in this study. First, nitrate triggered an up-regulation of steroidogenic function resulting in increased gonadal synthesis of sex steroids. Second, nitrate induced alterations to transport proteins hamper transport to the liver and concomitantly affect clearance. And lastly, elevated nitrate may impair liver function, thereby reducing its ability to clear these steroids from the blood.

The female fish in this study demonstrated increased plasma concentrations of androgens, as well as E_2. Considerable attention in the literature evaluating the effects of endocrine disrupting contaminants on aquatic animals has been directed at the estrogenic effects of compounds, because many effects reported in wildlife populations are a consequence of the feminization of males (Stoker et al., 2003; Sumpter 2005; Milnes et al., 2006). However, a growing literature recognizes that populations of female fish exposed to environmental contaminants exhibit masculinized features (Parrott et al., 2004). Toft et al. (2004) found that female mosquitofish (*Gambusia holbrooki*) exposed to paper mill effluent exhibited masculinized anal fins, and exhibited lower fecundity (number of embryos per unit of female size) than reference fish. 17β-trenbolone is an anabolic steroid used to promote growth in beef cattle and has shown strong androgenic activity, and is thought to be the cause of reproductive alterations in fish living downstream from animal feedlot operations (Jegou et al., 2001; Wilson et al., 2002; Orlando et al., 2002). It is unclear what effects elevated androgens, or estrogens for that matter, have on Siberian sturgeon reproduction, and this lab is currently investigating the mechanisms involved.

In aquaculture systems, nitrate has been neglected as a material water quality hazard. Commercial aquaculture operations have traditionally used large influxes of water to maintain water chemistry, and it is not uncommon to have water exchanges of 100% or more per day.

Consequently, nitrate has not traditionally been a concern in commercial aquaculture since this flush rate is sufficient to maintain relatively low nitrate concentrations. Water is rapidly becoming recognized as a valuable and limited resource, and legislative mandate is becoming more stringent in its limits of the amount of water which may be consumed or discharged. As aquaculture attempts to keep pace with global demand, the growing number of aquaculture operations will be forced to utilize recirculating aquaculture technology, and significantly reduce the heavy water usage in current practice. Nitrification systems are well understood in aquaculture, and are decidedly effective at reducing ammonia and nitrite to nitrate (Timmons, 2001). In recirculating aquaculture systems with limited water exchange, nitrate can rise to concentrations far in excess of those of natural environments, and it is unclear what impact these concentrations can have on species residing in these environments. Understanding the sublethal effects of exposure to nitrate is especially critical to sturgeon, whose economic viability relies heavily on proper egg production and reproductive performance.

Fish are highly sensitive to the chemical influences in their environment, and negative influences are often reflected in an acute stress response, indicated by elevations in concentrations of glucocorticoids (Guillette et al., 1997). Stress in fish, and the concomitant increase in plasma F concentrations, has been implicated in numerous physiological maladies, including reproductive impairment (Pankhurst and Van Der Kraak, 1997). Stress induced effects on reproduction include decreased plasma concentrations of sex steroids, depressed vitellogenin production and decreased gamete quality (Pankhurst and Van Der Kraak, 1997). Although plasma concentrations of sex steroids were significantly elevated in the groups of fish exposed to 57 mg/L nitrate-N, time 0 plasma F and glucose concentrations were not affected by nitrate

concentration in this study, indicating that the alterations to concentrations of plasma sex steroids were unlikely to be mediated by glucocorticoid action.

Induced stress in both experiments in this study, caused by confinement and associated blood sampling stressors, caused a dramatic increase in plasma F concentrations after 1-h, with a significant decrease by the 6-h sampling period; this response was not influenced by nitrate concentration in this study. Previous studies with gilthead sea bream (*Sparus aurata*) have shown a decreased acute stress response in chronically stressed fish, speculating that the reduced plasma F response likely resulted from negative feedback of mild but chronically elevated F caused by the confinement stressor on the hypothalamic-pituitary-interrenal axis (Barton et al., 2005). Since the initial blood samples (time 0) were taken generally within 30 s of capture, it is likely initial concentrations of plasma F seen in this study (≈ 6 ng/ml) are representative of basal plasma F concentrations of captive sturgeon in our facility. Previous studies with Siberian sturgeon exposed to severe hypoxic stress, demonstrated peak plasma F concentrations of 35 ng/ml (Maxime et al., 1995). Peak concentrations of plasma F in our study rose to over 40 ng/ml in one experiment, and nearly 70 ng/ml in the second experiment, demonstrating the plasticity of physiological response for this species. Nitrate in this study was shown to alter at least one component of the stress response, defined by plasma glucose concentrations, during a 6-h period of confinement stress.

In conclusion, elevated nitrate is capable of altering the steroid profiles of cultured female Siberian sturgeon, and is able to alter the secondary stress response, defined by plasma glucose concentrations. We also show that responses to nitrate can change over time, and more work is necessary to uncover the mechanisms involved in steroid alterations seen in this study, as well as understand the impact these effects may have on reproductive performance.

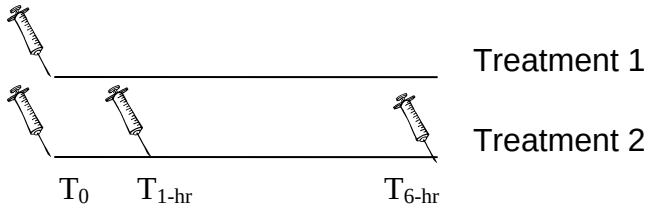
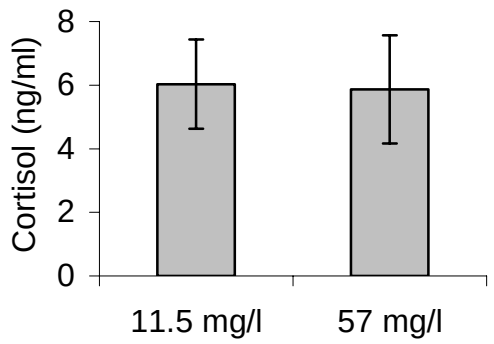


Figure 4-1. Blood sampling times for treatments 1 and 2 of fish held under confinement stress for 6-h.

A.



B.

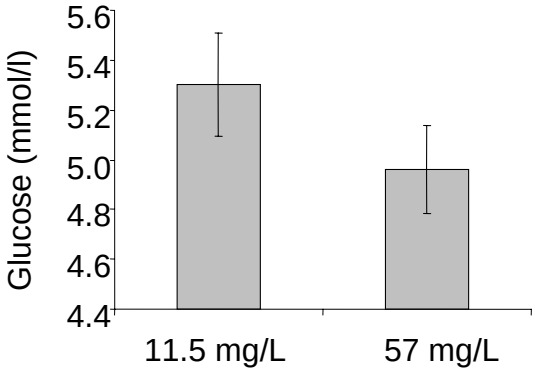


Figure 4-2. Plasma cortisol (A) and glucose (B) concentrations (mean ± 1 S.E.M.) in cultured female Siberian sturgeon (*Acipenser baeri)* exposed for 30 days to concentrations of 11.5 or 57 mg/L nitrate-N (n = 35 and n = 31 respectively). Means with no superscript are not significantly different ($p \geq 0.05$).

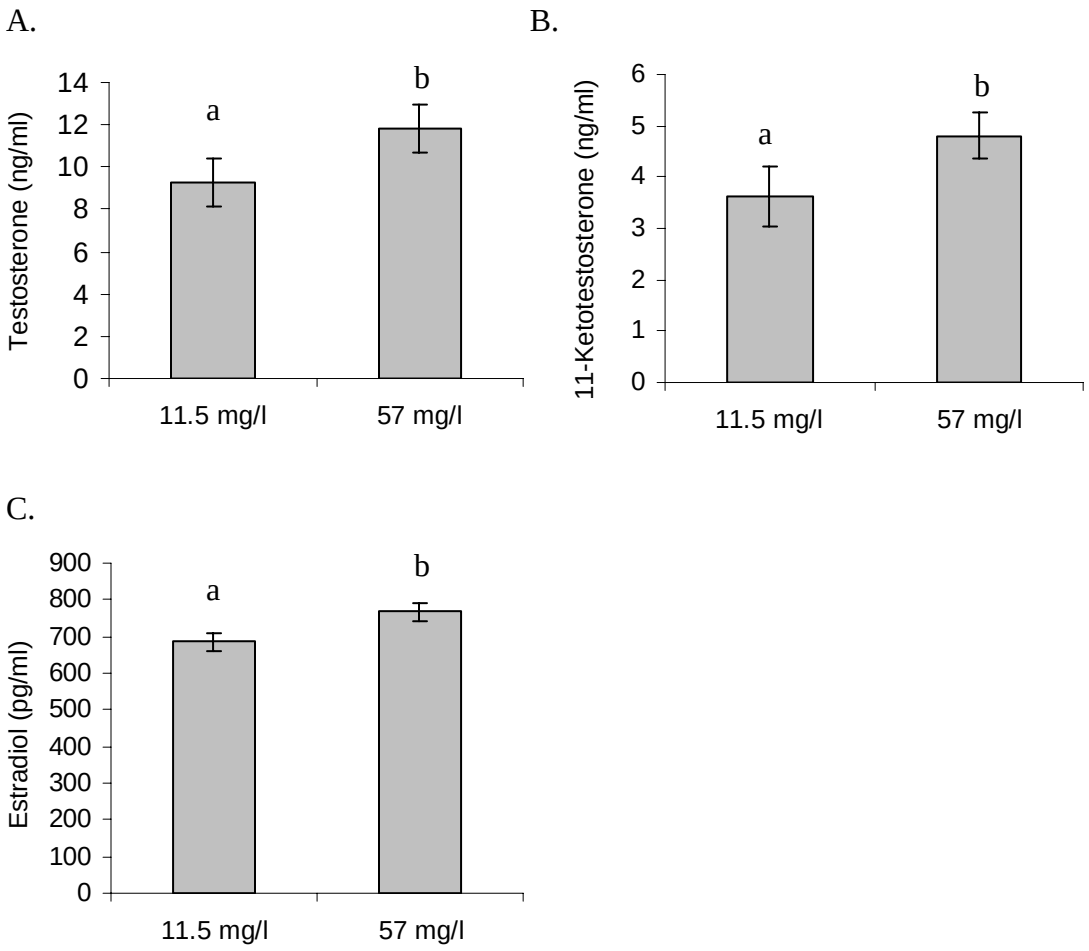


Figure 4-3. Plasma testosterone (A), 11-ketotestosterone (B) and estradiol (C) concentrations (mean ± 1 S.E.M.) in cultured female Siberian sturgeon (*Acipenser baeri)* exposed for 30 days to concentrations of 11.5 or 57 mg/L nitrate-N (n = 35 and n = 31 respectively). Superscripts designate significantly different values ($p \leq 0.05$).

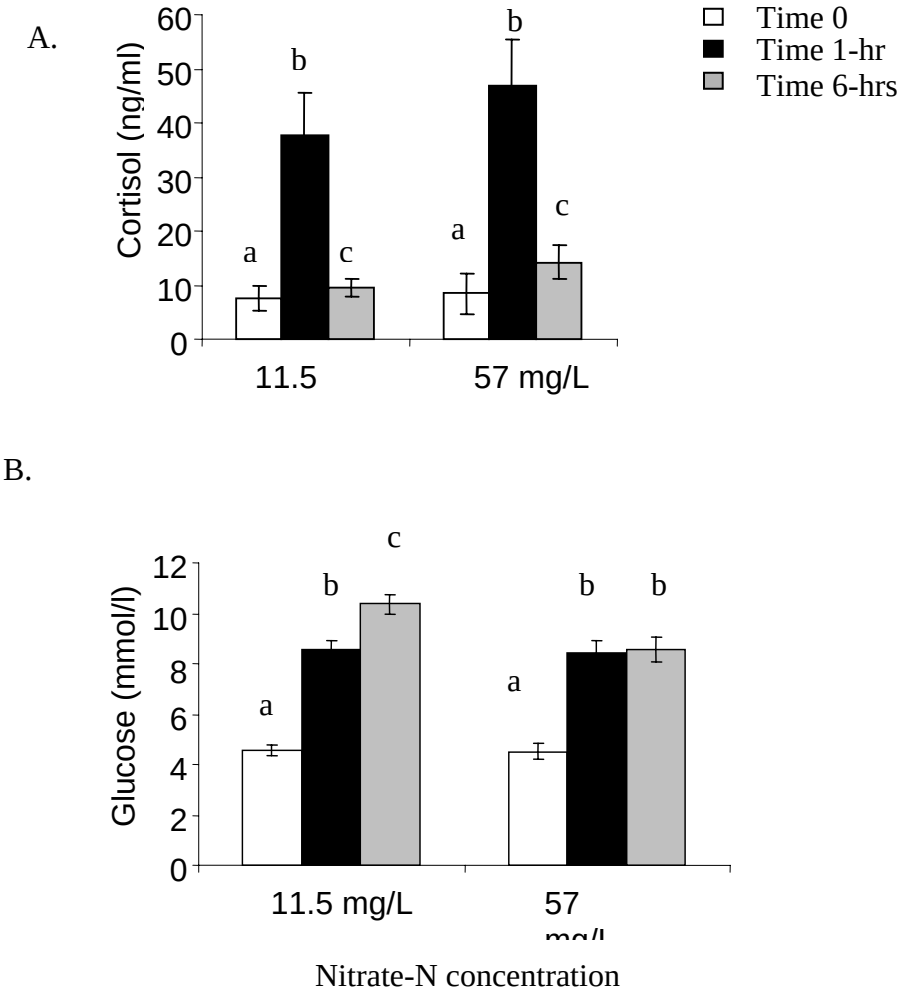


Figure 4-4. Plasma cortisol (A) and glucose (B) concentrations (mean ± 1 S.E.M.) in cultured female Siberian sturgeon (*Acipenser baeri)* exposed for 30 days to concentrations of 11.5 or 57 mg/L nitrate-N (n = 16 and n = 13 respectively). The fish were bled at time 0, 1-h and 6-h during a 6-h period of confinement stress. Means with the same superscript are not significantly different ($p \geq 0.05$).

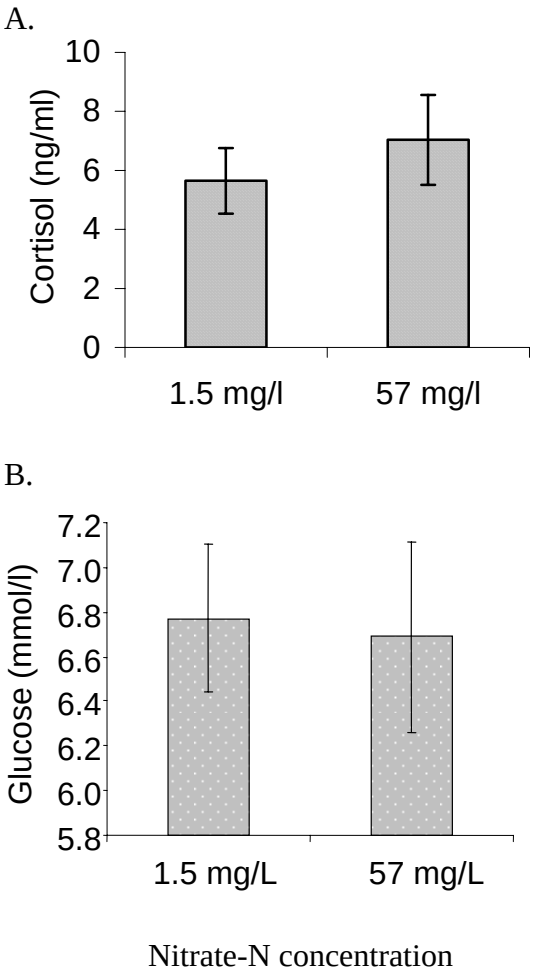


Figure 4-5. Plasma cortisol (A), glucose (B) testosterone concentrations (mean ± 1 S.E.M.) in cultured female Siberian sturgeon (*Acipenser baeri)* exposed for 30 days to concentrations of 1.5 or 57 mg/L nitrate-N (n = 36 for both nitrate groups). Means with no superscript are not significantly different ($p \geq 0.05$).

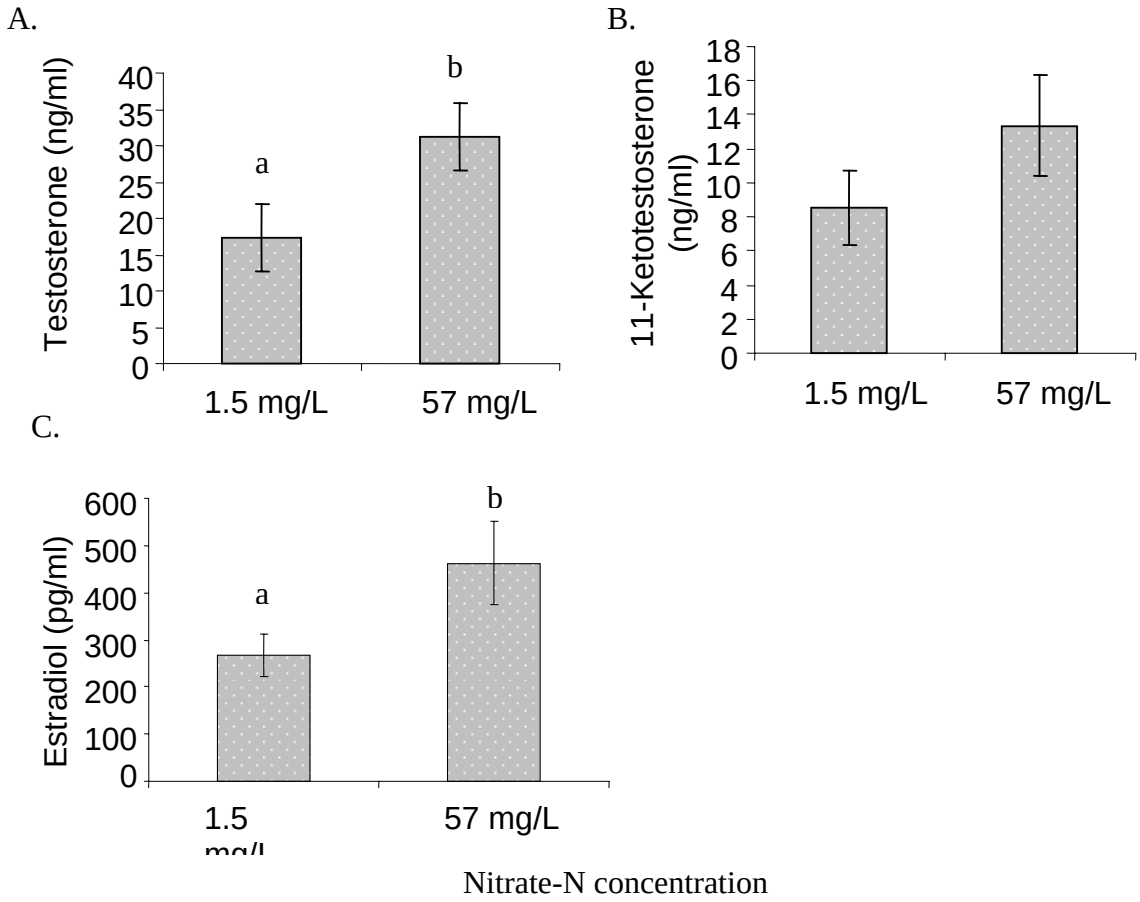


Figure 4-6. Plasma cortisol testosterone (A), 11-ketotestosterone (B) and estradiol-17β (C) concentrations (mean ± 1 S.E.M.) in cultured female Siberian sturgeon (*Acipenser baeri)* exposed for 30 days to concentrations of 1.5 or 57 mg/L nitrate-N (n = 36 for both nitrate groups). Superscripts designate significantly different values ($p \leq 0.05$).

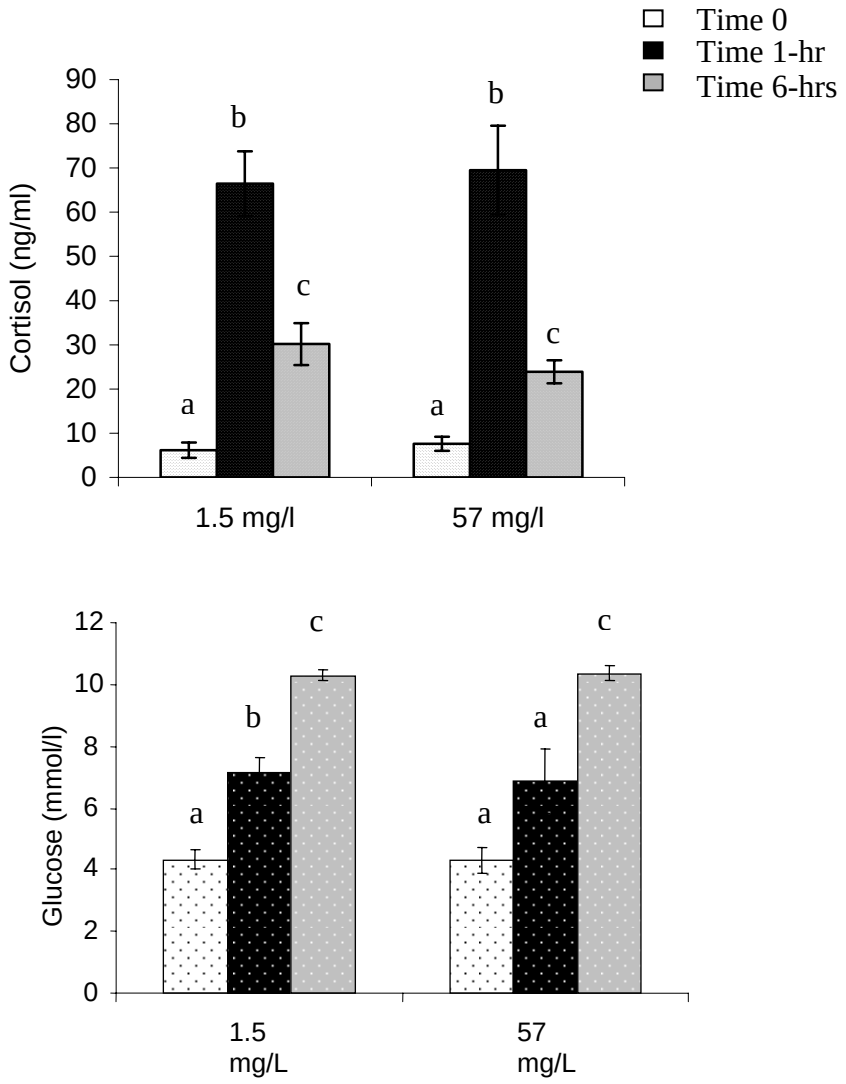


Figure 4-7. Plasma cortisol (A) and glucose (B) concentrations (mean ± 1 S.E.M.) in cultured female Siberian sturgeon (*Acipenser baeri)* exposed for 30 days to concentrations of 1.5 or 57 mg/L nitrate-N (n = 22 and n = 24 respectively). The fish were bled at time 0, 1-h and 6-h during a 6-h period of confinement stress. Means with the same superscript are not significantly different ($p \geq 0.05$).

CHAPTER 5
EFFECTS OF NITRATE ON STEROIDOGENIC GENE EXPRESSION IN CAPTIVE FEMALE SIBERIAN STURGEON

Introduction

Environmental contaminants capable of altering steroidogenic regulation and function are well documented in the literature for both terrestrial and aquatic inhabitants (Guillette and Gunderson, 2001; Mills and Chichester, 2005; Sumpter, 2005; Edwards et al., 2006c). These endocrine disrupting contaminants (EDCs) can exert their effects through numerous physiological mechanisms including mimicking naturally occurring steroids, altering hormone synthesis and degradation and interacting directly with steroid receptors (vom Saal et al., 1995; Rooney and Guillette, 2000). In the latter case, EDCs can either stimulate (Parks et al., 2001) or inhibit (Kelce et al., 1995) the expression of the target genes for that receptor. The endocrine system is responsible for numerous physiological processes, and as such, perturbations to this system have the potential to deleteriously affect reproductive and developmental performance of the affected organism.

Stress has also been shown to alter endocrine function, and is generally negatively correlated with concentrations of sex steroids (Pankhurst and Van Der Kraak, 1997; Orlando et al., 2002). Cortisol, a predominant glucocorticoid, is the most commonly accepted plasma indicator of the degree to which an animal is stressed and has been associated with inhibitory effects on reproduction (Pankhurst and Van Der Kraak, 1997). Commonly studied stressors in fishes include capture and confinement or handling and alterations to various environmental parameters such as temperature, pH or salinity (Pankhurst and Dedual, 1994). Certain contaminants, however, have also been shown to increase plasma glucocorticoid concentrations, further contributing to the suppression of circulating sex steroids (Schreck and Lorz, 1978).

In the United States, the input of nitrogen from terrestrial agriculture has increased 20-fold in the past 50 years (Pucket, 1995). Aquatic nitrate concentrations of over 100 mg/L have been reported in some locations (Kross et al., 1993; Rouse et al., 1999), a ten-fold increase over the U.S. drinking water standards of 10 mg/L NO_3-N (EPA, 1996). A growing body of literature implicates agricultural non-point source pollution as the leading cause of these elevations in freshwater systems, posing a direct health risk to both humans and wildlife (Sampat, 2000). A global pollutant of aquatic habitats, the ubiquitous presence of nitrate has only recently begun to receive attention for its ability to alter endocrine function, and now joins the list of environmental contaminants implicated in reproductive dysgenesis (see review by Guillette and Edwards, 2005). Unlike most environmental endocrine disrupting contaminants, nitrate is unique in that it exists naturally at low concentrations in the aquatic environment as the degradative end product of nitrification. Therefore, the physiological disruptive actions of nitrate stem from its relative concentration, as well as its interactions within the environment in which it persists (Edwards et al., 2006a).

The seafood trade deficit in the United States is exceeding eight billion dollars annually, a natural resource deficit second only to oil and natural gas in magnitude. With the oceans at or exceeding their maximum sustainable yields for 75% of commercially relevant species, aquaculture, or the culture of fish and other aquatic organisms, has been proposed as the only viable alternative to keep pace with global demand (FAO, 2004). Like seafood, water is also becoming a limited and increasingly valuable resource, and the necessary increase in aquaculture operations will not be afforded the liberal quantities of water permitted to established facilities.

Although recirculating aquaculture facilities, which recycle and reuse a significant portion of their water, are becoming increasingly common, the limiting factor for water exchange

for most of these facilities is nitrate. Work is ongoing to develop technologies to reduce nitrate in commercial aquaculture, but it is still unclear what concentrations of nitrate are safe, especially for sensitive physiological systems such as the endocrine system which have been shown to be vulnerable to the effects of nitrate (Suzuki et al., 2003; van Rijn et al., 2006).

Sturgeon species are ideally suited to serve as models to study the endocrine disruptive effects of elevated nitrate exposure. Many species are commercially viable, highly endangered and have documented sensitivities to environmental contaminants, including nitrate (Akimova and Ruban, 1995; Dwyer et al., 2005; Hamlin, 2006). The Caspian Sea, which houses some of the most endangered sturgeon species, is becoming increasingly affected by contaminants (Birstein, 1993; Stone, 2002) many of which are implicated in the disruption of reproduction in sturgeon species (Akimova and Ruban, 1995).

It has been proposed that aquaculture, incorporating the development of captive broodstock programs, could be the best solution to reduce fishing pressures, facilitating recovery of wild populations (Williot et al., 2002). The economic viability of sturgeon culture rests squarely with the successful production of eggs, or caviar, the commercial hallmark of this family of fishes. Therefore, environmental contaminants, that have the potential to alter reproductive endpoints such as egg production, are critical areas of investigation for threatened species whose promise in aquaculture relies almost entirely on proper egg development.

In many aquatic animals, including most fish, nitrate enters the bloodstream by crossing the gill epithelia, either by diffusion or against a concentration gradient by substituting for chloride, and accumulating in extracellular fluid (Lee and Prichard, 1985; Jensen, 1995). Ingested nitrate is readily absorbed by the proximal small intestine in mammals (Walker, 1996), or can also be converted to nitrite, although the degree and mechanism of the latter has been a

significant point of debate (Hartman, 1982). Concentrations of excess nitrite can cause the potentially fatal methemoglobinemia, or brown blood disease in fishes, caused by an inability to reversibly carry oxygen in the blood (Scott and Crunkilton, 2000). Both nitrate and nitrite are capable of generating nitric oxide (NO) (Meyer, 1995; Cadenas et al., 2000; Lepore, 2000). Nitric oxide has been shown to inhibit steroidogenesis through its interactions with steroidogenic acute regulatory protein (StAR) or the enzyme cytochrome P450 side chain cleavage ($P450_{SCC}$) (White et al., 1987).

In the mitochondria of steroidogenic cells, free cholesterol, the precursor for steroidogenesis, is transported across the mitochondrial membrane by StAR. This cholesterol is then converted to pregnenolone by the $P450_{SCC}$ enzyme (Stocco, 1999). Pregnenolone is subsequently converted to progesterone by mitochondrial 3β hydroxysteroid dehydrogenase (3β-HSD) (Stocco, 1999). Progesterone then exits the mitochondria and depending on the tissue, will be converted to either mineralcorticoids, glucocorticoids, progestins, androgens or estrogens in the smooth endoplasmic reticulum (Norris, 1997). Noticeably absent from nitrate studies describing the mechanisms of altered steroid concentrations, are studies of enzymes and receptors involved in regulating the earliest stages of steroidogenesis. In fact, the majority of steroidogenic research has focused on enzymes and receptors further downstream from the conversions of cholesterol to pregnenolone (Goto-Kazeto et al., 2004).

The goal of this study is to examine nitrate-induced alterations in endocrine function and identify mechanisms through which environmental exposure to nitrate alters steroidogenesis at the molecular level. These mechanisms will be investigated by comparing the mRNA expression of a regulatory enzyme functioning at an early stage of steroidogenesis ($P450_{SCC}$) as well as receptor proteins at the end of the steroidogenic cascade for both sex steroids and

glucocorticoids, estrogen receptor β (ERβ) and glucocorticoid receptor (GR), the mRNA expression patterns of which have not been previously characterized in sturgeon.

Methods

Fish and Experimental Systems

Siberian sturgeon were collected from four 30,000 liter tanks, from separate commercial recirculating aquaculture systems at Mote Marine Laboratory's Aquaculture Park (Commercial Sturgeon Demonstration Project) in Sarasota, FL. The fish were 4.5 years old and weighed an average of 6.14 ± 1.10 kg. Water chemistry in each of these systems was analyzed weekly for ammonia, nitrite, nitrate, and pH prior to commencement of the experiments. Dissolved oxygen and temperature were monitored continuously with stationary probes, which were spot-checked bi-weekly for calibration with portable probes. Hardness, alkalinity and chloride were analyzed the day prior to commencement of the experiment.

Surgical Sexing and Tissue Collection

The sturgeon were pulled by hand at the side of the tank and immediately held down on a padded V-shaped surgical table. Pulling the fish from the tank by hand (versus netting) decreased the likelihood of stressing fish remaining in the tank and allowed for more immediate access to the fish for sampling.

For surgical sexing, the fish were anesthetized in a 5 - 8° C water bath containing carbon dioxide (CO_2) gas; CO_2 was used because it is a low regulatory priority anesthetic for fish that are grown for food production and requires no withdrawal period; the sturgeon used in this study were part of a commercial food production program. Pure oxygen gas administered through a fine air stone was used to maintain a dissolved oxygen concentration of 9.0 - 13.0 mg/L, and sodium bicarbonate was added to maintain a pH of 6.8 - 7.6 in the bath throughout the procedure.

Fish generally took 3 - 5 minutes for full anesthetization. A 2.5 – 3.5 cm incision was made on the ventral side of the fish, approximately 8 cm anterior to the vent, along the median axis to allow inspection of the gonads on either side of the fish for sex determination and tissue collection. A piece of gonad approximately 5 mm^3 was removed with a biopsy forcep (Ethicon Inc., Somerville, New Jersey), flash frozen in liquid nitrogen and stored at -80°C. The fish was sutured closed with coated vicryl absorbable suture (Ethicon Inc., Somerville, New Jersey).

Treatments and Experimental Conditions

Two treatments were established which sampled fish from each of four commercial culture tanks (30,000 L each) located in separate recirculating systems at Mote Marine Laboratory's Aquaculture Park in Sarasota, FL. Two of the culture tanks were held at a nitrate concentration of 1.5 mg/L nitrate-N (6.5 mg/L total nitrate) for one month, and two tanks were held at 57 mg/L (250 mg/L total nitrate). Nitrate concentrations were achieved by adjusting the freshwater input to each system, typical of commercial aquaculture practices. A nitrate concentration of 57 mg/L nitrate-N was chosen as the upper limit in this study, as this is the maximum concentration deemed safe, defined by feeding behavior and mortality, at Mote's Commercial Sturgeon Demonstration Project. The lower concentration of 1.5 mg/L nitrate-N was chosen because it reflects ecologically relevant exposures. Eight fish were sampled from each of the four commercial recirculating culture tanks (N = 16 per nitrate treatment).

RNA Isolation and Primer Design

Frozen gonadal tissues were weighed and immediately homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA) at a ratio of 1 ml TRIzol / 100 mg tissue. Total RNA was isolated by collecting the aqueous phase of a chloroform/phenol extraction and precipitated in isopropanol. The pellet was washed in 80% ethanol and then dissolved in DEPC treated water. An SV Total RNA Isolation System kit (Promega, Madison, WI) was used to purify the samples and perform

a DNase treatment . The quality and concentration of the total RNA was determined with agarose gel electrophoresis and spectrophotometer, respectively. First strand cDNA was synthesized with 2 μg total RNA with Oligo $(dT)_{12-18}$ Primer (Invitrogen) and SuperScript III RNase H^- Reverse Transcriptase.

Degenerate primers for L8 (a ribosomal protein used for normalization of mRNA levels), glucocorticoid receptor (GR), $P450_{SCC}$, and ERβ were designed from conserved regions of the respective genes from other species. The PCR primers were used to amplify fragments of the sturgeon cDNA. Amplified cDNA were purified by Wizard SV Gel and PCR Clean-up System (Promega) and cloned by pGEM-T Vector System (Promega). Cloned plasmids were isolated by Wizard Plus SV Miniprep DNA Purification System (Promega). We used the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) to sequence the amplifed fragments which were analyzed with an ABI PRISM 3100. BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) was used to check for nucleotide and amino acid homology. Primer Express (Applied Biosystems, Foster City, CA) was used to design the real-time PCR primers (Table 5-1).

Quantitative Real-Time PCR

Quantitative real-time PCR (Q-PCR) was conduced using SYBR Green PCR Master Mix using a MyiQ Single Color Real-Time PCR Detection System (Bio-Rad) in a reaction volume of 15μl following the manufacturer's protocol as previously described by this lab (Katsu et al., 2004). Conditions for Q-PCR for all genes were 3 min at 95°C and 40 cycles of 95°C for 10 seconds and 1 min. at the best annealing temperature for each gene. The best annealing temperature for $P450_{SCC}$ was 60.6°C, with L8, ERβ and GR running at an annealing temperature of 65°C. Starting quantities of cDNA (copies/ml) for each gene were calculated according to

(Yin, 2001), based on optical density and molecular weight values. The expression of mRNA of the samples was calculated from a standard curve created from a serially diluted sample. Samples were run in triplicate and were normalized for ribosomal L8 expression.

Sequence Data

The sequence data were analyzed using CLC Free Workbench (CLC Bio A/S, Cambridge, MA), and homologous sequences of their deduced amino acid sequences were searched by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The amino acid sequences were aligned using ClustalX (Thompson et al., 1997). Genebank accession numbers for the amino acid sequences of RPL8 are Q6P0V6 (zebrafish), P41116 (*X. leaves*), XP_416772 (Chicken), P62918 (Mouse) and P62917 (Human); those of GR are BAE92737 (zebrafish), P49844 (*X. laevis*), XP_420437 (chicken), NP_032199 (mouse) and P04150 (human); those of ER-beta are NP_851297 (zebrafish), NP_001035101 (*X. tropicalis*), NP_990125 (chicken), NP_034287 (mouse) and NP_001428 (human); those of P450 SCC are XP_691817 (zebrafish), NP_001001756 (chicken), Q9QZ82 (mouse) and AAH32329 (human). The Conserved Domains in amino acid sequences were searched by CD-search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

Statistical Analyses

Statistical analyses were performed using StatView for Windows (SAS Institute, Cary, NC, USA). Initial comparisons were made to determine significance within treatments. F-tests were conducted to test variances among treatment groups for homogeneity. If variance was heterogenous, data were $\log_{10}$ transformed to achieve homogeneity of variance, however, all reported mean ($\pm$ 1 SE) values are from non-transformed data. The relative expression of each gene was computed as a ratio with L8 and then multiplied by a consistent multiplier of 10 to ensure all values were greater than one prior to analyses of variance (ANOVA). Figures,

however, display original values. If significance was determined ($p < 0.05$). Fisher's protected least-significant difference was used to determine differences among treatment means.

Results

Sequence Data

Nucleotide and deduced amino acid sequences of RPL8 (L8), $P450_{SCC,}$ ERβ and GR are shown in Figures 5-1 to 5-4. Cloned cDNAs are 309, 584, 698 and 845 base pairs encoding 102, 194, 232 and 281 amino acids, and are similar to L8, GR, ERβ and $P450_{SCC}$, respectively (Figs. 5-1 to 5-8). These are partial cDNA sequences and it is 40, 26, 42 and 55% of the length of the zebrafish coding region for L8, GR, ER-β and $P450_{SCC}$, respectively. Cloned L8 included a partial conserved domain of ribosomal protein L2 C-terminal domain, and revealed higher than 97% of sequence identity among the vertebrates (Fig. 5-5). Cloned $P450_{SCC}$ encoded a part of conserved region for cytochrome P450s, and revealed 77, 67, 49 and 51% of sequence identity compared with zebrafish, chicken, mouse and human, respectively (Fig. 5-6).

Partially cloned GR included a complete hinge region, and a partial DNA- and ligand-binding domain (Fig. 5-7). Sturgeon GR showed 74, 67, 59, 67 and 65% of sequence identity with GR cloned from zebrafish, *Xenopus leavis*, chicken, mouse and human, respectively (Fig. 5-7). Cloned partial cDNA for ERβ included a partial hinge region and ligand binding domain, and revealed 71, 58, 57, 59 and 57% of sequence identity with ERβ of zebrafish, *Xenopus tropicalis*, chicken, mouse and human, respectively (Fig. 5-8).

Water Chemistry

Water chemistry parameters were tested the day of experimentation and were as follows: unionized ammonia (NH_3) ≤ 5.35 µg/L, nitrite ≤ 0.20 mg/L; pH 7.6, alkalinity 240 mg/L, chloride 90 mg/L, total hardness 240 mg/L and calcium hardness 135 mg/L. Dissolved oxygen

concentrations were maintained at ≥ 95% saturation throughout the trial and temperature was 23.5 °C.

Steroidogenic Gene Expression and Hormone Regressions from Previous Studies

Expression levels, normalized to L8 expression, of all genes evaluated were statistically similar between the fish residing in the 1.5 or 57 mg/L nitrate-N (Figs. 5-9 to 5-11). As expected, the expression of L8 was not significant for either treatment. Additionally, there was no tank effect among treatments. Mean expression levels for $P450_{SCC}$ were 0.027 (± 0.007) and 0.026 (± 0.006) for the 1.5 and 57 mg/L nitrate-N treatments respectively (Fig. 5-8). Mean expression levels for GR averaged 0.359 (± 0.056) and 0.341 (± 0.035) for the 1.5 and 57 mg/L nitrate-N treatments respectively (Fig. 5-9). Mean expression level for ERβ was 0.440 (±0.109) and 0.583 (± 0.160) for the 1.5 and 57 mg/L nitrate-N concentrations respectively (Fig. 5-10).

Simple regression analyses of mRNA expression levels (normalized to L8) of $P450_{SCC}$, ERβ and GR, as well as sex steroid and stress hormone plasma concentrations from Chapter 4 are summarized in Tables 5-2 and 5-3 as well as Figs. 5-12 to 5-15. Fish exposed to 1.5 mg/L NO_3-N demonstrated significant regressions ($p \leq 0.05$) for the following comparisons: GR vs. ERβ; GR vs. glucose; and T vs. 11-KT. Fish exposed to 57 mg/L NO_3-N demonstrated significant regressions for the following comparisons: ERβ vs. $P450_{SCC}$; ERβ vs. 11-KT; $P450_{SCC}$ vs. T; $P450_{SCC}$ vs. 11-KT.

Discussion

This is the first study to successfully clone and describe the mRNA expression patterns of sturgeon $P450_{SCC}$, ERβ and GR, key constituents in steroidogenic and stress receptor functioning. These genes represent both early ($P450_{SCC}$) and late (ERβ and GR) steroidogenic endpoints, with their expressions offering insight into several steroidogenic pathways.

In mammals, two ERs have been identified, in contrast to teleosts in which there are three known ERs, ERα and two isoforms of ERβ (Filby and Tyler, 2005). Although both ERα and ERβ are found in the gonads of fish and mammals, there is currently no agreement regarding the relative importance of one form over the other (Hall et al., 2001). ERβ has been shown to attenuate the ligand stimulated transcriptional activity of ERα, and has been shown to heterodimerize with ERα in vitro, suggesting that relative expression levels of the receptors could dictate cellular sensitivities to estrogens (Hall et al., 2001).

ERβ is most strongly expressed in the gonad in most fishes. In a study of largemouth bass (*Micropterus salmoides*) the gonadal mRNA expression of ERβ was many fold greater than ERα, however its relative expression was strongly dependent upon time of the year (Sabo-Attwood et al., 2004). This study also showed that ERα was more strongly expressed in the liver, but only for certain periods of the year. In rivulus (*Rivulus marmoratus*) the greatest expression of ERβ is found in the gonad and it has been shown that environmental pollutants can dramatically alter ER expression in this species (Seo et al., 2006). Rivulus has both hermaphroditic and primary male forms, and it has been shown that expression levels of ERβ can vary dramatically depending on the form (Orlando et. al., 2006). ERβ has been shown to be preferentially sensitive to synthetic antiestrogens and phytoestrogens versus ERα (Bodo and Rissman, 2006). Taken together, these data demonstrate the plasticity of ERβ mRNA expression and its capacity to be altered by environmental variables.

The fish in this study were part of a larger body of work examining several endocrine endpoints associated with nitrate exposure. In Chapter 4, we documented a significant rise in plasma concentrations of sex steroids under conditions of elevated nitrate. In that study, I offered three possible explanations for the observed rise in plasma sex steroid concentrations,

which included increased steroidogenesis and a concomitant increase in gonadal synthesis of sex steroid hormones, alterations in transport proteins or reductions in liver clearance. The enzyme $P450_{SCC}$ is regarded as the chronically regulated rate-limiting step in steroidogenesis (Miller, 2002) and functions at the early stages of steroidogenesis. The $P450_{SCC}$ enzyme is expressed very early in development; in mice expression begins at embryonic day 11 (Hsu et al., 2006). During these early embryonic stages, mice with targeted disruption of the $P450_{SCC}$ gene produce no steroids and have severe adrenal defects, and die shortly after birth; zebrafish with blocked $P450_{SCC}$ function do not survive as well (Hsu et al., 2006).

In general, gonadotropins regulate $P450_{SCC}$ expression, however, sex steroids have been found to alter its expression in several tissues (Von Hofsten et al., 2002). In Arctic char (*Salvelinus alpinus*) 11-KT has been shown to up-regulate $P450_{SCC}$ expression in the gonads (Von Hofsten et al., 2002). Although nitrate exposure did not appear to alter the mRNA expression of $P450_{SCC}$ in sturgeon in this study, there was a significantly positive correlation with $P450_{SCC}$ and both androgens (Chapter 4) in fish exposed to 57 mg/L NO_3-N, that was not apparent in fish exposed to 1.5 mg/L NO_3-N. Given this difference, I hypothesize that the sex steroids at the upper nitrate concentration, that were significantly elevated compared to the population of fish exposed to low nitrate, approached a threshold for feed back; that is, the binding of a critical number of receptors sufficient to trigger a response, and this elevated gene expression. It is logical to suggest, that although the fish in this study possessed vitellogenic oocytes, they were nonetheless early in their development, and it is possible that the fish in the 1.5 mg/L NO_3-N concentration would experience an elevation in sex steroid hormones concomitant with progressive egg development, and once these sex steroids reached a critical concentration, they too would demonstrate similar correlations. It is also possible that nitrate is

affecting an unknown mechanism, that itself regulates both $P450_{SCC}$ and sex steroid expression, and that their correlation is not necessarily directly causative.

Interestingly, there was a positive correlation between ERβ and 11-KT in the fish exposed to 57 mg/L NO_3-N that was not evident in fish exposed to 1.5 mg/L NO_3-N. It has been shown in female sturgeon that both T and 11-KT rise significantly during vitellogenesis, and often peak just prior to final maturation (Barannikova et al., 2004). It is possible that under a normal reproductive cycle, that during a key period of development in Siberian sturgeon, androgens of ovarian origin rise, providing a precursor for estrogen synthesis, and thus, serving as a signal for the production of aromatase to facilitate the conversion of androgens to estrogens.

The estrogen receptor protein expression examined in this study represents an endpoint regulated far downstream, via negative feedback, in the steroidogenic pathway. That we did not observe an increase in mRNA expression for a chronically regulated upstream enzyme, nor for downstream estrogenic receptors, suggests that sex steroid elevations were not likely due to increased gonadal output. It is more likely then, that the discord between plasma sex steroid concentrations and mRNA expression patterns could be explained by altered hepatic metabolism, either via alterations in transport proteins to the liver, or by direct action on the liver itself.

Although these results do not provide a mechanism for hepatic or transport protein failure, they do support the need for future studies clarifying liver performance under high nitrate conditions. Thibaut and Porte (2004) found significantly reduced metabolic liver clearance when carp (*C. carpio*) were exposed to estrogenic nonylphenol and androgenic fenarimol at concentrations as low as 10 μM and 50 μM, respectively. Several other studies have shown that altered plasma sex steroid concentrations, induced by xenobiotics, could be caused by altered hydroxylase enzyme activity in the liver (see review by Guillette and Gunderson, 2001).

NO, derived from nitrate or nitrite, has been shown to have inhibitory effects on steroidogenesis via its actions on StAR or $P450_{SCC}$ by binding to the heme groups of these compounds (White et al., 1987). Heme groups characterize all enzymes of the P450 family, and have been shown to be susceptible to chemical perturbation (White et al., 1987; Walsh and Stocco, 2000; Danielson, 2002). These studies provide a possible mechanism for nitrate induced hepatic alterations by inhibiting enzymatic action of the various P450s in the liver responsible for clearance (Guillette and Edwards, 2005).

This study is unique in several regards. It is the first study to evaluate the steroidogenic effects of nitrate exposure in a commercially viable and ecologically threatened species, habituated to a warm environment under commercial culture conditions. Of significant importance is the fact that this study used nitrate produced through nitrification as its source. Most studies examining nitrate exposure use a purified aquatic medium dosed with various nitrate salts (e.g. $NaNO_3$, KNO_3). Nitrate produced through nitrification brings with it a host of metabolites and oxidative end products not present in a purified medium, and is more relevant to ecological exposure. This is of particular importance because it has been shown that the nitrate medium itself can significantly alter its toxic effects, even if the same source of nitrate (i.e. $NaNO_3$) is used. Edwards et al. (2006a), found that Southern Toad (*Bufo terrestris*) tadpoles exposed to various concentrations of nitrate responded differently depending on the source of freshwater used, and this difference could not be attributed to differential electrolyte balances since both sources were equivalent.

Although we did not observe nitrate induced alterations in mRNA gene expression patterns of $P450_{SCC}$, ERβ or GR in this experiment, it is important to note that these animals were exposed to the nitrate concentrations for 30 days, and it is probable that the fish were adapted to

the nitrate concentrations in terms of gene expression, since most alterations in gene expression are observable hours or days after a disrupting event. However, a goal of this study was to understand the implications of long-term exposure to elevated nitrate, and these adaptive and persistent mRNA expression patterns are relevant to aquaculture environments.

It is now known that a major function of glucocorticoids (GCs), including cortisol, is to protect against over stimulation by host defenses in a stress event (Li and Sanchez, 2005). GCs regulate numerous biological processes and play diverse roles in growth, development and maintenance of stress related homeostasis (Sapolsky et al., 2000). GCs effectuate their responses by their association with glucocorticoid receptors (GRs), and altered GRs have been implicated as a causative factor in several pathologic states (Barden, 2004; Marchetti et al., 2005). That GR-deficient mice die within a few hours after birth clearly shows that proper GR function is essential for survival (Cole et al., 1995).

Although nitrate did not alter the mRNA expression of GR in this study, there was a positive correlation between GR and both ERβ and glucose. There is no evidence in the literature of an overt regulatory mechanism for GR induction of either ERβ or glucose, or a mechanism by which glucose alters GR or ERβ expression, and it is possible this relationship is the result of an unknown or unapparent factor that is co-regulating these genes. However, it has been shown recently that glucose has the ability to regulate hepatic gene expression in a transcriptional manner, through the carbohydrate responsive element binding protein (ChREBP) (Dentin et al., 2006). In addition, glucose has been shown to directly up-regulate the mRNA expression of β-defensin-1, an immune system peptide, in cultured human renal cells (Malik and Al-Kafaji, 2006). Therefore, although the relationship between glucose and GR mRNA

expression is not yet clear, given that glucose has been shown to regulate gene expression in other systems, it is possible that glucose could regulate the expression patterns of these receptors.

Cortisol bio-synthesis commences with the stimulation of interrenal tissues by adrenocorticotropic hormone, resulting in an enzymatic conversion of cholesterol which progresses through the steroidogenic cascade through a series of enzymatic steps, including the cytochrome P450 family of proteins. It was recently shown in rainbow trout (*O. mykiss*) that xenobiotic stressors that activate aryl hydrocarbon signaling, impair the corticosteroid response to stress by inhibiting both StAR and $P450_{SCC}$ (Aluru and Vijayan, 2006). Other studies have also documented the impairment of the adaptive stress response by decreasing the capacity for interrenal cortisol production (Wilson et al., 1998; Hontela, 2005). In Chapter 4 it was shown that basal cortisol production was not increased in animals exposed to elevated nitrate for 30 days. Expectedly, we did not observe a change in mRNA expression for GR in animals exposed to elevated nitrate, indicating nitrate may not alter the enzymes involved in the adaptive stress response long term as these animals are likely adapted to the elevated nitrate at the tissue (interrenal) level, although the question of hepatic alteration and clearance still remains a concern.

This study contributes a better mechanistic understanding of the endocrine disruptive effects of nitrate exposure. Future studies of the endocrinological effects of nitrate should focus on mechanisms of hepatic alteration including examining enzymes involved in clearance, expression of gonadal and hepatic StAR protein and vitellogenin production, as well as transport protein kinetics.

Table 5-1. Forward and reverse primers used for quantitative real-time PCR

Gene	Forward Primer (5' – 3') Reverse Primer (3' – 5')	Product Size (bp)
L8	CCGGTGACCGTGGTAAACTG TCAGGGTTGTGGGAGATGACA	67
$P450_{SCC}$	AGCCTCAGCGTCTCCTTTAT CCCTGTTGTGGACCATGTT	159
ERβ	TGGTCAGCTGGGCCAAA CCAATAGGCATACCTGGTCATACA	69
GR	CAAGCAACACCGCTACCAGAT CGTTAGCTGTGGCATCGATTT	66

Table 5-2. Regression data mRNA expression patterns for P450 side chain cleavage enzyme ($P450_{SCC}$), estrogen receptor β (ERβ), glucocorticoid receptor (GR), testosterone (T), 11-ketotestosterone (11KT), 17β-estradiol (E_2) cortisol and glucose in sturgeon exposed to 1.5 and 57 mg/L NO_3-N. Bold numbers represent significant, positive correlations.

1.5 mg/L NO_3-N

	$P450_{SCC}$	ERβ	GR
ERβ	p = 0.4821 r^2 = 0.064		
GR	p = 0.3927 r^2 = 0.093	**p = 0.02** **r^2 = 0.471**	
T	p = 0.2849 r^2 = 0.161	p = 0.3249 r^2 = 0.121	p = 0.3923 r^2 = 0.093
11-KT	p = 0.3640 r^2 = 0.119	p = 0.9435 r^2 = 0.001	p = 0.1477 r^2 = 0.243
E_2	p = 0.0704 r^2 = 0.512	p = 0.7351 r^2 = 0.021	p = 0.6785 r^2 = 0.031
Cortisol	p = 0.7455 r^2 = 0.014	p = 0.8008 r^2 = 0.007	p = 0.5109 r^2 = 0.049
Glucose	p = 0.2303 r^2 = 0.198	p = 0.2263 r^2 = 0.074	**p = 0.035** **r^2 = 0.444**

57.0 mg/L NO_3-N

	$P450_{SCC}$	ERβ	GR
ERβ	**p = 0.0278** **r^2 = 0.320**		
GR	p = 0.3069 r^2 = 0.080	p = 0.4833 r^2 = 0.039	
T	**p = 0.0002** **r^2 = 0.673**	p = 0.0827 r^2 = 0.214	p = 0.9835 r^2 = 0.000
11-KT	**p = 0.0019** **r^2 = 0.567**	**p = 0.0193** **r^2 = 0.378**	p = 0.4818 r^2 = 0.042
E_2	p = 0.6361 r^2 = 0.026	p = 0.0678 r^2 = 0.324	p = 0.2330 r^2 = 0.060
Cortisol	p = 0.9510 r^2 = 0.000	p = 0.8467 r^2 = 0.004	p = 0.7247 r^2 = 0.013
Glucose	p = 0.1735 r^2 = 0.149	p = 0.6392 r^2 = 0.019	p = 0.7834 r^2 = 0.007

Table 5-3. Regression data for testosterone (T), 11-ketotestosterone (11KT), 17β-estradiol (E_2) cortisol and glucose in sturgeon exposed to 1.5 and 57 mg/L NO_3-N from Chapter 4. Bold numbers represent significant, positive correlations.

1.5 mg/L NO_3-N

	T	11-KT	E_2	Cortisol
11-KT	p = 0.0588 r^2 = 0.377			
E_2	p = 0.9919 r^2 = 0.000	p = 0.8984 r^2 = 0.003		
Cortisol	p = 0.5528 r^2 = 0.046	p = 7108 r^2 = 0.018	p = 0.4648 r^2 = 0.092	
Glucose	p = 0.6245 r^2 = 0.031	p = 0.4601 r^2 = 0.070	p = 0.5398 r^2 = 0.066	p = 0.4326 r^2 = 0.079

57.0 mg/L NO_3-N

	T	11-KT	E_2	Cortisol
11-KT	**p = 0.0001** **r^2 = 0.819**			
E_2	p = 0.9221 r^2 = 0.001	p = 0.4658 r^2 = 0.061		
Cortisol	p = 0.5190 r^2 = 0.043	p = 0.4652 r^2 = 0.061	p = 0.1247 r^2 = 0.347	
Glucose	p = 0.0563 r^2 = 0.271	p = 0.1029 r^2 = 0.223	p = 0.3525 r^2 = 0.109	p = 0.3397 r^2 = 0.091

```
CTCAGCTGAATATTGGCAATGTTCTCCCAGTTGGCACCATGCCTGAAGGTACCATTATTT    60
  Q  L  N  I  G  N  V  L  P  V  G  T  M  P  E  G  T  I  I  C    20
GCTGCCTGGAAGAGAAGCCCGGTGACCGTGGTAAACTGGCCCGTGCCTCTGGGAACTACG   120
  C  L  E  E  K  P  G  D  R  G  K  L  A  R  A  S  G  N  Y  A    40
CCACTGTCATCTCCCACAACCCTGAAACTAAGAAATCCCGCGTGAAGCTGCCATCCGGGT   180
  T  V  I  S  H  N  P  E  T  K  K  S  R  V  K  L  P  S  G  S    60
CCAAGAAAGTAATCTCCTCTGCCAACAGAGCCGTAGTCGGTGTTGTTGCTGGTGGTGGTC   240
  K  K  V  I  S  S  A  N  R  A  V  V  G  V  V  A  G  G  G  R    80
GTATTGACAAACCAATCCTGAAGGCGGGTCGAGCCTATCACAAATACAAGGCCAAGAGAA   300
  I  D  K  P  I  L  K  A  G  R  A  Y  H  K  Y  K  A  K  R  N   100
ACTGCTGGC                                                      309
  C  W                                                         102
```

Figure 5-1. Nucleotide and deduced amino acid sequences of Siberian sturgeon ribosomal protein L8 (RPL8). Partial cDNA of RPL8 was 309 base pairs encoding 102 amino acids.

```
CACAAACAGATAGAGAGGAGTGGGAAAGGAAGCTGGACGGCAGATCTTTCACATGAGCTC    60
H  K  Q  I  E  R  S  G  K  G  S  W  T  A  D  L  S  H  E  L     20
TTCAGATTTGCACTTGAGTCGGTGAGCCACGTGCTGTATGGGGAGCGGCTGGGATTGCTG   120
F  R  F  A  L  E  S  V  S  H  V  L  Y  G  E  R  L  G  L  L     40
CAGGACCACATCGACCCTGATACCCAGAAGTTCATCGACTGCATCACCCTGATGTTCAAT   180
Q  D  H  I  D  P  D  T  Q  K  F  I  D  C  I  T  L  M  F  N     60
ACCACGTCACCCATGCTGTACATCCCGCCTGCCCTACTGCGGAGAGTCGGGGCCAAGGTG   240
T  T  S  P  M  L  Y  I  P  P  A  L  L  R  R  V  G  A  K  V     80
TGGCGAGACCACGTGGAGGCCTGGGACGCCATCTTCAGTCACGCTGACCGATGCATTCAG   300
W  R  D  H  V  E  A  W  D  A  I  F  S  H  A  D  R  C  I  Q    100
AACATCTACAGGAAGTTACGTCAGTCTCCTGAAAGTGAGGGGAAGTACCCTGGAGTCCTG   360
N  I  Y  R  K  L  R  Q  S  P  E  S  E  G  K  Y  P  G  V  L    120
GCTAGCCTTCTCATGCTGGACAAGCTGTCCATTGAAGACATCAAGGCCAACGTGACTGAG   420
A  S  L  L  M  L  D  K  L  S  I  E  D  I  K  A  N  V  T  E    140
CTAATGGCCGGAGGGGTTGACACTACTTCCATTACCCTGTTGTGGACCATGTATGAACTT   480
L  M  A  G  G  V  D  T  T  S  I  T  L  L  W  T  M  Y  E  L    160
GCCAGATACCCCGACCTGCAGGAACAGCTGCGGGCTGAGGTTCAGGATGCCTGGGCCTCT   540
A  R  Y  P  D  L  Q  E  Q  L  R  A  E  V  Q  D  A  W  A  S    180
TCACAGGGGGACATGATCAAGATGTTAAAGTCAATTCCTTTGGTTAAAGGAGCCATAAAG   600
S  Q  G  D  M  I  K  M  L  K  S  I  P  L  V  K  G  A  I  K    200
GAGACGCTGAGGCTGCACCCAGTTGCTGTGAGCTTGCAAAGGTATATAACTGAGGATATT   660
E  T  L  R  L  H  P  V  A  V  S  L  Q  R  Y  I  T  E  D  I    220
GTGATCCAAAACTACCACATTCCATCAGGGACTCTGGTGCAGCTAGGGCTCTACGCTATG   720
V  I  Q  N  Y  H  I  P  S  G  T  L  V  Q  L  G  L  Y  A  M    240
GGGCGGAATCCACAGATTTTCCCAAGACCTCTGCAATATAACCCGGCCCGCTGGCTCAAA   780
G  R  N  P  Q  I  F  P  R  P  L  Q  Y  N  P  A  R  W  L  K    260
GGGGAGAGCCACTATTTCAAAAGCCTTAGCTTTGGATTCGGTCCCCGGCAGTGTCTGGGC   840
G  E  S  H  Y  F  K  S  L  S  F  G  F  G  P  R  Q  C  L  G    280
CGCAG                                                          845
R                                                              281
```

Figure 5-2. Nucleotide and deduced amino acid sequences of Siberian sturgeon $P450_{SCC.}$ Partial cDNA of $P450_{SCC}$ was 845 base pairs encoding 281 amino acids.

```
GAGCGTTCTAATTATCGCATTGTACGACACAGGCGTCTTTCTCAAGGCCAAGTGCAGCCC    60
E  R  S  N  Y  R  I  V  R  H  R  R  L  S  Q  G  Q  V  Q  P     20
AGTAGTAAAGCCAGCAAAACCAATGAAAGTGGCTTACTGCAGACAAGGAGGATTCACTTC   120
S  S  K  A  S  K  T  N  E  S  G  L  L  Q  T  R  R  I  H  F     40
ACTTCTCTGAGCCCTGAAATGCTCATGTCTTCAGTAATAGAGGCTGAACCGCCTGAGATT   180
T  S  L  S  P  E  M  L  M  S  S  V  I  E  A  E  P  P  E  I     60
TATTTGATGAGCTATCTCATGAAGCCATTCACTGAGGCCACCGTGATGACATCATTAACC   240
Y  L  M  S  Y  L  M  K  P  F  T  E  A  T  V  M  T  S  L  T T   80
ACCCTTGCAGACAAGGAACTCGTTTACATGGTCAGCTGGGCCAAAAAAATTCCAGGGTTT   300
L  A  D  K  E  L  V  Y  M  V  S  W  A  K  K  I  P  G  F  V     100
GTGGAGCTCGGTGTGTATGACCAGGTATGCCTATTGGAGTGTTGCTGGTTAGAGGTGCTG   360
E  L  G  V  Y  D  Q  V  C  L  L  E  C  C  W  L  E  V  L  M     120
ATGGTAGGGCTGATGTGGAGATCTATTAATCATCCAGGGAATCTCGTCTTTGCATCTGAC   420
V  G  L  M  W  R  S  I  N  H  P  G  N  L  V  F  A  S  D  L     140
CTTATTTTAAACAGGGACGACGGGAACTGCGTGGAAGGATTAGTGGAGGTTTTCGACATG   480
I  L  N  R  D  D  G  N  C  V  E  G  L  V  E  V  F  D  M  L     160
CTTTTGGCTATAACTTCAAAGTTTCGAGAGCTGAATCTGCAGCGAGAGGAGTATCTCTGC   540
L  A  I  T  S  K  F  R  E  L  N  L  Q  R  E  E  Y  L  C  L     180
CTCAAGGTCATGGTCCTCCTCAACTCCACTATGTTCCCCGGTCCCTCAGAGAAGCCAGAA   600
K  V  M  V  L  L  N  S  T  M  F  P  G  P  S  E  K  P  E  K     200
AAAAGTGAAAGTAGAGATAATCTGCTTAAACTTCTGGATGCAATCACCGATGCTTTAGTC   660
S  E  S  R  D  N  L  L  K  L  L  D  A  I  T  D  A  L  V  W     220
TGGGTTATTTCGAAGAAAGGACTCTCTTTACAGCAGCA                         698
V  I  S  K  K  G  L  S  L  Q  Q                                232
```

Figure 5-3. Nucleotide and deduced amino acid sequences of Siberian sturgeon ERβ. Partial cDNA of ERβ was 698 base pairs encoding 232 amino acids.

```
TGAACTTAGAAGCACGGAAAACAAAGAAGCTCAACAAATTGAAGGGAATTCAGGCGCCCG    60
  N  L  E  A  R  K  T  K  K  L  N  K  L  K  G  I  Q  A  P  V    20

TTGAGCAAGCAACACCGCTACCAGATGAGCGGTCACAGGCGCTGGTCCCCAAATCGATGC   120
  E  Q  A  T  P  L  P  D  E  R  S  Q  A  L  V  P  K  S  M  P    40

CACAGCTAACGCCAACCATGCTGTCGCTCTTGGAGGCCATCGAGCCAGAAATTATCTACT   180
  Q  L  T  P  T  M  L  S  L  L  E  A  I  E  P  E  I  I  Y  S    60

CGGGATACGACAGCACCATACCTGACACGTCCACGCGCCTTATGAGCACACTGAACAGGC   240
  G  Y  D  S  T  I  P  D  T  S  T  R  L  M  S  T  L  N  R  L    80

TAGGGGGAAGACAAGTGGTAGCTGCAGTAAAGTGGGCAAAGTCATTACCAGGGTTTAGAA   300
  G  G  R  Q  V  V  A  A  V  K  W  A  K  S  L  P  G  F  R  S   100

GCCTGCACCTTGATGATCAGATGACCCTGCTGCAGTGTTCCTGGCTGTTTCTCATGTCTT   360
  L  H  L  D  D  Q  M  T  L  L  Q  C  S  W  L  F  L  M  S  F   120

TTAGTCTGGGTTGGAGATCCTACAAGCAGTCTAATGGAAGCATGTTGTGCTTTGCACCAG   420
  S  L  G  W  R  S  Y  K  Q  S  N  G  S  M  L  C  F  A  P  D   140

ACCTAGTCATAAACGACGAGAGAATGAAGCTCCCTTACATGTTTGAACAGTGTGAACAAA   480
  L  V  I  N  D  E  R  M  K  L  P  Y  M  F  E  Q  C  E  Q  M   160

TGCTGAAGATTTCAAACGAGTTAGTACGACTTCAGCTTTCATATGATGAATACCTCTGCA   540
  L  K  I  S  N  E  L  V  R  L  Q  L  S  Y  D  E  Y  L  C  M   180

TGAAGGTTCTGTTGCTGCTCAGTTCAGTTCCTAAAGAGGGTCTG                   584
  K  V  L  L  L  L  S  S  V  P  K  E  G  L                     194
```

Figure 5-4. Nucleotide and deduced amino acid sequences of Siberian sturgeon GR. Partial cDNA of GR was 584 base pairs encoding 194 amino acids.

```
Sturgeon    QLNIGNVLPVGTMPEGTIICCLEEKPGDRGKLARASGNYATVISHNPETKKSRVKLPSGS  60
Zebrafish   QLNIGNVLPVGTMPEGTIVCCLEEKPGDRGKLARASGNYATVISHNPETKKSRVKLPSGS 154
X.laevis    QLNIGNVLPVGTMPEGTIVCCVEEKPGDRGKLARASGNYATVISHNPETKKTRVKLPSGS 154
Chicken     QLNIGNVLPVGTMPEGTIVCCLEEKPGDRGKLARASGNYATVISHNPETKKTRVKLPSGS 145
Mouse       QLNIGNVLPVGTMPEGTIVCCLEEKPGDRGKLARASGNYATVISHNPETKKTRVKLPSGS 154
Human       QLNIGNVLPVGTMPEGTIVCCLEEKPGDRGKLARASGNYATVISHNPETKKTRVKLPSGS 154
            ******************:**:******************************:********

Sturgeon    KKVISSANRAVVGVVAGGGRIDKPILKAGRAYHKYKAKRNCW 102  Identity
Zebrafish   KKVISSANRAVVGVVAGGGRIDKPILKAGRAYHKYKAKRNCW 196     99%
X.laevis    KKVISSANRAIVGVVAGGGRIDKPILKAGRAYHKYKAKRNCW 196     96%
Chicken     KKVISSANRAVVGIVAGGGRIDKPILKAGRAYHKYKAKRNCW 187     97%
Mouse       KKVISSANRAVVGVVAGGGRIDKPILKAGRAYHKYKAKRNCW 196     98%
Human       KKVISSANRAVVGVVAGGGRIDKPILKAGRAYHKYKAKRNCW 196     98%
            **********:**:****************************
```

Figure 5-5. Sequence comparison of deduced amino acid sequences for ribosomal protein L8 (RPL8). Asterisk indicates position, which has a single, fully conserved residue. Colon and period indicates position, which are fully conserved “strong” and “weaker” groups.

```
Sturgeon    HKQIERSGKGSWTADLSHELFRFALESVSHVLYGERLGLLQDHIDPDTQKFIDCITLMFN   60
Zebrafish   NKKIERSGQNQWTTDLSHELFKFALESVSAVLYGERLGLLLDYIDPDSQRFIDCITLMFK  238
Chicken     RAQVQQSGRERWTADFSHELFRFALESVCHVLYGERLGLLQDFVDPEAQQFIDAVTLMFH  234
Mouse       HRRIKQQNSGNFSGVISDDLFRFSFESISSVIFGERMGMLEEIVDPEAQRFINAVYQMFH  239
Human       HRRIKKAGSGNYSGDISDDLFRFAFESITNVIFGERQGMLEEVVNPEAQRFIDAIYQMFH  242
            . :::: .   ::  :*.:**:*::**:  *::*** *:* : ::*::*:**:.:  **:

Sturgeon    TTSPMLYIPPALLRRVGAKVWRDHVEAWDAIFSHADRCIQNIYRKLRQSPESEGKYPGVL  120
Zebrafish   TTSPMLYLPPGLLRPIRSKIWRNHVEAWDGIFNQADRCIQNIYRQLRKNPEGNGKYTGVL  298
Chicken     TTSPMLYVPPALLRHLNTKTWRDHVHAWDAIFTQADKCIQNVYRDIRLQRKSTEEHTGIL  294
Mouse       TSVPMLNLPPDFFRLLRTKTWKDHAAAWDVIFNKADEYTQNFYWDLRQK-RDFSQYPGVL  298
Human       TSVPMLNLPPDLFRLFRTKTWKDHVAAWDVIFSKADIYTQNFYWELRQKGSVHHDYRGIL  302
            *: *** :** ::* . :* *::*. *** **.:**   **.* .:* .     .: *:*

Sturgeon    ASLLMLDKLSIEDIKANVTELMAGGVDTTSITLLWTMYELARYPDLQEQLRAEVQDAWAS  180
Zebrafish   ASLLMLDKLSIEDIKASVTELMAGGVDTTAITLLWTLYELARNPDLQEEIRAEISAARIA  358
Chicken     FSLLVQDKLPLDDIKASVTEMMAGGVDTTSMTLQWAMLELARSPGIQERLRAEVLAAKQE  354
Mouse       YSLLGGNKLPFKNIQANITEMLAGGVDTTSMTLQWNLYEMAHNLKVQEMLRAEVLAARRQ  358
Human       YRLLGDSKMSFEDIKANVTEMLAGGVDTTSMTLQWHLYEMARNLKVQDMLRAEVLAARHQ  362
              **  .*:.:.:*:*.:**::********:** * : *:*:   :*: :***:  *

Sturgeon    SQGDMIKMLKSIPLVKGAIKETLRLHPVAVSLQRYITEDIVIQNYHIPSGTLVQLGLYAM  240
Zebrafish   SKGDMVQMLKMIPLVKGTLKETLRLHPVAVSLQRYITEDIVIQKYHIPAGTLVQLGLYAM  418
Chicken     AQGDRVKMLKSIRLLKAAIKETLRLHPVAVTLQRYTTQEVILQDYRIPPKTLVQVGLYAM  414
Mouse       AQGDMAKMVQLVPLLKASIKETLRLHPISVTLQRYTVNDLVLRNYKIPAKTLVQVASFAM  418
Human       AQGDMATMLQLVPLLKASIKETLRLHPISVTLQRYLVNDLVLRDYMIPAKTLVQVAIYAL  422
            ::**   *:: : *:*.::********::*:**** .:::::::.* **. ****:. :*:

Sturgeon    GRNPQIFPRPLQYNPARWLKG--ESHYFKSLSFGFGPRQCLGR  281  Identity
Zebrafish   GRDHQVFPNPEQYLPSRWVNS--QNHYFKSLSFGFGPRQCLGR  459    77%
Chicken     GRDPEVFPKPEQFNPERWLVM--GSKHFKGLSFGFGPRQCLGR  455    67%
Mouse       GRDPGFFPNPNKFDPTRWLEKSQNTTHFRYLGFGWGVRQCLGR  461    49%
Human       GREPTFFFDPENFDPTRWLSKDKNITYFRNLGFGWGVRQCLGR  465    51%
            **:  .*  * :: * **:       :*: *.**:* ******
```

Figure 5-6. Sequence comparison of deduced amino acid sequences for $P450_{SCC}$. Asterisk indicates position, which has a single, fully conserved residue. Colon and period indicates position, which are fully conserved "strong" and "weaker" groups.

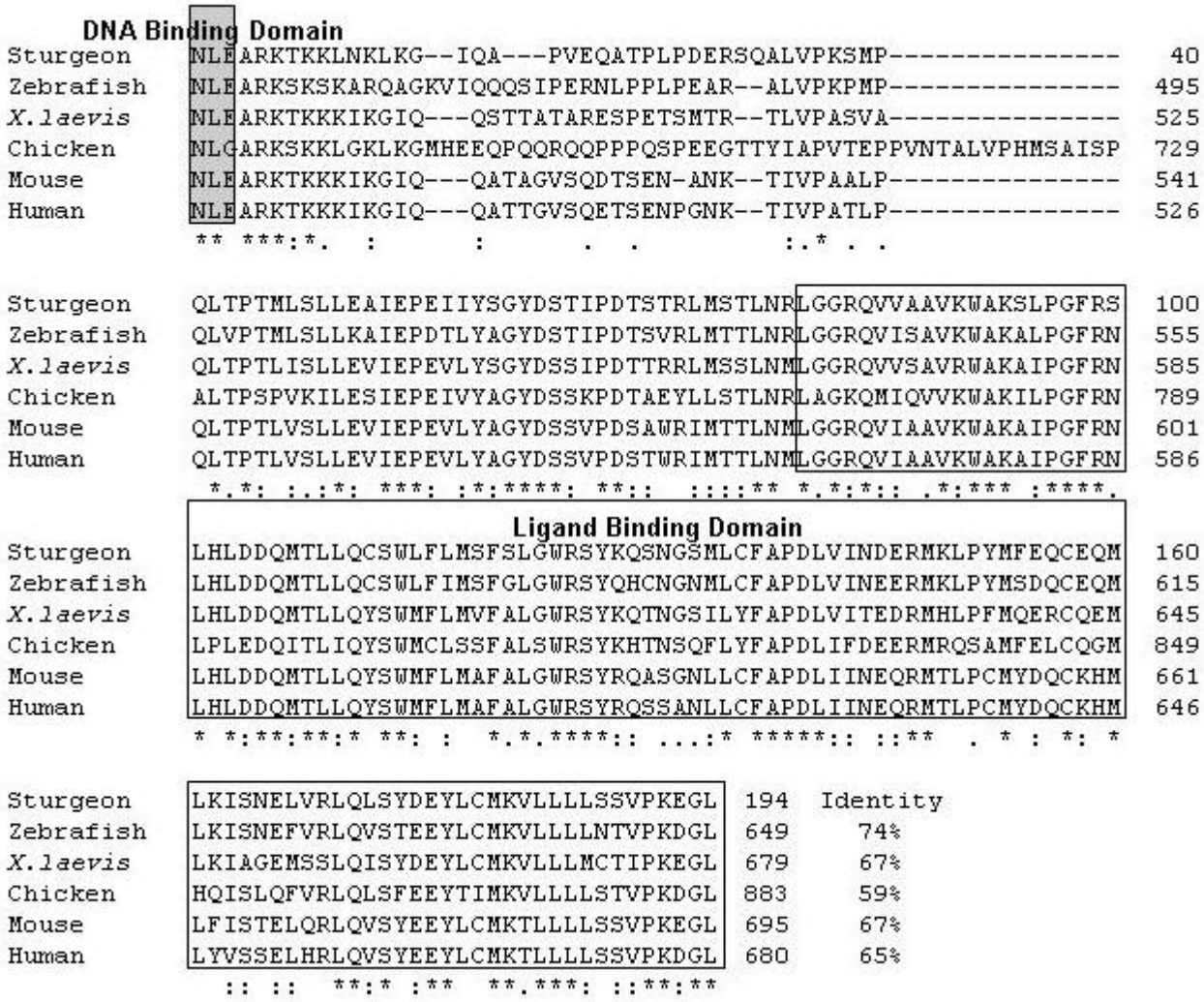


Figure 5-7. Sequence comparison of deduced amino acid sequences for GR. The open and filled box indicates the ligand and DNA binding domain of nuclear receptor subfamily, respectively. Asterisk indicates position, which has a single, fully conserved residue. Colon and period indicates position, which are fully conserved "strong" and "weaker" groups.

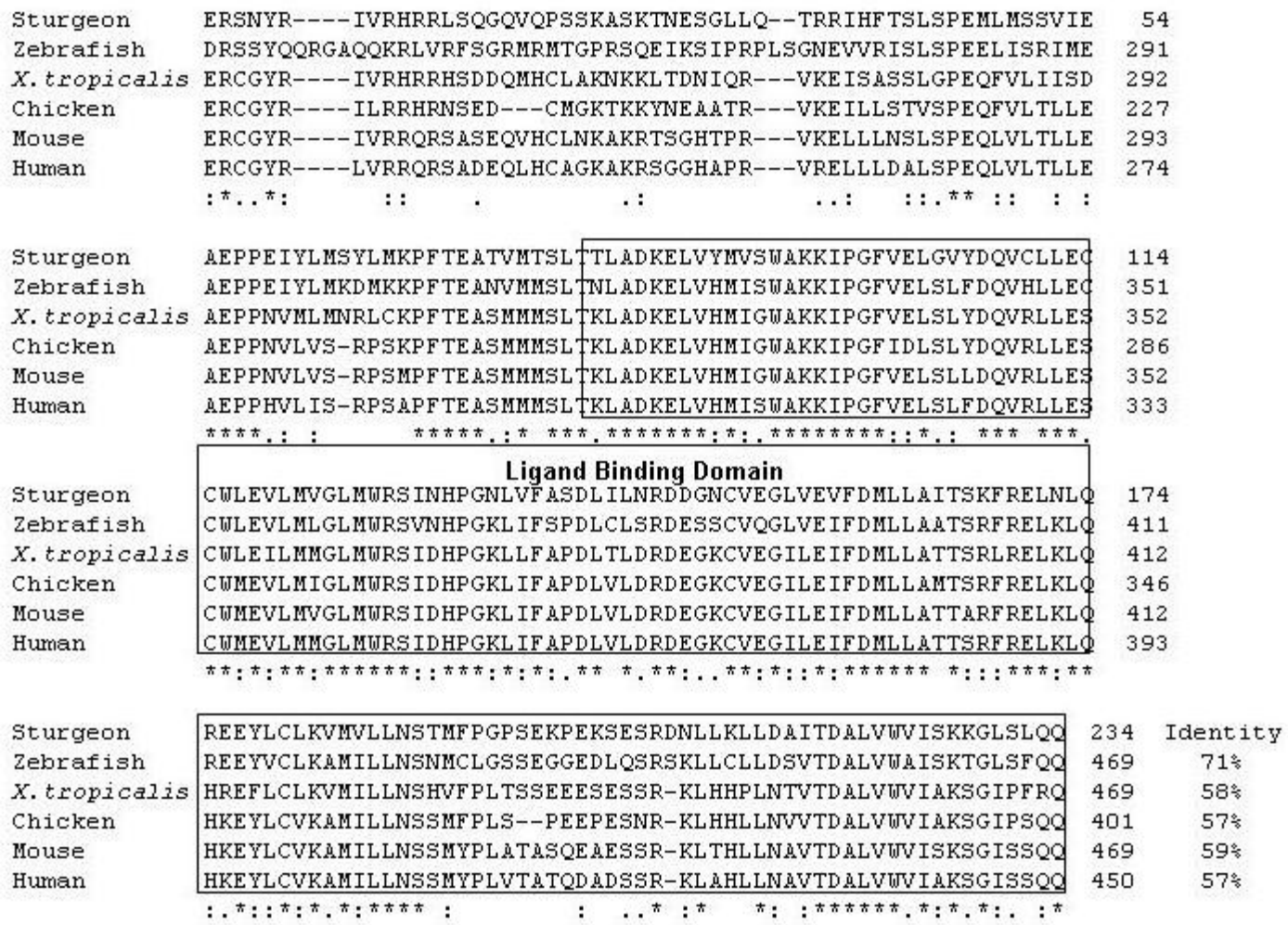


Figure 5-8. Sequence comparison of deduced amino acid sequences for ERβ. The open and filled box indicates the ligand and DNA binding domain of nuclear receptor subfamily, respectively. Asterisk indicates position, which has a single, fully conserved residue. Colon and period indicates position, which are fully conserved "strong" and "weaker" groups.

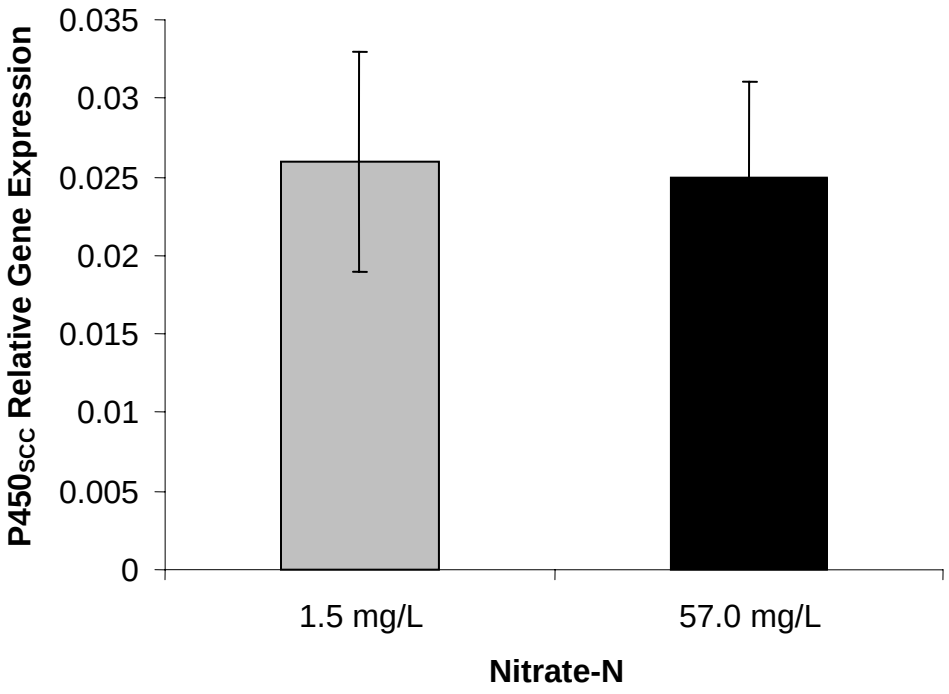


Figure 5-9. Mean (± SE) expression of $P450_{SCC}$ mRNA in 4.5 year-old Siberian sturgeon.

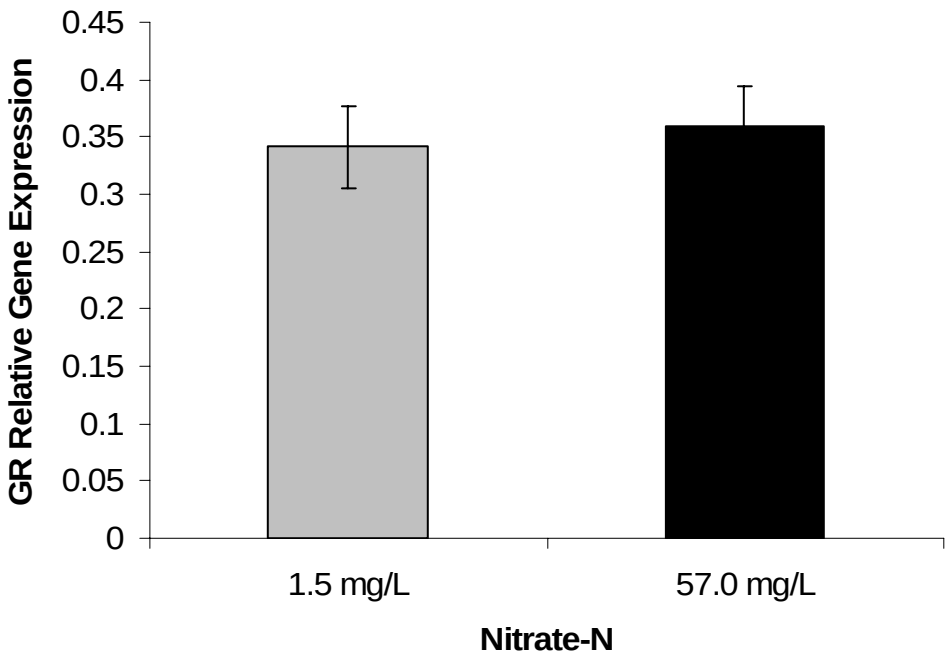


Figure 5-10. Mean (± SE) expression of glucocorticoid (GR) receptor mRNA in 4.5 year-old Siberian sturgeon.

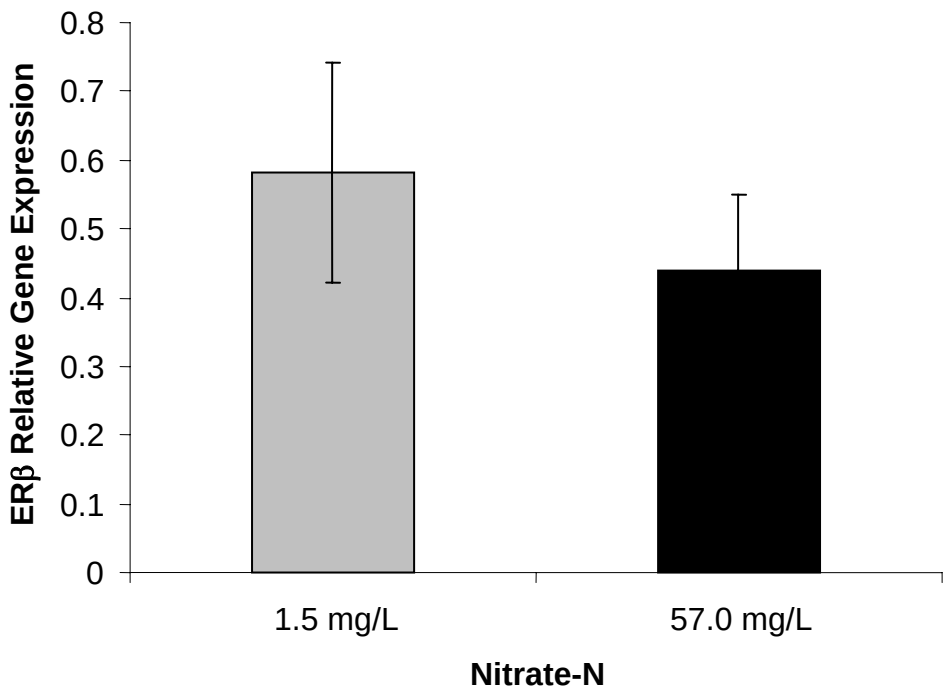


Figure 5-11. Mean (± SE) expression of estrogen receptor-β (ERβ) mRNA in 4.5 year-old Siberian sturgeon.

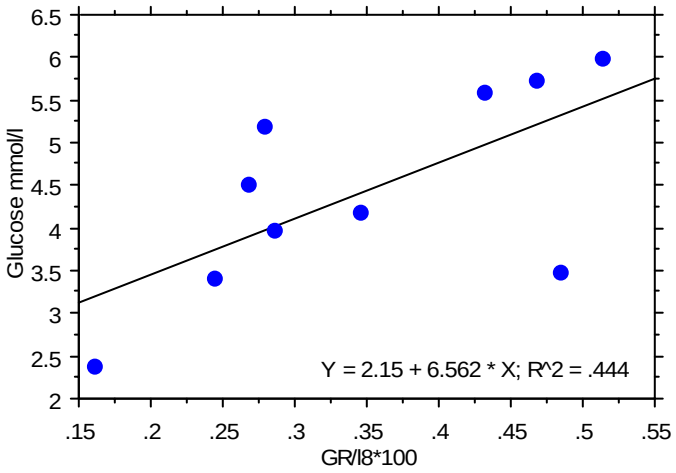


Figure 5-12. Linear regression of glucose (mmol/L) vs GR mRNA (normalized to L8 expression) for fish exposed to 1.5 mg/L nitrate-N.

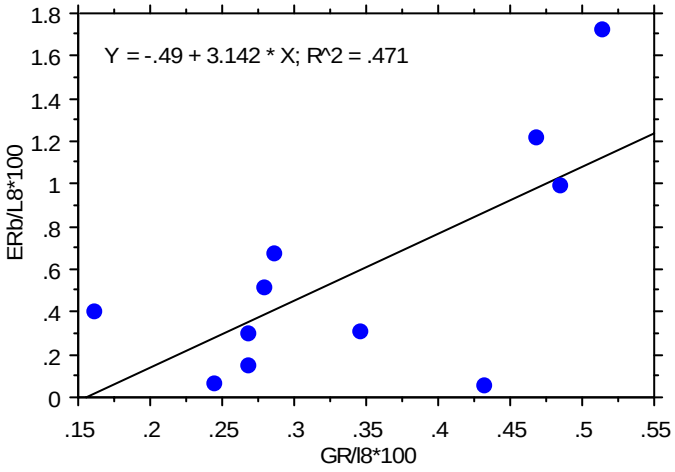


Figure 5-13. Linear regression of ERβ mRNA and GR mRNA (normalized to L8 expression) for fish exposed to 1.5 mg/L nitrate-N.

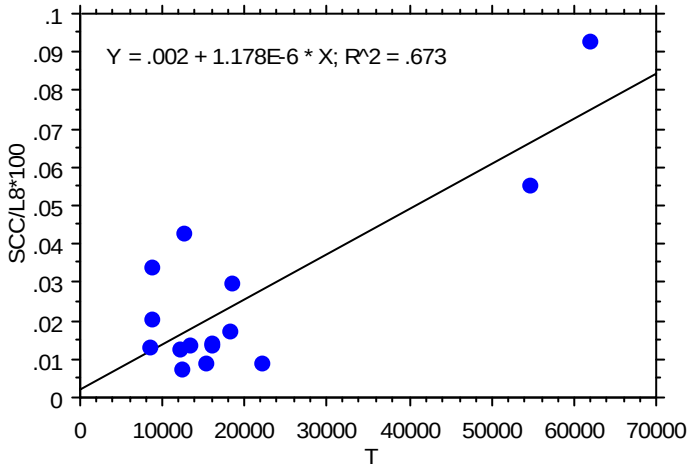


Figure 5-14. Linear regression of $P450_{SCC}$ mRNA (normalized to L8 expression) and T for fish exposed to 57 mg/L nitrate-N. The significance of this regression is driven primarily by the two extraneous points.

.

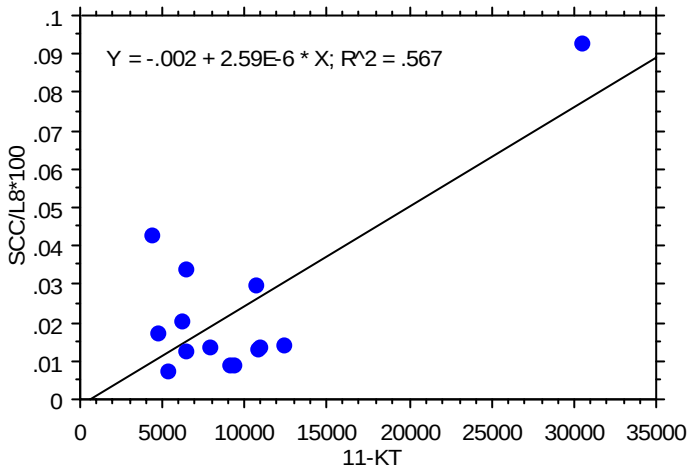


Figure 5-15. Linear regression of $P450_{SCC}$ mRNA (normalized to L8 expression) and 11-KT for fish exposed to 57 mg/L nitrate-N. The significance of this regression is driven primarily by the extraneous point.

.

CHAPTER 6
SUMMARY AND FUTURE DIRECTIONS

Summary

The role of pesticides in the reproductive impairment of wildlife was first made public in Rachel Carson's *Silent Spring* in 1962 (Carson, 1962), and since then a host of man-made chemicals ranging from surfactants to polychlorinated biphenyls (PCBs) have been implicated in countless developmental and reproductive abnormalities (Colborn et al., 1993). Noticeably absent from most of these studies are the affects of naturally occurring compounds, which can become elevated well beyond naturally occurring background concentrations from anthropogenic impact. The EPA drinking water limit for nitrate is 10 mg/L NO_3-N, however, many rural drinking water wells exceed this standard. For example, in Iowa 18% of drinking wells above the EPA standard were recorded (Kross et al., 1993). Natural water bodies can exceed 100 mg/L nitrate (Rouse et al., 1999) and although elevated nitrate is becoming a ubiquitous component of aquatic ecosystems, only recently has it been considered for its role in altering reproductive and developmental physiology (Guillette and Edwards, 2005).

Results from Chapter 2 showed sturgeon to be especially sensitive to nitrate toxicosis, and unexpectedly, this sensitivity increases as the fish grows. This could have serious implications for mature and reproductively active animals that could be far more sensitive than the ontogenetic size classes tested in that study. This is especially alarming since nitrate concentrations in natural water bodies in Florida have been documented approaching the upper nitrate concentration of 57 ppm NO_3-N, shown in Chapter 4 to significantly alter plasma concentrations of sex steroids in maturing female sturgeon (Katz et al., 1999; Katz, 2004). Although sturgeon were more sensitive to nitrate toxicosis than many species reported to date, it should be noted that the toxic effects of nitrate have been examined in only a handful of aquatic

species. Although sturgeon may be well suited to serve as sentinel species for nitrate induced reproductive impairment, it is highly likely that other species will be concomitantly affected by elevated nitrate exposure.

In Chapter 3 we observed a significant elevation in plasma T concentration under periods of greatest stress, defined by plasma cortisol. Plasma T concentrations have been shown to be uniquely sensitive to environmental alterations (Milnes et al., 2006). Unexpectedly, the positive correlation between T and cortisol was not apparent in studies outlined in Chapter 4. The reproduction system is characterized by cyclic changes, modulated by hypothalamic releasing hormones, pituitary gonadotropes and gonadal steroids. These cycles can be influenced by environmental cues such as temperature, photoperiod and other factors (Norris, 1997; Kim et al., 1998). Experiments in Chapter 4 were conducted at a different time of the year, in animals that were one year older and more reproductively mature. It is possible conditions present in the second series of experiments were not conducive to the stress induced alterations observed in Chapter 3.

Consistent between these studies (i.e. Chapters 3 and 4), however, was that induced stress did not result in reductions in plasma sex steroid concentrations. Chapter 4 examined the effects of nitrate on various steroid endpoints. These data describe nitrate-induced elevations in plasma concentrations of T, 11-KT and E_2 in animals exposed to 57 mg/L NO_3-N. Although endocrine disrupting contaminants usually reduce plasma sex steroid concentrations, as opposed to the plasma sex steroid elevations observed in this study, other studies of aquatic animals have shown elevations in plasma sex steroid concentrations following chemical exposure. A study of American alligators showed significant elevations in plasma T concentrations in alligators exposed to a little as 0.01 ppb toxaphene (Milnes, 2005). It is difficult to predict what the

observed elevations in plasma concentrations of sex steroids would impose on reproductive performance. Altered circulating concentrations would likely alter feedback mechanisms on the hypothalamo-pituitary axis as well as negative feedback on the gonad and other tissues responsive to sex steroids, such as the liver and female reproductive tract. Persistent elevation in hormones could be countered by adaptation but the long term implications of chronic elevation in hormones is not favorable. It is unclear if nitrate induced elevations in sex steroids observed in Chapter 4 are due to a generalized stress response, as Chapter 3 data suggest, but given that plasma cortisol concentrations remained unaffected by nitrate, renders this explanation suspect.

In aquaculture altered reproductive performance, of a species such as sturgeon whose economic viability relies almost entirely on the successful culture of eggs (caviar), could significantly diminish profit margins and reduce their potential value. Even in warm captive environments, sturgeon can take 4-6 years to reach reproductive maturity, and even slight delays in maturation can have significant financial impacts. From an ecological perspective, the cost of altered reproductive performance, in a wild sturgeon that can take 10-20 years or more to reach sexual maturity (Detlaff et al., 1993), is that much greater.

Fertilizers applied near water bodies coupled with spring rainstorms, contributes to an aquatic nitrate pulse that overlaps the breeding season of many sturgeon species (Detlaff et al., 1993; Barbeau, 2004). Although speculative, given the global increases in aquatic concentrations of nitrate, and the fact that pollution has been cited as a significant cause of reductions in sturgeon populations in the Caspian, it is reasonable to hypothesize that nitrate could contribute to observed population declines. Although it is possible that the observed elevations in circulating concentrations of sex steroids seen in these studies imparts a

physiological advantage, accelerating the reproductive process, data from Chapter 5 renders this conclusion unlikely.

In Chapter 5, mRNA expression patterns of various enzyme and receptor proteins involved in the steroidogenic cascade were examined. These data showed no nitrate-induced alterations in mRNA expression patterns as would be expected given the elevations in hormone end products seen in Chapter 4. This was the first study to clone and describe the mRNA expression patterns of key upstream and downstream constituents in the steroidogenic pathway. We observed significant correlations with steroidogenic enzymes and hormone end products, which appeared to be nitrate dependent, notable between $P450_{SCC}$ and both androgens in fish exposed to 57 mg/L NO_3-N. I hypothesize that this association may be the result of sex steroids that were sufficiently elevated following exposure to the upper nitrate concentration, that they reached the threshold needed to induce feed back mechanisms and alter gene expression.

Taken together, these data suggest that the observed elevations in plasma sex steroid concentrations are unlikely due to an up-regulation of gonadal synthesis. Figure 6-1 revisits the summary figure first introduced in Chapter 1 (Figure 1-1), outlining hormone production and cycling. This updated figure reflects possible mechanisms of disruption based on the data collected for this dissertation. Elevated plasma concentrations of sex steroids could result from several physiological mechanisms, including an upregulation of gonadal synthesis, alterations in hepatic biotransformation and clearance, or altered plasma storage associated with steroid binding proteins. An upregulation of gonadal synthesis was predicted to be associated with an upregulation in mRNA expression of various steroidogenic endpoints, such as the enzymes essential for this process. An increase in mRNA concentration was not observed for P450scc, suggesting that our prediction was false. Although this is only one enzyme in the steroidogenic

pathway, it plays an initial role and is considered a rate-limiting step. Thus, alternative mechanisms to explain the elevated plasma sex steroid concentrations, such as hepatic biotransformation and clearance or altered concentrations of steroid binding proteins, need to be examined in the future. As the fish used in this study were part of a commercial aquaculture facility, it was not possible in these series of studies to kill the fish to examine hepatic enzyme activity.

The hypothesis that nitrate can influence vertebrate reproduction by altering nitric oxide (NO) regulation has been proposed by several authors (Vanvoorhis et al., 1994; DelPunta, 1996; Panesar and Chan, 2000; Guillette and Edwards, 2005). It appears NO reduces the synthesis of steroid hormones by inhibiting steroidogenic enzymes, notably the P450 family of enzymes. Given that the liver relies heavily on P450 enzymes for proper function, it seems a likely possibility that alterations in hepatic function could explain the discord between elevations in concentrations of sex steroids and the unremarkable mRNA expression patterns observed in sturgeon cultured in high and low nitrate environments.

Future Directions

Determining the cause of nitrate induced increases in concentrations of circulating sex steroids will be critical to understanding the effects of nitrate on reproductive physiology. Due to the pervasive role of P450 enzymes in hepatic function, nitrate induced hepatic alteration is proposed as the most likely cause of elevated sex steroid concentrations in sturgeon cultured in elevated nitrate environments. Therefore, future studies should focus on defining the liver's role in sex steroid clearance in sturgeon exposed to nitrate. Since the liver is responsible for the production of vitellogenin, a protein necessary for oocyte growth and development, its production and possible alteration should also be examined. Nitrate effects should also be

examined in male sturgeon, as well as other commercially relevant species to determine if the results observed in this work can be observed in other species.

Perhaps most importantly, understanding the biological significance of nitrate induced elevations in concentrations of sex steroids in terms of reproductive performance, including time to maturation, egg size, fecundity and larval viability is critical to determining the ecological significance of nitrate's effects.

Conclusions

In summary, these data suggest that steroids should not serve as the exclusive endpoint for evidence of nitrate induced endocrine disruptions. These endpoints should also include steroidogenic enzymes and steroid receptors, and possibly hepatic enzymes and receptors as well. Understanding the role of nitrate in sturgeon reproduction will be dependent upon future studies uncovering the biological and reproductive implications of hormonal and molecular effects uncovered in these studies.

In aquaculture, nitrate has been overlooked as a material water quality hazard, largely because most aquaculture facilities use large quantities of water, which keeps nitrate concentrations well below that which will elicit observable effects, such as mortality. The data obtained in these studies suggest that indeed nitrate is a material water quality hazard, and that easily observable effects such as mortality can no longer be considered valid endpoints to define safe concentrations of nitrate in aquaculture. Sublethal effects of nitrate exposure, such as endocrine disruption, must now be considered in the effective management of sturgeon populations in both natural and captive environments.

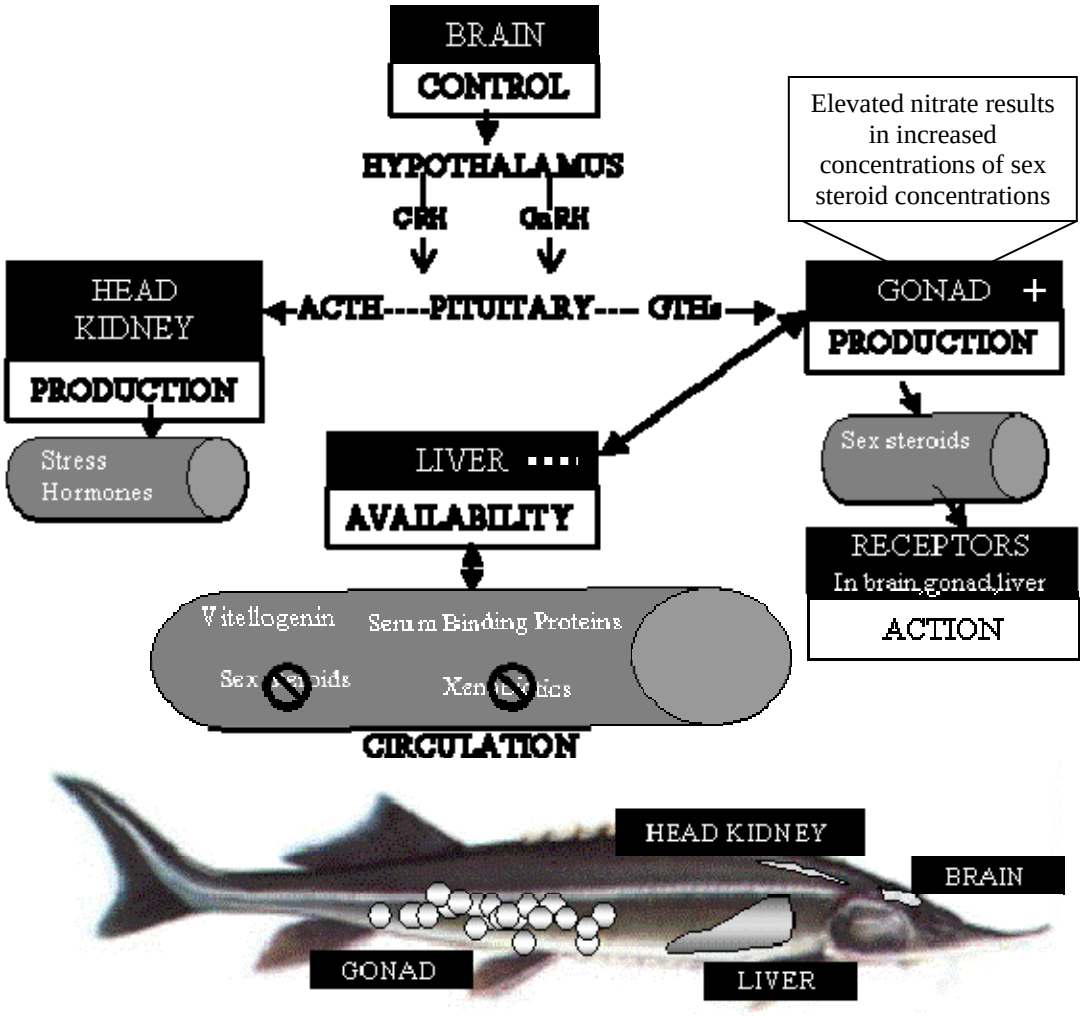


Figure 6-1. Possible alterations in nitrate induced elevations of sex steroid concentrations in Siberian sturgeon. (+) represents a possible up-regulation and (--) represents a possible mechanism of disruption. Studies of mRNA expression patterns suggest sex steroid elevations are not the result of increased gonadal synthesis, but that another mechanism is involved. Based on nitrate's documented role in altering P450 enzymes and function, it is hypothesized here that the liver, which relies heavily on P450 enzymes for proper function, is altered. It is also possible, that serum binding proteins, are altered by nitrate's effects.

LIST OF REFERENCES

Akimova, N.V., Ruban, G.I., 1995. A classification of reproductive disturbances in sturgeons (Acipenseridae) caused by an anthropogenic impact. J. Ichthyol. 36, 61-76.

Alonso, A., Camargo, J.A., 2003. Short-term toxicity of ammonia, nitrite and nitrate to the aquatic snail *Potamopyrgus antipodarum* (Hydrobiidae, Mollusca). Bull. Environ. Contam. Toxicol. 70, 1006-1012.

Aluru, N., Vijayan, M.M., 2006. Aryl hydrocarbon receptor activation impairs cortisol response to stress in rainbow trout by disrupting the rate-limiting steps in steroidogenesis. Endocrinology 147, 1895-1903.

Attayde, J.L., Hansson, L.A., 1999. Effects of nutrient recycling by zooplankton and fish on phytoplankton communities. Oecologia 121, 47-54.

Barannikova, I.A., Bayunova, L.V., Dyubin, V.P., Saenko, I.I., Semenkova, T.B., 2000. Serum cortisol levels and function of interregnal gland during life cycle of sturgeon, *Acipenser gueldenstaedti*. Vopr. Ikhtyologii 40, 379-388.

Barannikova, I.A., Bayunova, L.V., Semenkova, T.B., 2004. Serum levels of testosterone, 11-ketotestosterone and oestradiol-17β in three species of sturgeon during gonadal development and final maturation induced by hormonal treatment. J. Fish Biol. 64, 1330-1338.

Barbeau, T.R., 2004. Influence of insulin-like growth factor-1, steroids, and nitrate on reproduction in amphibians. PhD dissertation, University of Florida, Gainesville FL.

Barden, N., 2004. Implications of the hypothalamic-pituitary-adrenal axis in the physiopathology of depression. J. Phychiatry Neurosci. 29, 185-193.

Barton, B.A., 2002. Stress in fishes; a diversity of responses with particular reference to changes in circulating corticosteroids. Integr. Comp. Biol., 42, 517-525.

Barton, B.A., Iwama, G.K., 1991. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Ann. Rev. Fish Dis. 1, 3-26.

Barton, B.A., Rahn, A.B., Feist, G., Bollig, H., Schreck, C.B., 1998. Physiological stress responses of the freshwater chondrostean paddlefish (*Polydon spathula*) to acute physical disturbances. Comp. Biochem. Physiol. A 120, 355-363.

Barton, B.L., Schreck, C.B., 1987. Metabolic cost of acute physical stress in juvenile steelhead. Trans. Amer. Fish. Soc. 116, 257-263.

Barton, B.A., Schreck, C.B., Sigismondi, L.A., 1986. Multiple acute disturbances evoke cumulative physiological stress responses in juvenile Chinook salmon. Trans. Amer. Fish. Soc. 115, 245-251.

Bayne, B.L., 1985. Responses to environmental stress: tolerance, resistance and adaptation. In *Marine Biology of Polar Regions and Effects of Stress on Marine Organisms*, (ed. J.S. Gray and M.E. Christiansen), pp. 331-349. Wiley, New York.

Bayunova, L., Barannikova, I., Semenkova, T., 2002. Sturgeon stress reactions in aquaculture. J. Appl. Ichthyol. 18, 397-404.

Beamesderfer, R.C.P., Farr, R.A., 1997. Alternatives for the protection and restoration of sturgeons and their habitat. Environ. Biol. Fishes 48, 407-417.

Belanger, J.M., Son, J.H., Laugero, K.D., Moberg, G.P., Doroshov, S.I., Lankford, S.E., Cech, J.J., Jr., 2001. Effects of short-term management stress and ACTH injections on plasma cortisol levels in cultured white sturgeon, *Acipenser transmontanous*. Aquaculture 1-2, 165-176.

Bernier, N.J., Bedard, N., Peter, R.E., 2004. Effects of cortisol on food intake, growth, and forebrain neuropeptide Y and corticotropin-releasing factor gene expression in goldfish. Gen. Comp. Endocrinol. 135, 230-240.

Birstein, V.J., 1993. Sturgeons and paddlefishes: threatened fishes in need of conservation. Conserv. Biol. 7, 773-787.

Bodo, C., Rissman, E.F., 2006. New roles for estrogen receptor β in behavior and neuroendocrinology. Front. Neuroendocrinol. 27, 217-232.

Bodwell, J.E., Kingsley, L.A., Hamilton, J.W., 2004. Arsenic at very low concentrations alters glucocorticoid receptor (GR)-mediated gene activation but not GR-mediated gene repression: complex dose-response effects are closely correlated with levels of activated GR and require a functional GR DNA binding domain, Chem. Res. Toxicol. 17, 1064-1076.

Bohl, M., 1977. Some initial aquaculture experiments in recirculating water systems. Aquaculture 11, 323-328.

Brion, F., Tyler, C.R., Palazzi, X., Laillet, B., Porcher, J.M., Garric, J., Flammarion, P., 2004. Impacts of 17β-estradiol, including environmentally relevant concentrations, on reproduction after exposure during embryo-larval-juvenile and adult-life stages in zebrafish (*Danio rerio*). Aquatic Toxicol. 68, 193-217.

Bromage, N.R., Shepard, C.J, Roberts, J., 1988. Farming systems and husbandry practice. In *Intensive Fish Farming*, (ed. C.J. Shepard and N.R. Bromage), pp. 92-95. Oxford BSP Professional.

Brownell, C.L., 1980. Water quality requirements for first-feeding in marine fish larvae. 1. Ammonia, nitrite and nitrate. J. Exp. Mar. Biol. Ecol. 44, 269-283.

Buikema, A.L.Jr., Niederlehner, B.R., Cairns, J.Jr., 1982. Biological monitoring part IV-toxicity testing. Water Res. 16, 239-262.

Bulger, W.H., Kupfer, D., 1985. Estrogenic activity of pesticides and other xenobiotics on the uterus and male reproductive tract. In *Endocrine toxicology*, (ed. J.A., Thomas, K.S., Korach, and J. A., McLachlan), pp 1-33. Raven Press, New York.

Bury, N.R., Sturm, A., Le Rouzic, P., Lethimonier, C., Ducouret, B., Guiguen, Y., Robinson-Rechavi, M., Laudet, V., Rafestin-Oblin, M.E., Prunet, P., 2003. Evedence for two distinct functional glucocorticoid receptors in teleost fish. J. Mol. Endocrinol. 31, 141-156.

Cadenas, E., Poderoso, J.J., Antunes, F., Boveris, A., 2000. Analysis of the pathways of nitric oxide utilization in mitochondria. Free Radical Res. 33, 747-756.

Camargo, J.A., Ward, J.V., 1992. Short-term toxicity of sodium nitrate ($NaNO_3$) to non-target freshwater invertebrates. Chemosphere 24, 23-28.

Campbell, P.M., Pottinger, T.G., Sumpter, J.P., 1994. Preliminary evidence that chronic confinement stress reduces the quality of gametes produced by brown trout and rainbow trout. Aquaculture 120, 151-169.

Capriulo, G.M., Smith, G., Troy, R., Wikfors, G.H., Pellet, J., Yarish, C., 2002. The planktonic food web structure of a temperate zone estuary, and its alteration due to eutorphication. Hydorbiologia 475, 263-333.

Carragher, J.F., Pankhurst, N.W., 1991. Stress and reproduction in a commercially important marine fish, *Pagrus auratus* (Sparidae). In *Proceedings of the Fourth International Symposium on Reproductive Physiology of Fish* (ed. A.P. Scott, J.P. Sumpter, D.E. Kime, M.S. Rolfe) pp. 253-255. FishSymp 91, Sheffield.

Carragher, J.F., Sumpter, J.P., 1990. The effect of cortisol on the secretion of sex steroids from cultured ovarian follicles of rainbow trout. Gen. Comp. Endocrinol. 77, 403-407.

Carragher, J.F., Sumpter, J.P., Pottinger, T.G., Pickering, A.D., 1989. The deleterious effects of cortisol implantation on reproductive function in two species of trout, *Salmo trutta* L. and *Salmo gairdneri* Richardson. Gen. Comp. Endocrinol. 76, 310-321.

Carson, R., 1962. *Silent Spring*. The Riverside Press, Cambridge.

Cavalli, R.O., Wasielesky, W., Franco, C.S., Miranda, K., 1996. Evaluation of the short-term toxicity of ammonia, nitrite and nitrate to *Penaeus paulensis* (Crustacea, Decapoda) broodstock. Arch Biol. Tech. 39, 567-575.

Chebanov, M.S., Karnaukhov, G.I., Galich, E.V., Chmir, Y.N., 2002. Hatchery stock enhancement and conservation of sturgeon, with an emphasis on the Azov Sea populations. J. Appl. Ichthyol. 18, 463-469.

Chow, C.K., Hong, C.B., 2002. Dietary vitamin E and selenium and toxicity of nitrite and nitrate. Toxicology 180, 195-207.

Colborn, T., Clement, C., 1992. *Chemically-induced Alterations in Sexual and Functional Development: The Wildlife/Human Connection*. Princeton Sci. Publ. Co. Inc., Princeton.

Colborn, T., Vom Saal, F.S., Soto, A.M., 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ. Health Perspec. 101, 378-384.

Cole, T.J., Blendy J.S., Monaghan, A.P., Krieglstein, K., Schmid, W., Aguzzi, A., 1995. Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. Genes Dev. 9, 1608-1621.

Colt, J., Tchobanoglous, G., 1976. Evaluation of the short-term toxicity of nitrogenous compounds to channel catfish, *Ictalurus punctatus*. Aquaculture 8, 209-224.

Comly, H.H., 1945. Cyanosis in infants caused by nitrate in well water. J. Amer. Med. Assoc. 129, 112-116.

Consten, D., Lambert, J.G.D., Komen, H., Goos, H.J.Th., 2002. Corticosteroids affect the testicular androgen production in male common carp (*Cyprinus carpio* L.). Biol. Reprod. 66, 106-111.

Conte, F.S., 2004. Stress and the welfare of cultured fish. Appl. Anim. Behav. Sci. 86, 205-223.

Cooke, P.S., Holsberger, D.R., Witorsch, R.J., Sylvester, P.W., Meredith, J.M., Treinen, K.A., Chapin, R.E., 2004. Thyroid hormone, glucocorticoids, and prolactin at the nexus of physiology, reproduction, and toxicology. Toxicol. Appl. Pharmacol. 194, 309-335.

Danielson, P.B., 2002. The cytochrome P450 superfamily: biochemistry, evolution and drug metabolism in humans. Curr. Drug and Metab. 3, 561-597.

DelPunta, K., Charreau, E.H., Pignataro, O.P., 1996. Nitric oxide inhibits Leydig cell steroidogenesis. Endocrinology 137, 5337-5343.

Denslow, N.D., Lee, S.H., Bowman, C.J., Hemmer, M.J., Folmar, L.C., 2001. Multiple responses in gene expression in fish treated with estrogen. Comp. Biochem. Physiol. B. 129, 277-282.

Dentin, R., Denechaud, P.D., Benhamed, F., Girard, J., Postic, C., 2006. Hepatic gene regulation by glucose and polyunsaturated fatty acids: a role for ChREBP. J. Nutr. 136, 1145-1149.

Detlaff, T.A., Hinsvurg, A.S., Schmalhausen, O.I., 1993. *Sturgeon Fishes. Developmental Biology and Aquaculture*. Springer-Verlag, Berlin.

Di Marco, P., McKenzie, D.J., Mandich, A., Bronzi, P., Cataldi, E., Cataudella, S., 1999. Influence of sampling conditions on blood chemistry values of Adriatic sturgeon *Acipenser naccarii (*Bonaparte, 1836). J. Appl. Ichthyol. 15, 73-77.

Dowden, B.F., Bennet, H.J., 1965. Toxicity of selected chemicals to certain animals. J. Wat. Pollut. Control Fed. 37, 1308-1316.

Dwyer, F.J., Mayer, F.L., Sappington, L.C., Buckler, D.R., Bridges, C.M., Greer, I.E., Hardesty, D.K., Henke, C.E., Ingersoll, C.G., Kunz, J.L., Whites, D.W., Augspurger, T., Mount, D.R., Hattala, K., Neuderfer, G.N., 2005. Assessing contaminant sensitivity of endangered and threatened aquatic species: Part I. Acute toxicity of five chemicals. Arch. Environ. Contam. Toxicol. 48, 143-154.

Edwards, T.M., 2006. Environmental influences on mosquitofish reproduction. PhD Dissertation, University of Florida, Gainesville, FL.

Edwards, T.M., McCoy, K.A., Barbeau, T., McCoy, M.W., Thro, J.M., Guillette, L.J.Jr., 2006a. Environmental context determines nitrate toxicity in Southern toad (*Bufo terrestris*) tadpoles. Aquatic Toxicol. 78, 50-58.

Edwards, T.M., Miller, H.D., Guillette, L.J.Jr., 2006b. Water quality influences reproduction in female mosquitofish (*Gambusia holbrooki*) from eight Florida springs. Env. Health Perspect. 114, 69-75.

Edwards, T.M., Moore, B.C., Guillette, L.J.Jr., 2006c. Reproductive dysgenesis in wildlife: a comparative view. Int. J. Androl. 29, 109-121.

Environmental Protection Agency (US EPA), 1986. *Quality criteria for water*, Washington, DC.

Environmental Protection Agency (US EPA), 1996. *Drinking Water Regulations and Health Advisories*. Washington, DC.

Esch, G.W., Hazen, T.C., 1978. Thermal ecology and stress: a case history for red-sore disease in largemouth bass. DOE (U.S. Department of Energy) Symposium Series 48, 331-363.

Food and Agriculture Organization of the United Nations (FAO), 2004. The State of the World Fisheries and Oceans, Rome Italy.

Filby, A.L., Tyler, C.R., 2005. Molecular characterization of estrogen receptors 1, 2a, 2b, and their tissue and ontogenetic expression profiles in fathead minnow (Pimephales promales). Biol. Reprod. 73, 648-662.

Flik, G., Klaren, P.H.M., Van den Burg, E.H., Metz, J.R., Huising, M.O., 2006. CRF and stress in fish. Gen. Comp. Endorinol. 146, 36-44.

Folmar, L.C., Denslow, N.D., Kroll, K., Orlando, E.F., Enblom, J., Marcino, J., Metcalfe, C., Guillette, L.J. Jr., 2001. Altered serum sex steroids and vitellogenin induction in walleye (Stizostedion vitreum) collected near a metropolitan sewage treatment plant. Environ. Contam. Toxicol. 40, 392-398.

Foo, T.W., Lam, T.J., 1993a. Retardation of ovarian growth and depression of serum steroid levels in the tilapia *Oreochromis mossambicus*, by cortisol implantation. Aquaculture 115, 133-143.

Foo, T.W., Lam, T.J., 1993b. Serum cortisol response to handling stress and the effect of cortisol implantation on testosterone levels in the tilapia *Oreochromis mossambicus*. Aquaculture 115, 145-158.

Francis, K.T., 1981. The relationship between high and low trait psychological stress, serum testosterone, and serum cortisol. Experientia 37, 1296-1297.

Gisbert, E., Rodriquez, A., Cardona, L., Huertas, M., Gallardo, M.A., Sarasquete, C., Sala-Rabanal, M., Ibarz, A., Sanchez, J., Castello-Orvay, F., 2004. Recovery of Siberian sturgeon yearlings after an acute exposure to environmental nitrite: changes in the plasmatic ionic balance, Na+ - K+ ATPase activity, and gill histology. Aquaculture 239, 141-154.

Gisbert, E., Williot, P., 2002. Advances in the larval rearing of Siberian sturgeon. J. Fish Biol. 60, 1071-1092.

Goetz, FW., Norberg, B., McCauley, L.A.R., Iliev, DB., 2004. Characterization of the cod (Gadus morhua) steroidogenic acute regulatory protein (StAR) shed light on StAR gene structure in fish. Comp. Biochem. Physiol. 137, 351-362.

Goto-Kazeto, R., Kight, K.E., Zohar, Y., Place, A.R., Trant, J.M., 2004. Localization and expression of aromatase mRNA in adult zebrafish. Gen. Comp. Endocrinol. 139, 72-84.

Gray, G.D., Smith, E.R., Damassa, D.A., Ehrenkranz, J.R.L., Davidson, J.M., 1978. Neuroendocrine mechanisms mediating the suppression of circulating testosterone levels associated with chronic stress in male rats. Neuroendocrinol. 25, 247-256.

Greenwood, A.K., Butler, P.C., White, R.B., DeMarco, U., Pearce, D., Fernald, R.D., 2003. Multiple corticosteroid receptors in a teleost fish: distinct sequences, expression patterns, and transcriptional activities. Endocrinology 144, 4226-4236.

Guillette, L.J., 1999. Serum concentrations of various environmental contaminants and their relationship to sex steroid concentrations and phallus size in Juvenile American alligators. Arch. Environ. Contam. Toxicol. 36, 447-455.

Guillette, L.J., 2000. Contaminant-induced endocrine disruption in wildlife. Growth Hormone & IGF Research, Supplement B, S45-S50.

Guillette, L.J., Brock, J.W., Rooney, A.A., Woodward, A.R., 1999. Serum concentrations of various environmental contaminants and their relationship to sex steroid concentrations and phallus size in juvenile American alligators. Arch. Environ. Contam. Toxicol. 36, 447-455.

Guillette, L.J., Crain, D.A., 2000. *Endocrine disrupting contaminants: An evolutionary perspective*. Francis and Taylor, Inc., Philadelphia.

Guillette, L.J., Crain, D.A., Gunderson, M.P., Kools, S.A.E., Milnes, M.R., Orlando, E.F., Rooney, A.A., Woodward, A.R., 2000. Alligators and endocrine disrupting contaminants: a current perspective. Amer. Zool. 40, 438-452.

Guillette, L.J., Crain, D.A., Pickford, D.B., 1995. Organization versus activiation: the role of endocrine-disrupting contaminants (EDCs) during embryonic development in wildlife. Environ. Health Perspect. 103, 157-164.

Guillette, L.J., Crain, D.A., Rooney, A.A., Woodward, A.R., 1997. Effect of acute stress on plasma concentrations of sex and stress hormones in juvenile alligators living in control and contaminated lakes. J. Herpetol. 31, 347-353.

Guillette, L.J., Edwards, T.M., 2005. Is nitrate an ecologically relevant endocrine disruptor in vertebrates? Integr. Comp. Biol. 45, 19-27.

Guillette, L.J., Gunderson, M.P., 2001. Alterations in development of reproductive and endocrine systems of wildlife populations exposed to endocrine-disrupting contaminants. Reproduction 122, 857-864.

Hall, J.M., Couse, J.F., Korach, K.S., 2001. The multifaceted mechanisms of estradiol and estrogen receptor signaling. J. Biol. Chem. 276, 36869-36872.

Hamlin, 2006. Nitrate toxicity in Siberian sturgeon (*Acipenser baeri*). Aquaculture 253, 688-693.

Harris, J., Bird, D.J., 2000. Modulation of the fish immune system by hormones. Vet. Immunol. Immunopathol. 77, 163-176.

Hartman, P.E., 1982. Nitrates and nitrites: ingestion, pharmacodynamics, and toxicology. In *Chemical Mutagens, Principles and Methods for Their Detection* (ed. F.J. de Serres, A. Holleander), pp. 211-294, New York.

Hellal-Levy, C., Fagart, J., Souque, A., Rafestin-Oblin, M.E., 2000. Mechanistic aspects of mineralcorticoid receptor activation. Kidney Int. 57, 1250-1255.

Heugens, E.H.W., Hendriks, A.J., Dekker, T., van Straalen, N.M., Admiral, W., 2001. A review of the effects of multiple stressors on aquatic organisms and analysis of uncertainty factors for use in risk assessment. Crit. Rev. Toxicol. 31, 247-284.

Hill, M.J., 1999. Nitrate toxicity: myth or reality? Br. J. Nut. 81, 343-344.

Hogasen, H.R., 1995. Changes in blood composition during sampling by caudal vein puncture or caudal transaction of the teleost *Salvelinus alpinus*. Comp. Biochem. Physiol. 111, 99-105.

Hontela, A., 2005. Stress and the hypothalamo-pituitary-interrenal axis: adrenal toxicology-effects of environmental pollutants on the structure and function of the HPI axis. *In Biochemical and Molecular Biology of Fishes: Vol. 6 Environmental Toxicology* (ed. T.W. Moon, T.P. Mommsen), pp. 331-363. Elsevier, Amsterdam.

Hrubec, T.C., 1996. Nitrate toxicity: A potential problem of recirculating systems. Aquacul. Eng. Soc. Proc. 1, 41-48.

Hsu, J.H., Hsu, N.C., Hu, M.C., Chung, B.C., 2006. Steroidogenesis in zebrafish and mouse models. Mol. Cell. Endocrinol. 248, 160-163.

Huertas, M., Gisbert, E., Rodriguez, A., Cardona, L., Williot, P., Castello-Orvay, F., 2002. Acute exposure of Siberian sturgeon (*Acipenser baeri*, Brandt) yearlings to nitrite: median-lethal concentration (LC_{50}) determination, haematological changes and nitrite accumulation in selected tissues. Aquat. Toxicol. 57, 257-266.

Hurvitz, A., Degani, G., Goldberg, D., Yom Din, Sl., Jackson, K., Levavi-Sivan, B., 2005. Cloning of FSHβ, LHβ, and glycoprotein α subunits from the Russian sturgeon (*Acipenser gueldenstaedti*), β-subunit mRNA expression, gonad development, and steroid levels in immature fish. Gen. Comp. Endocrinol. 140, 61-73.

Idler, D.R., Sangalang, G.B., 1970. Steroids of a chondrostean: in-vitro steroidogenesis in yellow bodies isolated from kidneys and along the posterior cardinal veins of the American Atlantic sturgeon, Acipenser oxyrhynchus Mitchill. J. Endocrinol. 48, 627-637.

Iguchi, T., Sato, T., 2000. Endocrine disruption and developmental abnormalities of female reproduction. Amer. Zool. 40, 402-411.

Inai, Y., Nagai, K., Ukena, K., Oishi, T., Tsutsui, K., 2003. Seasonal changes in neurosteroid concentrations in the amphibian brain and environmental factors regulating their changes. Brain Res. 959, 214-225.

Jardine, J.J., Van Der Kraak, G.J., Munkittrick, K.R., 1996. Capture and confinement stress in white sucker exposed to bleached kraft pulp mill effluent. Ecotoxicol. Environ. Safety 33, 287-298.

Jegou, B., Sots, A., Sundlof, S., Stephany, R., Meyer, H., Lefffers, H., 2001. Existing guidelines for the use of meat hormones and other food additives in Europe and USA. APMIS (Suppl.) 103, S551-S556.

Jensen, F.P., 1995. Uptake and effects of nitrite and nitrate in animals. In *Nitrate Metabolism and Excretion*, (ed. P.J. Walsh and P. Wright), pp. 289-303. CRC Press, Inc. Boca Raton, FL.

Jensen, F.B., 1996. Uptake, elimination and effects of nitrite and nitrate in freshwater crayfish. Aquat. Toxicol. 34, 95-104.

Jobling, S., Casey, D., Rodgers-Gray, T., Oehlmann, J., Schulte-Oehlamann, U., Pawlowski, S., Baunbeck, T., Turner, A.P., Tyler, C.R., 2003. Comparative responses of mulluscs and fish to environmental estrogens and an estrogenic effluent. Aquatic Toxicol. 65, 205-220.

Jobling, S., Beresford, N., Nolan, M., Rodgers-Gray, T.P., Brighty, G., Sumpter, J., Tyler, C.R., 2002. Altered sexual maturation and gamete production in wild roach (*Rutilus rutilus*) living in rivers that receive treated sewage effluents. Biol. Repro. 66, 272-281.

Jobling, S., Nolan, M., Tyler, C.R., Brighty, G., Sumpter, J.P., 1998. Widespread sexual disruption in wild fish. Environ. Sci. Technol. 32, 2498-2506.

Jobling, S., Reynolds, T., White, R., Parker, M.G., Sumpter, J.P., 1995. A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. Environ. Health Perspec. 103, 582-587.

Jobling, S., Sheahan, D., Osborne, J.A., Matthiessen, P., Sumpter, J.P., 1996. Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals. Environ. Toxicol. Chem. 15, 194-202.

Johansson, M., Nilsson, S., Lund, B.O., 1998. Interactions between methylsulfonyl PCBs and the glucocorticoid receptor. Environ. Health Perspect. 106, 769-772.

Katsu, Y., Bermudez, D.S., Braun, E.L., Helbing, C., Miyagawa, S., Gunderson, M.P., Kohno, S., Bryan, T.A., Guillette, L.J.Jr., Iguchi, T., 2004. Molecular cloning of the estrogen and progesterone receptors of the American alligator. Gen. Comp. Endocrinol. 136, 122-133.

Katz, B.G., 2004. Sources of nitrate contamination and age of water in large karstic springs of Florida. Environ. Geol. 46, 689-706.

Katz, B.G., Hornsby, D., Bohlke, J.F., Mokray, M.F., 1999. Sources and chronology of nitrate contamination in spring waters, Suwanee River basin, Florida. Water-Resources Investigations Report No. 99-4252. US Geological survey, Tallahassee, Florida.

Kelce, W.R., Stone, C.R., Laws, S.C., Gray, L.E., Kemppalnen, J.A., Wilson, E.M., 1995. Persistent DDT metabolite p,p'-DDE is a potent androgen receptor antagonist. Nature 375, 581-585.

Kentouri, M., Leon, L., Tort, L., Divanach, P., 1994. Experimental methodology in aquaculture: modification of the feeding rate of the gilthead sea bream *Sparus aurata* at a self-feeder after weighing. Aquaculture 119, 191-200.

Kim, J.W., Im, W.B, Choi, H.H., 1998. Seasonal fluctuations in pituitary gland and plasma levels of gonadotropic hormones in *Rana*. Gen. Comp. Endocrinol. 109, 13-23.

Kime, D.E., 1997. The steroids. In Fundamentals of Comparative Vertebrate Endocrinology, (ed. I. Chester-Jones, P. Ingleton and J.G. Phillips), pp. 3-56. Plenum Press, New York

Kime, D.E., 1999. A strategy for assessing the effects of xenobiotics on fish reproduction. Sci. Tot. Environ. 225, 3-11.

Knepp, G.L., Arkin, G.F., 1973. Ammonia toxicity levels and nitrate tolerance of channel catfish. Prog. Fish-Cult. 35, 221-224.

Knobil, E., Neill, J., 1994. *The physiology of Reproduction* (2nd ed.) Raven, New York.

Krulich, L., Hefco, E., Illner, P., Read, C.B., 1974. The effects of acute stress on the secretion of LH, FSH and GH in the normal male rat, with comments on their statistical evaluation. Neuroendocrinology 16, 293-311.

Kross, B.C., Hallberg, G.R., Bruner, D.R., Cherryholmes, K., Johnson, J.K., 1993. The nitrate contamination of private well water in Iowa. Am. J. Public Health 83, 270-272.

Lai, K.M., Scrimshaw, M.D., Lester, J.N., 2002. The effects of natural and synthetic steroid estrogens in relation to their environmental occurrence. Crit. Rev. Toxicol. 32, 113-132.

Lankford, S.E., Adams, T.E., Cech, Jr., 2003. Time of day and water temperature modify the physiological stress response in green sturgeon, *Acipenser medirostris*. Comp. Biochem. Physiol., A 135, 291-302.

Leatherland, J.F., 1999. Stress, cortisol and reproductive dysfunction in salmonids: Fact or fallacy? Bull. Europ. Assoc. Fish Pathol. 19, 254-257.

Lee, S.H., Pritchard, J.B., 1985. Bicarbonate-chloride exchange in gill plasma membranes of blue crab. Am. J. Physiol. 249, 544-550.

Lepore, D.A., 2000. Nitric oxide synthase-independent generation of nitric oxide in muscle ischemia-reperfusion injury. Nitric Oxide-Biol. Chem. 4, 541-545.

Li, D.P., Sanchez, E.R., 2005. Glucorticoid receptor and heat shock factor 1: Novel mechanism of reciprocal regulation. Vit. Hormones 71, 239.

Lloyd, R., 1979. The use of concentration response relationship in assessing acute fish toxicity data. International Workshop on the Application of Hazard Evaluation Programs for Chemicals in the Aquatic Environment, Waterville valley, N.H..

Macek, K.J., Birge, W., Mayer, F.L., Buikema, A.L., Jr., Maki, A.W., 1978. Discussion session synopsis of the use of aquatic toxicity tests for evaluation of the effects of toxic substances. In *Estimating the Hazard of Chemical Substances to Aquatic Life*. (ed. J. Cairns, K.L. Dickson and A.W. Maki) pp. 27-32. Philadelphia, PA.

Malik, A.N., Kafaji, G.A., 2007. Glucose regulation of β-defensin-1 mRNA in human renal cells. Biochem. Biophys. Res. Commun. 353, 318-323.

Marchetti, B., Serra, P.A., Tirolo, C., L'Episcopo, F., Caniglia, S., Gennuso, F., Testa, N., Miele, E., Desole, S., Barden, N., Morale, M.C., 2005. Glucocorticoid receptor-nitric oxide crosstalk and vulnerability to experimental parkinsonism: pivotal role for glia-neuron interations. Brain Res. Rev. 48, 302-321.

Marco, A., Blaustein, A.R., 1999. The effects of nitrite on behavior and metamorphosis in Cascades frogs (*Rana cascadae*). Environ. Toxicol. Chem. 18, 946-949.

Marco, A., Quilchano, C., Blaustein, A.R., 1999. Sensitivity to nitrate and nitrite in pond-breeding amphibians from the Pacific Northwest, USA. Environ. Toxicol. Chem. 18, 2836-2839.

Maxime, V., Nonnotte, G., Peyraud, C., Williot, P., Truchot, J.P., 1995. Circulatory and respiratory effects of an hypoxic stress in the Siberian sturgeon. Resp. Physiol. 100, 203-212.

Meyer, D., 1995. Enzymatic/non-enzymatic formation of nitric oxide. Nat. Med. 1, 1103

McKim, J.M., 1985. Early life stage toxicity tests. In *Fundamentals of Aquatic toxicology Methods and Applications* (ed. G.M. Rand and S.R. Petrocelli, S.R.), pp. 58-95. Hemisphere publishing.

McLachlan, J.A., 2001. Environmental Signaling: What embryos and evolution teach us about endocrine disrupting chemicals. Endocrine Rev. 22, 319-341.

McMaster, 2001. A review of the evidence for endocrine disruption in Canadian aquatic ecosystems. Water Qual. Res. J. Canada 36, 215-231.

Meade, J.W., 1985. Allowable ammonia for fish culture. Prog. Fish-Cult. 47, 135-145.

Meade, M.E., Watts, S.A., 1995. Toxicity of ammonia, nitrite, and nitrate to juvenile Australian crayfish, *Cherax quadricarinatus*. J. Shellfish Res. 14, 341-346.

Meyer, D., 1995. Enzymatic/non-enzymatic formation of nitric oxide. *Nature Medicine* 1, 1103.

Miller, W.L., 2002. Androgen biosynthesis from cholesterol to DHEA. Mol. Cell Endocrinol. 198, 7-14.

Mills, L.J., Chichester, C., 2005. Review of evidence: Are endocrine-disrupting chemicals in the aquatic environment impacting fish populations? Sci. Tot. Environ. 343, 1-34.

Milnes, M.R., 2005. Effects of environmental contaminants on development, sexual differentiation, and steroidogenesis in *Alligator mississippiensis*. PhD Dissertation, University of Florida, Gainesville, FL.

Milnes, M.R., Allen, D., Bryan, T., Sedacca, C.D., Guillette, L.J. Jr., 2004. Developmental effects of embryonic exposure to toxaphene in the American alligator (*Alligator mississippiensis*). Comp. Biochem. Physiol. C 138, 81-87.

Milnes, M.R., Bermudez, D.S., Bryan, T.A., Edwards, T.M., Gunderson, M.P., Larkin, I.L.V., Moore, B.C., Guillette, L.J., 2006. Contaminant-induced feminization and demasculinization of nonmammalian vertebrate males in aquatic environments. Environ. Res. 100, 3-17.

Mommsen, T.P., Vijayan, M.M., Moon, T.W., 1999. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Rev. Fish Biol. Fish. 9, 211-268.

Munkittrick, K.R., McMaster, M.E., Portt, C.B., Van Der Kraak, G.J., Smith, I.R., Dixon, D.G., 1992. Changes in maturity, plasma sex steroid levels, hepatic mixed function oxygenase activity, and presence of external lesions in lake whitefish (*Coregonus clupeaformis*) exposed to bleached kraft mill effluent. Can. J. Fish. Aquat. Sci. 49, 1560-1569.

Munkittrick, K.R., Van Der Kraak, G.J., McMaster, M.E., Portt, C.B., van den Heuval, M.R., Servos, M.R., 1994. Survey of receiving-water environmental impacts associated with discharges from pulp mills: 2. Gonad size, liver size, hepatic EROD activity and plasma sex steroid levels in white sucker. Environ. Toxicol. Chem. 13, 1089-1101.

Norris, D.O., 1997. *Vertebrate Endocrinology*. Academic Press, San Diego, CA.

Odermatt, A., Gumy, C., Atanasov, A.G., Dzyakanchuk, A.A., 2006. Disruption of glucocorticoid action by environmental chemicals: potential mechanisms and relevance. J. Ster. Biochem. Mol. Biol. 102, 222-231.

Orlando, E.F., Binczik, G.A., Kroll, K.J., Guillette, L.J., 2002. The contaminant-associated stress response and its relationship to plasma stress and sex steroid concentrations in the Florida gar, *Lepisosteus platyrhincus*. Env. Sci. 9, 329-342.

Orlando, E.F., Davis, W.P., Guillette, L.J.Jr., 2002. Aromatase activity in the ovary and brain of the eastern mosquitofish (*Gambusia holbrooki*) exposed to paper mill effluent. Environ. Health Perspect. 110, 429-433.

Orlando, E.F., Katsu, Y., Miyagawa, S., Iguchi, T., 2006. Cloning and defferential expression of estrogen receptor and aromatase genes in the self-fertilizing hermaphrodite and male mangrove rivulus, *Kryptolebias marmoratus*. J. Mol. Endocrinol. 37, 353-365.

Overli, O., Winberg, S., Pottinger, T.G., 2005. Behavioral and neuroendocrine correlates of selection for stress resposiveness in rainbow trout- a review. Integr. Comp. Biol. 45, 463-474.

Panesar, N.S., 2000. Is steroid deficiency the cause of tolerance in nitrate therapy? Med. Hypotheses 55, 310-313.

Panesar, N.S., Chan, K.W., 2000. Decreased steroid hormone synthesis from inorganic nitrite and nitrate: Studies *in vitro* and *in vivo*. Toxicol. Appl. Pharmacol. 169, 222-230.

Pankhurst, N.W., Dedual, M., 1994. Effects of capture and recovery on plasma levels of cortisol, lactate and gonadal steroids in a natural population of rainbow trout. J. Fish Biol. 45, 1013-1025.

Pankhurst, N.W., Van Der Kraak, G., 1997. Effects of stress on reproduction and growth of fish. In *Fish Stress and Health in Aquaculture* (ed. G.K. Iwama, A.D. Pickering, J.P. Sumpter and C.B. Schreck), pp. 73-95. Cambridge University Press, Campbridge.

Pankhurst, N.W., Van Der Kraak, G.J., Peter, R.E., 1995. Evidence that the inhibitory effects of stress on reproduction in teleost fish are not mediated by the action of cortisol on ovarian steroidogenesis. Gen. Comp. Endocrinol. 99, 249-257.

Parish, P.R., 1985. Acute toxicity tests. In *Fundamentals of Aquatic toxicology Methods and Applications* (ed. G.M. Rand and S.R. Petrocelli), pp. 31-56. Hemisphere publishing.

Parks, L.G., Lambright, C.S., Orlando, E.F., Guillette, L.J., Ankley, G.T., Gray, L.E., 2001. Masculinization of female mosquitofish in kraft mill effluent-contaminated fenholloway river water is associated with androgen receptor agonist activity. Toxicol. Sci. 62, 257-267.

Parrott, J.L., Wood, C.S., Boutot, P., Dunn, S., 2004. Changes in growth, secondary sex characteristics, and reproduction of fathead minnows exposed for a life cycle to bleached sulfite mill effluent. J. Toxicol. Environ. Health 67, 1755-1764.

Pickering, A.D., Pottinger, T.G., 1985. Factors influencing blood cortisol levels of brown trout under intensive culture conditions. In *Current Trends in Comparative Endocrinology, vol. 2*, (ed. B. Lofts and W.N. Holmes), pp. 1239-1242. Hong Kong Univ. Press, Hong Kong.

Pickering, A.D., Pottinger, T.G., Carragher, J., Sumpter, J.P., 1987. The effects of acute and chronic stress on the levels of reproductive hormones in the plasma of mature brown trout, *Salmo trutta* L.. Gen. Comp. Endocrinol. 68, 249-259.

Pierce, R.H., Weeks, J.M., Prappas, J.M., 1993. Nitrate toxicity to five species of marine fish. J. World. Aquacult. Soc. 24, 105-107.

Pottinger, T.G., Carrick, T.R., Hughes, S.E., Balm, P.H.M., 1996. Testosterone, 11-ketotestosterone, and estradiol-17β modify baseline and stress-induced interrinal and corticotrope activity in trout. Gen. Comp. Endocrinol. 104, 284-295.

Pottinger, T.G., Yeomans, W.E., Carrick, T.R., 1999. Plasma cortisol and 17β-oestradiol levels in roach exposed to acute and chronic stress. J. Fish Biol. 54, 525-532.

Pozzi, AG., Lantos, CP., Ceballos, NR., 2002. Effect of salt acclimatization on 3β-hydroxysteroid dehydrogenase/isomerase activity in the interrenal of *Bufo arenarum*. Gen. Comp. Endocrinol. 126, 68-74.

Prunet, P., Sturm, A., Milla, S., 2006. Multiple corticosteroid receptors in fish: From old ideas to new concepts. Gen. Comp. Endocrinol. 147, 17-23.

Pucket, L.J., 1995. Identifying the major sources of nutrient water pollution. Environ. Sci. Technol. 29, 408-414.

Querat, B., Sellouk, A., Salmon, C., 2000. Phylogenetic analysis of the vertebrate glycoprotein hormone family including new sequences of sturgeon (*Acipenser baeri*) beta subunits of the two gonadotropins and the thyroid-stimulating hormone. Biol. Reprod. 63, 222-228.

Rooney, A.A., Guillette, L.J.Jr., 2000. Contaminant interactions with steroid receptors: Evidence for receptor binding. In *Environmental Endocrine Disruptors: An Evolutionary Perspective* (ed. L.J. Guillette and A.D. Crain), pp. 82-125. Taylor and Francis, New York.

Rosenberger, D.R., Long, E., Bogardus, R., Farbenbloom, E., Hitch, R., Hitch, S., 1978. Considerations in conducting bioassays. Dredged Material Research Program, Technical report D-78-23, Charleston, Illinois.

Rottlant, J., Balm, P.H.M., Ruane, N.M., Perez-Sanchez, J., Wendelaar Bonga, S.E., Tort, L., 2000. Pituitary proopiomelanocortin-derived peptides and hypothalamus-pituitary-interrenal axis activity in gilthead sea bream (*Sparus aurata*) during prolonged crowding stress: differential regulation of adrenocorticotropin hormone and α-melanocyte-stimulating hormone release by corticotropin-releasing hormone. Gen. Comp. Endocrinol. 119, 152-163.

Rottlant, J., Tort, L., 1997. Cortisol and glucose responses after acute stress by net handling in the sparid red porgy previously subjected to crowding stress. J. Fish Biol. 51, 21-28.

Rouse, J.D., Bishop, C.A., Struger, J., 1999. Nitrogen pollution: an assessment of its threat to amphibian survival. Environ. Health Perspect. 107, 799-803.

Ruane, N.M., Carballo, E.C., Komen, J., 2002. Increased stocking density influences the acute physiological stress response of common carp *Cyprinus carpio* (L.). Aquacult. Res. 33, 777-784

Rubin, A.J., Elmaraghy, G.A., 1977. Studies on the toxicity of ammonia, nitrate and their mixtures to guppy fry. Water Res. 11, 927-935.

Russo, R.C., 1985. Ammonia, nitrite, and nitrate. In *Fundamentals of Aquatic toxicology Methods and Applications* (ed. G.M. Rand and S.R. Petrocelli), pp. 455-471. Hemisphere publishing.

Sabo-Attwood, T., Kroll, K.J., Denslow, N.D., 2004. Differential expression of largemouth bass (*Micropterus salmoides*) estrogen receptor isotypes alpha, beta, and gamma by estradiol. Mol. Cell. Endocrinol. 218, 107-118.

Saez, J.M., Morera, A.M., Haour, F., Evain, D., 1977. Effects of *in vivo* administration of dexamethasone, corticotrophin and human chorionic gonadotropin on steroidogenesis and protein and DNA synthesis of testicular interstitial cells in prepubertal rats. Endocrinol. 101, 1256-1263.

Sampat, P., 2000. *Deep Trouble: The Hidden Threat of Groundwater Pollution*. Worldwatch Institute, Washington, DC.

Sapolsky, R.M., 1985. Stress-induced suppression of testicular function in the wild baboon: role of glucocorticoids. Endocrinol. 116, 2273-2278.

Sapolsky, R.M., Romero, L.M., Munck, A.U., 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory and preparative actions. Endocrinol. Rev. 21, 55-89.

Schreck, C.B., Contreras-Sanchez, W., Fitzpatrick, M.S., 2001. Effects of stress on fish reproduction, gamete quality, and progeny. Aquaculture 197, 3-24.

Selye, H., 1936. A syndrome produced by diverse nocuous agents. Nature 138, 32.

Selye, H., 1956. *The stress of life*. McGraw-Hill, New York.

Semenkova, T., Barannikova, I., Kime, D.E., McAllister, B.G., Bayunova, L., Dyubin, V., 2002. Sex steroid profiles in female and male stellate sturgeon (*Acipenser stellatus* Pallas) during final maturation induced by hormonal treatment. J. Appl. Ichthyol. 18, 375-381.

Semenkova, T.B., Bayunova, L.V., Boev, A.A., Dyubin, V.P., 1999. Effects of stress on serum cortisol levels of sturgeon in aquaculture. J. Appl. Ichthyol. 15, 270-272.

Seo, J.S., Lee, Y.M., Jung, S.O., Kim, I.C., Yoon, Y.D., Lee J.S., 2006. Nonylphenol modulates expression of androgen receptor and estrogen receptor genes differently in gender types of the hermaphroditic fish Rivulus marmoratus. Biochem. Biophysic. Res. Comm. 346, 213-223.

Schreck, C.B., 1990. Physiological, behavioral and performance indicators of stress. Am. Fish. Soc. 8, 29-37.

Schreck, C.B., Lorz, H.H., 1978. Stress response of coho salmon, (*Onchorynchus kisutch*) elicited by cadmium and copper and potential use of cortisol as an indicator of stress. J. Fish. Res. Board Can. 35, 1124-1129.

Schulz, R.W., Miura, T., 2002. Spermatogenesis and its endocrine regulation. Fish Physiol. Biochem. 26, 43-56.

Schuytema, G.S., Nebeker, A.V., 1999. Comparative toxicity of ammonium and nitrate compounds to Pacific treefrog and African clawed frog tadpoles. Environ. Toxicol. Chem. 18, 2251-2257.

Scott, G., Crunkilton, R.L., 2000. Acute and chronic toxicity of nitrate to fathead minnows (*Pimephales promelas*), *Ceriodaphnia dubia*, and *Daphnia magna*. Environ. Toxicol. Chem. 19, 2918-2922.

Sharma, B., Ahlert, R.C., 1977. Nitrification and nitrogen removal. Water Res. 11, 897-925.

Shimura, R., Ijiri, K., Mizuno, R., Nagoaka, S., 2002. Aquatic animal research in space station and its issues – focus on support technology on nitrate toxicity. In *Space Life Sciences: Biological Research and Space Radiation,* Vol. 30, pp. 803-808. Pergamon-Elsevier Science, Lt. Oxford, England.

Sprague, J.B, 1985. Factors that modify toxicity. In *Fundamentals of Aquatic toxicology Methods and Applications* (ed. G.M. Rand and S.R. Petrocelli), pp. 124-163. Hemisphere publishing, Washington.

Stacey, N.E., MacKenzie, D.S., Marchant, T.A., Kyle, A.L., Peter, R.E., 1984. Endocrine changes during natural spawning in the white sucker *Catostomus commersoni*. I. Gonadotropin, growth hormone, and thyroid hormones. Gen. Comp. Endocrinol. 56, 333-348.

Stocco, D.M., 1999. Steroidogenic acute regulatory (StAR) protein: what's new? BioEssays 21, 768-775.

Stocco, DM., 2001. Tracking the role of StAR in the sky of the new millennium. Mol. Endocrinol. 15, 1245-1254.

Stocco, D.M, Wang X., Jo, Y., Manna, R.R., 2005. Multiple signaling pathways regularing steroidogenesis and steroidogenic acute regulatory protein expression: more complicated that we thought. Mol. Endocrinol. 19, 2647-269.

Stoker, C., Rey, F., Rodriguez, H., Ramos, J.G., Sirosky, P., Larriera, A., Luque, E.H., Munoz-de-toro, M., 2003. Sex reversal effects on Caiman latirostris exposed to exvironmentally relevant doses of the xenoestrogen bisphenol A. Gen. Comp. Endocrinol. 133, 287-296.

Stone, R., 2002. Caspian ecology teeters on the brink. Science 295, 393-572.

Sumpter, J.P., 1997. The endocrinology of stress. In *Fish Stress and Health in Aquaculture* (ed. G.K. Iwata, A.D. Pickering, J.P. Sumpter and C.B. Schreck), pp. 278 Cambridge University Press, New York.

Sumpter, J.P., 2005. Endocrine Disrupters in the aquatic environment: An overview. Acta Hydrochim. Hydrobiol. 33, 9-16.

Sumpter, J.P., Carragher, J.F., Pottinger, T.G., Pickering, A.D., 1987. Interaction of stress and reproduction in trout. In *Reproductive Physiology of Fish* (ed. D.R. Idler, L.W. Crim and J.M. Walsh), pp. 299-302. Memorial Univ. of Newfoundland, St. John's.

Sumpter, J.P., Dye, H.M., Benfey, T.J., 1986. The effects of stress on plasma ACTH, -MSH, and cortisol levels in salmonid fishes. Gen. Comp. Endocrinol. 62, 377-385.

Suzuki, Y., Maruyama, T., Numata, H., Sato, H., Asakawa, M., 2003. Performance of a closed recirculating system with foam separation, nitrification and denitrification untis for intensive culture of eel: towards zero emission. Aquacul. Eng. 29, 165-182.

Takase, M., Ukena, K., Yamazaki, T., Kominami, S., Tsutsui, K., 1999. Pregnenolone, pregnenolone sulfate, and cytochrome P450 side-chain cleavage enzyme in the amphibian brain and their seasonal changes. Endocrinology 140, 1936-1944.

Terova, G., Gornati, R., Rimoldi, S., Bernardini, G., Saroglia, M., 2005. Quantification of a glucocorticoid receptor in sea bass (*Dicentrarchus labrax*, L.) reared at high stocking density. Gene 357, 144-151.

Thibaut, R., Porte, C., 2002. Effects of endocrine disrupters on sex steroid synthesis and metabolism pathways in fish. J. Steroid Biochem. Mol. Biol. 92, 485-494.

Thomas, P., 1990. Molecular and biochemical responses of fish to stressors and their potential use in environmental monitoring. Amer. Fish. Soc. Symp. 8, 9-28.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nuc. Acids Res. 2s4, 4876-4882.

Tilak, K.S., Lakshmi, S.J., Susan, T.A., 2002. The toxicity of ammonia, nitrite and nitrate to the fish, *Catla catla* (Hamilton). J. Environ. Biol. 23, 147-149.

Timmons, M.B., Ebeling, J.M., Wheaton, F.W., Summerfelt, S.T., Vinci, B.J., 2001. *Recirculating Aquaculture Systems*. Cayuga Aqua Ventures, Ithaca NY.

Toft, G., Baatrup, E., Guillette, L.J., 2004. Altered social behavior and sexual characteristics in mosquitofish (*Gambusia holbrooki*) living downstream of a paper mill. Aquat. Toxicol. 70, 213-222.

Toft, G., Edwards, T.M., Baatrup, E., Guillette, L.J., 2003. Disturbed sexual characteristics in male mosquitofish (*Gambusia holbrooki*) from a lake contaminated with endocrine disruptors. Environ. Health Perspect. 111, 695-701.

Toft, G., Guillette, L.J., Jr., 2005. Decreased sperm count and sexual behavior in mosquitofish exposed to water from a pesticide-contaminated lake. Ecotox. Environ. Safe 50, 15-20.

Tomasso, J.R., Carmichael, G.J., 1986. Acute Toxicity of ammonia, nitrite, and nitrate to the Guadalupe bass, *Micropterus treculi*. Bull. Environ. Contam. Toxicol. 36, 866-870.

Tort, L., Sunyer, J.O., Gomez, E., Molinero, A., 1996. Crowding stress induces changes in serum haemolytic and agglutinating activity in the gilthead sea bream *Sparus aurata*. Vet. Immunol. Immunopathol. 51, 179-188.

Trama, F.B., 1954. The acute toxicity of some common salts of sodium, potassium and calcium to the common bluegill (*Lepomis machrochirus Raninesque*). Proceedings of the Academy of Natural Sciences of Philadelphia 106, 185-205.

Tsai, S.J., Chen, J.C., 2002. Acute toxicity of nitrate on *Penaeus monodon* juveniles at different salinity levels. Aquaculture 213, 163-170.

Tyler, C.R., Jobling, S., Sumpter, J.P., 1998. Endocrine disruption in wildlife: A critical review of the evidence. Crit. Rev. Toxicol. 28, 319-361.

Tyler, C.R., Routledge, E.J., 1998. Oestrogenic effects in fish in English rivers with evidence of their causation. Pure. Appl. Chem. 70, 1795-1804.

Tyler, C.R., van Aerle, R., Hutchinson, T.H., Maddix, S., Trip, H., 1999. An in vivo testing system for endocrine disruptors in fish early life stages using induction of vitellogenin. Environ. Toxicol. Chem. 18, 337-347.

Van Der Kraak, G., Chang, J.P., Janz, D.M., 1998. In *The Physiology of Fishes* (ed. D.H. Evans) pp. 465-488. CRC Press, Boca Raton, FL.

Van Rijn, J., Tal, Y., Schreier, H.J., 2006. Denitrification in recirculating systems; theory and applications. Aquacul. Eng. 34, 364-376.

Vanvoorhis, B.J., Dunn, M.S., Snyder, G.D., Weiner, C.P., 1994. Nitric-oxide – an autocrine regulator of human granulosa-luteal cell steroidogenesis. Endocrinology 135, 1799-1806.

Vasudevan, N., Ogawa, S., Pfaff, D., 2002. Estrogen and thyroid hormone receptor interactions: physiological flexibility by molecular specificity. Physiol. Rev. 82, 923-944.

Vijayan, M.M., Leatherland, J.F., 1990. High stocking density affects cortisol secretion and tissue distribution in brook char, (*Salvelinus fontinalis*). J. Endocrinol. 124, 311-318.

vom Saal, F.S., Nagel, S.C., Palanza, P., Boechler, M., Parmigiani, S., Welshons, W.V., 1995. Estrogenic pesticides: binding relative to estradiol in MCF-7 cells and effects of exposure during fetal life on subsequent territorial behaviour in male mice. Toxicol. Lett. 77, 343-350.

von Hofsten, J., Karlsson, J., Jones, I., Olsson, P., 2002. Expression and regulation of Fushi Tarasu factor-1 and steroidogenic genes during reproduction in Arctic Char (*Salvelinus alpinus*). Biol. Reprod. 67, 1297-1304.

Waldman, J.R., Wirgin, I.I., 1997. Status and restoration options for Atlantic sturgeon in North America. Conserv. Biol. 12, 631-638.

Walker, R., 1996. The metabolism of dietary nitrites and nitrates. Biochem. Soc. Trans. 24, 780-785.

Walsh, L.P., Stocco, D.M., 2000. Effects of lindane on steroidogenesis and steroidogenic acute regulatory protein expression. Biol. Repro. 63, 1024-1033.

Waring, C.P., Poxton, M.G., Stagg, M.R., 1997. The physiological response of the turbot to multiple net confinement. Aquacult. Internat. 5, 1-12.

Wedemeyer, G.A., McLeay, D.J., 1991. In *Methods for determining the tolerance of fishes to environmental stressors*. (ed. A.D. Pickering), pp. 247-275. Academic Press, New York.

Wendelaar Bonga, S.E., 1997. The stress response in fish. Physiol. Rev. 77, 591-625.

Westin, D.T., 1974. Nitrate and nitrite toxicity to salmonoid fishes. Prog. Fish-Cult. 36, 86-89.

Weitzberg, E., Lundberg, J.O.N., 1998. Non-enzymatic nitric oxide production in humans. Nitric Oxide-Biol. Chem. 2, 17.

White, P.C., New, M.I., Dupont, B., 1987. Congenital adrenal hyperplasia. N. Engl. J. Med. 316, 1519-1524.

White, R., Jobling, S., Hoare, S.A., Sumpter, J.P., Parker, M.G., 1994. Environmentally persistent alkylphenolic compounds are estrogenic. Endocrinology 135, 175-182.

Williot, P., Arlati, G., Chebanov, M., Gulyas, T., Kasimov, R., Kirschbaum, F., Patriche, N., Pavlovskaya, L.P., Poliakova, L., Pourkazemi, M., Kim, Y., Zhuang, P., Zholdasova, I., 2002. Status and management of Eurasian sturgeon: an overview. Internat. Rev. Hydrobiol. 87, 483-506.

Wilson, J.M., Vijayan, M.M., Kennedy, C.J., Iwama, G.K., Moon, T.W., 1998. β-Naphthoflavone abolishes interrenal sensitivity to ACTH stimulation in rainbow trout. J. Endocrinol. 157, 63-70.

Wilson, V.S., Lambright, C, Ostby, J., Gray, L.E., Jr., 2002. In vitro and in vivo effects of 17β-trenbolone: A feedlot effluent contaminant. Toxicol. Sci. 70, 202-211.

Yin, J.L., Shackel, N.S., Zekry, A., McGuinness, P.H., Richards, C., van der Putten, K., McCaughan, G.W., Eris, J.M., Bishop, G.A., 2001. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) for measurement of cytokine and growth factor mRNA expression with fluorogenic probes or SYBR Green I. Immunol. Cell Biol. 79, 213-221.

Young, E., Abelson, J., Lightman, S.L., 2004. Cortisol pulsatility and its role in stress regulation and health. Front. Neuroendocrinol. 25, 69-76.

Young, G., Thorarensen, H., Davie, P.S., 1996. 11-Ketotestosterone suppresses interregnal activity in rainbow trout (Oncorhynchus mykiss). Gen Comp. Endocrinol. 103, 301-307.

Zweier, J.L., Samouilov, A., Kuppusamy, P., 1999. Non-enzymatic nitric oxide synthesis in biological systems. Biochem. Biophy. Acta. 1411, 250-262.

Zraly, Z., Bendova, J., Svecova, D., Faldikova, L., Veznik, Z., Zajicova, A., 1997. Effects of oral intake of nitrates on reproductive functions of bulls. Vet. Med. Czech. 42, 345-354.

BIOGRAPHICAL SKETCH

Heather J. Hamlin was born on November 22, 1972 in Bangor, Maine. She spent much of her childhood by the ocean, pawing through clumps of seaweed in search of ocean life that was unfortunate enough to be stranded by the outgoing tide. Heather graduated from Hampden Academy High School in 1991, and then began an associate's degree in legal technology, followed by a bachelor's degree in biology from the University of Maine at Orono. In 1996 she was accepted to the graduate program in Marine Bio-Resources at the University of Maine, where she completed her master's degree examining the culture and histology of haddock's early development.

After graduating in 1998, she worked as a biologist with the National Oceanic and Atmospheric Administration for a year before being hired as a senior biologist with Mote Marine Laboratory in Sarasota, FL, where she has worked since 1999.