Health Science Campus

FINAL APPROVAL OF THESIS
Master of Science in Biomedical Sciences

Nitrate Toxicity to Common Carp Measured Noninvasively by Novel Enzyme-linked Immunosorbent Assay for Cortisol

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In partial fulfillment of the requirements for the degree of
Master of Science in Biomedical Sciences

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Date of Defense: April 10, 2008
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Spring, 2008
Introduction

Endocrine activity in a broad array of species, including fishes, can be significantly altered under stressful circumstances contributed by both natural and anthropogenic factors (Carballo, Jimenez et al. 2005). Fishes subjected to various stressors exhibit primary stress responses similar to mammals, including the release of cortisol from interrenal tissue (equivalent of the mammalian adrenal cortex) (Manire, Rasmussen et al. 2007). Under chronic stress conditions (e.g., environmental deterioration), long-term mild to moderate cortisol hypersecretion may occur (Perez-Casanova, Rise et al. 2008). Specific physiological consequences of chronic stress include inhibition of innate immunity (Prophete, Carlson et al. 2006), growth (Shankar, Sudarashan et al. 2007) and reproduction (Berg, Westerlund et al. 2004). Quantification of fecal glucocorticoid (GC) metabolites has become a useful method for the noninvasive assessment of adrenocortical activity in temperamental, dangerous or poorly accessible species (Stead, Meltzer et al. 2000; Wasser, Hunt et al. 2000; Turner, Nemeth et al. 2003).

While GCs, and especially cortisol, have frequently been determined in plasma as an indicator of stress, blood sampling is itself invasive and stressful. In addition, fecal samples amortize hormone levels across time, eliminating the issue of potentially misleading acute hormonal spikes associated with sampling plasma (Turner et al., 2003). GCs are metabolized and excreted with both intact hormones and their metabolites present in feces (Touma, Sachser et al. 2003), which are relatively stable, making measurement of free hormone a direct and reliable endpoint. The aim of the first part of this study was to determine whether an enzyme-linked immunosorbent assay (ELISA) for cortisol could be applied to
measurement of fecal cortisol in fishes. Cortisol monitoring of fishes in both aquaculture and natural settings is a potentially valuable tool. Performance of the cortisol ELISA was validated by comparison to high performance liquid chromatography (HPLC), which has been used for measuring free glucocorticoids and glucocorticoid metabolites in feces of numerous mammalian species (Wasser et al., 2000) and more recently in fishes (Turner et al., 2003). Tests for parallelism and extraction efficiency were used to compare the cortisol ELISA and HPLC in fecal casts of parrotfishes.

The second part of this study examines the effect of elevated aquatic nitrate (NO$_3^-$) on the stress hormone cortisol in butterfly koi (Cyprinus carpio). Municipal and industrial wastes are concentrated sources of nitrogen compounds that are, to a large extent, released directly into surface waters (Shimura, Ma et al. 2004). Even when treated, this waste represents a heavy water pollution load since secondary treatment removes less than half of the nitrogen (Jittawattanarat, Kostarelos et al. 2007). Ammonium ions (NH$_4^+$) in the effluent of septic tanks may be rapidly converted to NO$_3^-$, which may penetrate some distance from the tank. Entire fish populations inhabiting a given site and exposed to repetitive or chronic stressors, such as NO$_3^-$, may be compromised (Chew, Poothodiyil et al. 2006). The expulsion of untreated sewage into aquatic environments represents a significant source of pollutants (Salo, Hebert et al. 2007). NO$_3^-$ can also be released into the environment via sedimentation (commercial dumping) and agricultural runoff associated with developed coastlines (Yeom, Lee et al. 2007). In a separate arena, there is interest in fish stress associated with aquaculture because of the need to minimize stress of crowding and poor water quality in the production of commercially valuable fish in artificial environments (Cuesta-Santos, Collazo et al. 2001).
As in the first study of this thesis, a modified ELISA was also used to measure cortisol levels in fish feces as an index of stress. Together, these two studies have shown that fecal cortisol can be reliably measured with this modified ELISA and is useful as a tool to measure endocrine disruptions due to NO$_3^-$.

**Literature**

Fish are exposed to stressors in natural environments as well as to anthropogenic conditions occurring in aquaculture and in the laboratory. The increasing contamination of both freshwater and marine ecosystems throughout the world by anthropogenic substances is one class of environmental stressor. The response of fish to such stressors involves all levels of organization, from the cell (Mezhoud, Praseuth et al. 2008), to the individual organism (Viarengo, Lowe et al. 2007), to the structure of the population (Yeom, Lee et al. 2007). Stress is most often associated with a negative perspective, as the word and concept in common use is generally connected with a system that is relentlessly challenged, and regularly fatigued. The stress response is vitally important and normal in all living organisms, ranging from induction of certain genes and proteins to a behavioral response, and allows animals to avoid or cope with challenges to homeostasis.

Stress is defined as the reaction of the cell, or organism, to any demand placed on it such that it causes an extension of a physiological status beyond the ordinary resting state (Sanchez, Piccini et al. 2008). The animal’s response to stressors may also be generally categorized into those that are either adaptive, which allows homeostatic recovery, or maladaptive, which compromise health. The stress response in teleost fish demonstrates several parallel
characteristics to that of the terrestrial vertebrates (Wendelaar Bonga 1997). Specifically, they exhibit the primary functions of the hypothalamic-chromaffin cell axis (equivalent of the hypothalamic-adrenal-medulla axis) and the hypothalamus-pituitary-interrenal axis (equivalent of the hypothalamus-pituitary-adrenal axis), as well as other functions involving stimulation of oxygen uptake and transfer, recruitment of energy substrates, reallocation of energy away from growth and reproduction, and the suppression of immune functions. As shown in Figure 1, stress signals the hypothalamus to produce corticotrophin releasing factor (CRF), which signals the pituitary to produce and release adrenocorticotropic hormone (ACTH), which in turn signals the interrenal glands to produce cortisol and release it into the blood stream.

Differences, however, are present and mostly associated with the aquatic environment. For example, endocrine disrupting compounds (EDCs) amplify the permeability of the surface epithelia of the gills to water and ions thereby inducing systemic disturbances. In fish, cortisol merges glucocorticoid- and mineralocorticoid-controlled events, where the latter is vital for the maintenance of hydromineral homeostasis, along with hormones such as prolactin (in freshwater) and growth hormone (in seawater).
Toxic stressor influences in fish are well-known more so than in mammals, mainly due to fish being exposed to aquatic pollutants by extensive and delicate respiratory exchange surfaces of the gills. Exposure to chemicals may also directly compromise the stress response by interfering with specific neuroendocrine control mechanisms (Sorensen, Overli et al. 2007). Because hydromineral disturbance is intrinsic to stress in fish, external factors such as water pH, mineral composition, and ionic calcium levels have a substantial impact on stressor intensity (Wendelaar Bonga 1997). Although the species studied in this thesis comprise a small and nonrepresentative sample of the almost 20,000 known teleost species, there are many indications that the stress response is variable and flexible in fish, in line with the great diversity of adaptations that enable these animals to live in a large variety of aquatic habitats.

**Physiological stress response**

In reaction to a stressor such as handling, crowding or exposure to aquatic pollutants, a fish will undergo a series of biochemical and physiological changes in an attempt to compensate for the challenge imposed upon it and, thereby cope with the stress (de la Torre, Ferrari et al. 2005). The stress response in fish has been broadly categorized into the primary, secondary and tertiary responses.

The primary response represents the perception of an altered state and initiates a neuroendocrine response that forms part of the generalized stress response in fish (Perez-Casanova, Rise et al. 2008). This response includes the rapid release of stress hormones, catecholamines and cortisol, into the circulation (Fig. 2). Catecholamines are released from the chromaffin tissue situated in the head kidney of teleosts, and from the endings of
adrenergic nerves (Rocha, Rantin et al. 2007). Cortisol is released from the interrenal tissue, located in the head (anterior) kidney, in response to several pituitary hormones, but most potently to adrenocorticotrophic hormone (ACTH; Rotllant, Guerreiro et al. 2005). For example, the approximate resting and stressed levels in the plasma of salmonids are < 3 and 20 - 70 nmoles/L for adrenaline, and < 10 and 40 - 200 ng/ml for cortisol, respectively. These values should serve as broad guidelines given that individual conditions, including species differences, genetic characteristics, rearing, and local environment will amend the plasma values for control and stressed states (Overli, Korzan et al. 2004).

As shown in Figure 2, Iwama et al., (2004) found plasma epinephrine has a very short half-life, whereas plasma cortisol levels stay elevated for longer periods after a stressful event. Plasma glucose levels rise gradually after exposure to a stressor, but remain elevated for
longer periods of time. For these reasons, cortisol was chosen as a biomarker to monitor the stress response in experimental fish. Note that the above response profile is subjected to several factors, including type of stressor, intensity and duration of the stressor, species of fish, nutritional status of the animal, age and health of the fish.

The secondary response comprises the various biochemical and physiological effects associated with stress, and mediated largely by the above stress hormones. The stress hormones activate a number of metabolic pathways that result in modifications in blood chemistry and hematology (Walsh, Mayer et al. 2003). The measurement of plasma glucose concentration has been used as an indicator of stressed states and probably the most commonly measured secondary (metabolic) response to stressors in fish (Barton, Bollig et al. 2000). The plasma glucose concentration in circulation is dependent upon glucose production and its clearance from the circulation. The production of glucose in response to stress mobilizes energy substrates to tissues such as brain, gills and muscles, in order to cope with the increased energy demand (Suski, Cooke et al. 2007). Liver is the main source of glucose production by glycogenolysis and/or gluconeogenesis (Aluru and Vijayan 2007). Adrenaline and cortisol have been shown to increase glucose production in fish and play an important role in the stress associated increase in plasma glucose concentration (Vijayan, Reddy et al. 1994). Although adrenaline is cleared rapidly from circulation after a stress (< 30 min), plasma glucose remains elevated for longer periods of time suggesting that cortisol plays a role in the long-term maintenance of glucose levels post-stress in fish (Vijayan, Reddy et al. 1994).
The tertiary response represents whole animal and population level changes associated with stress. If the fish or population is unable to acclimate or adapt to the stressor, whole animal changes may occur, including decreased reproductive capability and sexual development of juveniles (Cooke, Hinch et al. 2006). This might be associated with stress-mediated energy-reallocation that diverts energy away from vital life processes, such as reproduction and growth, in order to cope with the enhanced energy demand associated with stress (Consten, Keuning et al. 2002).

**Cellular stress response**

The generalized stress response at the cellular level is distinguished by a family of proteins referred to as heat shock proteins (HSPs, (Deane, Zhou et al. 2006). The HSPs are highly conserved cellular proteins that have been observed in all organisms (Feder and Hofmann 1999), including fish (Korkea-Aho, Vehniainen, et al. 2008). Extensive studies on various species have revealed three major families of HSPs: HSP90 (85-90 kDa), HSP70 (68-73 kDa), and low molecular weight HSPs (16-24 kDa). In the unstressed cell, there is a constant production of these proteins, which are required in various features of cellular homeostasis. Hsp70 assist in the folding of nascent polypeptide chains, act as a molecular chaperone, and mediate the repair and degradation of altered or denatured proteins (Feder and Hofmann 1999). HSP90 is active in supporting various components of cell signaling, including the cytoskeleton, enzymes, and steroid hormone receptors (Du, Li et al. 2008). The low molecular weight HSPs have diverse species-specific functions and unlike other HSPs, these proteins have no known function and seem to only be induced during stress (Kothary and Candido 1982).
The possible functions of HSPs in various aspects of fish physiology, including development, stress physiology, immunology, environmental physiology, and acclimation and stress tolerance have been well documented (Basu, Todgham et al. 2002). There is little understanding of the mechanisms underlying the relationships between the cellular stress response and the physiology of the fish, an area worthy of attention and further research. In fish, the induction of various HSP families have been reported in cell lines, primary cultures of cells, as well as in various tissues from whole animals (Basu, Nakano et al. 2001). While the majority of these studies have focused on the various effects of heat shock, there is increasing interest in the physiological and protective role of HSPs following exposure of fish to various environmental stressors (Iwama, Afonso et al. 2004). For example, increased levels of various HSPs have been measured in tissues of fish exposed to bacterial pathogens (Masada, LaPatra et al. 2002) and environmental contaminants, such as heavy metals (Deane and Woo 2006), industrial effluents (Porter and Janz 2003), pesticides (Eder, Kohler et al. 2007)), and polycyclic aromatic hydrocarbons (Hakkinen, Vehniainen et al. 2004). While many indicators of fish stress, such as plasma cortisol concentration, are altered by handling and sampling procedures, Vijayan et al. (1997) showed that handling stress does not significantly modify levels of hepatic HSP70 in rainbow trout (*Oncorhynchus mykiss*).

**Chloride cells**

The mitochondria-rich chloride cell is believed to be the principal site of *trans*-epithelial Ca\(^{2+}\) and Cl\(^{-}\) influxes and was first identified to describe the cells responsible for Cl\(^{-}\) secretion in seawater-adapted teleosts. The underlying mechanisms of both Na\(^{+}\) and Cl\(^{-}\) excretion across
the gills of marine teleost fish are now well established (Randall, Ip et al. 2004). Chloride cells perform an integral role in acid-base regulation. During conditions of alkalosis, the surface area of exposed chloride cells is increased, which serves to enhance base equivalent excretion as the rate of Cl⁻/HCO₃⁻ exchange is increased. On the contrary, during acidosis, the chloride cell surface area is decreased by a competitive increase of the adjacent pavement cells. This response reduces the number of functional Cl⁻/ HCO₃⁻ exchangers. The chloride cell is one of four differentiated cell types comprising the gill epithelium (Maina 1990). The other cell types are the pavement cells (also referred to as respiratory cells), mucus cells, and neuro-epithelial cells. In most teleost species that have been examined under normal conditions, the chloride cell occupies only a small fraction (generally less than 15%) of the total surface area of all epithelial cells exposed to the environment. The gill is composed of two distinct epithelial surfaces, the lamellar and filament epithelia. The chloride cells are largely confined to the trailing edge of the filament epithelium and in particular are concentrated in the inter-lamellar regions and at the junctions between the filament and lamella (Fig 3). The apical membrane of the chloride cell has a
distinct appearance that allows relatively simple designation of these cells from the surrounding pavement and mucus cells. Unlike the diversity of its surface structure, the microstructure of the chloride cell is well conserved among the various species (Karnaky, Ernst et al. 1976). The features of chloride cell microstructure are its abundance of mitochondria, an extensive tubular system emanating from the basolateral membrane, an array of sub-apical vesicles, and a large ovoid nucleus. Because the tubular/basolateral membranes are thought to be the sites of the transport enzyme Na$^+$, K$^+$-ATPase (Moreno, Cristobal et al. 2006), the chloride cell is also characterized by an abundance of this particular enzyme.

**Chloride cell and ionic regulation**

There is much ambiguity surrounding the role of the chloride cell in ionic regulation in freshwater fishes. In the freshwater environment, fish continually lose ions across their permeable body surfaces of which the gill, due to its large surface area, is the most noteworthy. Ionic equilibrium is achieved, however, as a result of a relatively equivalent absorption of ions (NO$_3^-$, NO$_2^-$, Ca$^{2+}$, Cl$^-$, Na$^+$) from the water. Although it has been widely assumed that the chloride cell is responsible for the uptake of Ca$^{2+}$, Cl$^-$, and Na$^+$ from the water, this hypothesis is based largely on indirect evidence and likely is an oversimplification (Marshall and Bryson 1998) provided by histological evidence for goldfish (*Carassius auratus*) chloride cells involved with Ca$^{2+}$ uptake in the surrounding area of chloride cell apical openings.
The nature of Cl⁻ uptake across the fish gill has not been firmly established. However, there is extensive indirect evidence suggesting that absorption from the water occurs via an apical membrane Cl⁻/HCO₃⁻ exchange (Grosell 2006). It is generally assumed that the chloride cell is the site of Ca²⁺ uptake in freshwater fish although direct evidence is still lacking (Zhang and Wang 2007). The indirect evidence, however, is convincing. For example, correlation analysis has revealed that inter- and intraspecific variation in the rates of Cl⁻ uptake among and within species can be accounted for entirely by similar variation in the surface area of chloride cell apical membranes exposed to the water (Jensen and Brahm 1995). The proliferation of branchial chloride cells in fish exposed to ion-poor water appears to be a response that increases the transporting capacity of the gill for both Ca²⁺ and Cl⁻ (Hwang and Lee 2007). In situ hybridization demonstrated the presence of messenger RNA (mRNA) for the Cl⁻/HCO₃⁻ anion exchanger in the trout gill upon exposure to decreasing ionic concentrations (Katoh, Tresguerres et al. 2006). Importantly, chloride cells seem to have a greater affinity for NO₃⁻ and NO₂⁻ than for Cl⁻ illuminating the toxicity for many species these ions induce in aquatic environments (Doblander and Lackner 1996).

**Chloride cells and gas transfer**

In surroundings that defy ionic regulation, such as exposure of fish to toxicants in aquatic environments, chloride cells proliferate on both the filamental and lamellae surfaces of the gill (Nebel, Romestand et al. 2005). Although the proliferation of lamellar chloride cells during ionoregulatory challenges is alleged to amplify the capacity of the gill to transport Ca²⁺ and Cl⁻, the proliferating cells might at the same time impair gas transfer due to a thickening of the lamellar blood-to-water diffusion barrier (Greco, Fenwick et al. 1996).
Chronic treatment of fish with hormones (including cortisol and growth hormone) elicits lamellar chloride cell proliferation and elevates the ion transport capacity of the gill for Ca\(^{2+}\) and NaCl (Sloman, Desforges et al. 2001). Therefore, both cortisol and growth hormone may serve as osmoregulatory hormones in freshwater fish in addition to their well-known role in the adaptation of euryhaline fish to seawater (Foskett, Bern et al. 1983). Chloride cell proliferation elicited by cortisol and/or ovine growth hormone caused a two-fold increase in the thickness of the lamellar diffusion barrier, where lamellar thickening was positively correlated to the extent of the chloride cell proliferation (Bindon, Gilmour et al. 1994).

Perry (1998) provided the first experimental evidence that respiratory gas transfer is impaired by chloride cell proliferation. In that study, two groups of rainbow trout were tested; one was acclimated to relatively ion-rich water (approximately 1 mmol NaCl/L), whereas the other was acclimated to relatively ion-poor water. Fish in ion-poor water displayed a pronounced proliferation of lamellar chloride cells and impairment of branchial oxygen transfer as indicated by the lowering of arterial PO\(_2\) and these fish were less tolerant of hypoxia. Subsequent studies investigating the relationship between chloride cell proliferation and gas transfer have confirmed these findings (Gilmour, Fenwick et al. 1995; Greco, Fenwick et al. 1996; Perry 1998).

In summary, chloride cell proliferation, although beneficial to ionic regulation is a detriment to gas transfer owing to an increase in the thickness of the lamellar blood-to-water diffusion barrier and appears to increase the rate at which noxious substances such as NO\(_3^-\) can penetrate the circulatory system of fish. Furthermore, the fact that chronically stressful
Nitrogen cycle

Briefly, the nitrogen cycle in an aquatic environment can be described as shown in Fig 4: fish excrete NH₃ as waste from their gills, kidneys and normal respiration, where decaying plant and animal material also contributes to NH₃ levels. A species of bacteria (*Nitrosomas*) converts this NH₃ into NO₂⁻. A second bacteria species (*Nitrobacter*) converts this NO₂⁻ into NO₃⁻. Algae and aquatic plants utilize NO₃⁻ to produce chlorophyll which is in turn consumed by fish. The “cycle” then repeats.

Nitrogen is introduced into the aquatic environment in a variety of ways. Every living organism, from fish to algae, all have great quantities of assimilated nitrogen in their cells as nitrogen is a fundamental ingredient for the formation of proteins and nucleic acids. There are two major sources for inorganic nitrogen: the atmosphere and introduced water. Atmospheric nitrogen (N₂) is integrated into aquarium water through nitrogen fixation by bacteria and cyanobacteria as ammonia (NH₃). Inorganic nitrogenous
compounds from municipal water systems also enter the aquatic environment. Even after employing extensive filtering systems such as reverse osmosis and deionization, trace quantities of nitrogen are still imported and accumulate over time.

**Sources of nitrogen**

In general, surface waters do not usually contain $\text{NO}_3^-$ in concentrations higher than 10 mg/L, and $\text{NO}_2^-$ concentrations rarely exceed 1 mg/L (Moore, Schindler et al. 2007). However, a steady increasing trend of $\text{NO}_3^-$ levels has been reported in recent years in some countries, both in surface and ground waters. For example, in the River Thames, England, $\text{NO}_3^-$ concentrations increased from an average of 4 mg/L in 1968 to an average of 9 mg/L for the last quarter of 1973 (Casey et al., 1979) to an average of 25 to 35 mg NO$_3^-$/L in 2006 (Neal, Jarvie et al. 2006). $\text{NO}_3^-$ concentrations are also increasing in many rivers that drain agricultural sections of the U.S. Midwest. For example, $\text{NO}_3^-$ concentrations ranged between <0.5 to 14 mg L$^{-1}$ at Walnut Creek, IA and 2.1 to 15 mg L$^{-1}$ at Squaw Creek, IA (Schilling and Spooner 2006). $\text{NO}_3^-$ concentrations decreased 1.2 mg L$^{-1}$ over 10 yr in the Walnut Creek watershed but increased 1.9 mg L$^{-1}$ over 10 yr in Squaw Creek, , and in certain small rivers the 45 mg/L limit set by the EPA is sometimes exceeded (Wang, Fox et al. 1999). A small increase in the NO$_3^-$ concentration of the Tamagawa River, Tokyo, Japan has also been reported, where from 1951-1965, the NO$_3^-$ concentration rose from 7.9 mg/L to 9.1 mg/L and to 14.1 mg/L in 2000 (Yukimi et al., 2000). Nitrogenous compounds can be formed depending upon the organisms present, the pH, and the available nutrients (trace elements and carbohydrates), and may be absorbed through the gill tissue of fish. Although the
outstanding feature of NO$_3^-$ toxicity is the development of methemoglobinaemia (metHb), NO$_3^-$ may also cause vasodilation which aggravates the effects of the metHb.

The NO$_2^-$ ion formed by reduction of NO$_3^-$ oxidizes the iron in the hemoglobin molecule from the ferrous to the ferric state. The resultant metHb is incapable of reversibly binding oxygen. Clinical signs of NO$_3^-$ toxicity appear when metHb values exceed about 20% (Jensen, 2003). Oxidation of hemoglobin to metHb by the NO$_2^-$ ion occurs at different rates for each animal species, but there is little difference between individuals of the same species (Huertas, Gisbert et al. 2002). Similarly, the reduction of metHb in erythrocytes mainly by the enzyme system, NADH-- metHb reductase, is characteristically different for each animal species. These physiological processes appear to be related, even though there is a large variation in the rate of formation of metHb and its subsequent reduction. This may explain, in part, the difference in species susceptibility and the variation in signs seen in NO$_3^-$ poisoning.

**Nitrogenous fertilizer**

The fact that plants cannot use soil nitrogen completely is of great significance as nitrogen utilization may vary from 25 to 85% depending on the crop and on agricultural techniques. Thus, to obtain maximum production, a great excess of nitrogen fertilizer must be applied to the soil and the resulting nitrogen runoff will be substantially increased. For example, as much as 55-60% of the nitrogen input in the Sangamon River feeding Lake Decatur, IL, was of fertilizer origin (Kohl et al., 1971). Nitrogen runoff is 3-10 times higher from fertilized areas than from unfertilized areas in the same region (Lee et al., 1970; Sawyer et al., 1947; and Sylvester et al., 1961). However, analysis of stream waters did not show a clear
relationship between the $\text{NO}_3^-$ concentrations in British rivers and the amounts of fertilizers used on adjacent land (Tomlinson, 1970).

**Municipal and industrial waste**

Raw, untreated sewage contains suspended solids, including metals, microorganisms, and a multitude of chemicals; in particular, a wide variety of anthropogenic organic pollutants, such as pesticides, pharmaceutical residues and polyaromatic hydrocarbons (PAHs), arising from the incomplete combustion of fossil fuels and accidental release of petroleum products of which many are noxious and stable in aquatic environments and have been documented to cause sex reversal in male fish as shown in Figure 5 by the induction of female proteins in the gonadal tissues (Rodgers-Gray, Jobling et al. 2001). The immune responses of fish are susceptible to environmental pollution and these have been useful as an indicator of aquatic environmental health (de la Torre, Salibian et al. 2007; Iwata, Kim et al. 2007). In vertebrates, including fish, the endocrine and immune systems are
interrelated and changes in hormone secretion in species can be influenced by changes in the functioning of the immune system. The presence of EDCs in the form of estrogens or estrogen-mimicking compounds in sewage effluents has been well documented. For example, exposure to sewage effluents have been correlated to shifts in hormone levels (Langer, Kocan et al. 2007; Maradonna and Carnevali 2007), delays in or inhibition of sexual maturation (Rogers-Gray et al., 2001), and induction of the yolk precursor protein, vitellogenin, commonly found only in the females of the species, leading to the demasculazition of male fish (Jackson, Hurvitz et al. 2006; Scott, Katsiadaki et al. 2006; Shang, Yu et al. 2006). These modifications also have indirect influences on the immune system of fish. A series of experiments investigated whether environmental estrogens in sewage effluent can influence the reproductive capabilities of adult male fathead minnows (*Pimephales promelas*) by interfering with their innate reproductive behaviors, including their capability to compete for mates and nesting sites (Martinovic, Hogarth et al. 2007). Fish were subjected to a control (non-treated group), effluent released by a sewage treatment plant, waterborne estradiol (E2), or a synthetic androgen for three weeks. Subsequently, the fish were housed with females and a nest, and their mating and nesting activities were observed for 5 d in either the presence or the absence of a competing control male, which was not exposed to any chemical treatment. The investigators found that males exposed to either stressor had increased amounts of circulating vitellogenin (p < 0.05) and showed decreased concentrations of 11-ketotestosterone (p < 0.05). A majority of contaminant-exposed males spawned effectively when a competing male was not present; however, contaminant-exposed males experienced virtually total reproductive failure when they were required to compete for mates and nesting sites.
Discharges of municipal and industrial wastes are concentrated sources of nitrogen compounds that are, to a large extent, released directly into surface waters. The amount of nitrogen in human wastes is estimated to be about 5 kg per person per year (Malisie, Prihandrijanti et al. 2007). Even if treated, this waste will represent a heavy water pollution load since secondary treatment removes less than half of the nitrogen (Kim, Park et al. 2004). Ammonium ions in the effluent of septic tanks may be rapidly converted to NO$_3^-$ which can enter underground water sources some distance from the tank. Sludge from treatment plants and septic tanks have also to be disposed of and represent another significant source of nitrogen pollution. The nitrogen content of industrial wastes is highly variable, where fuel and food processing industries and petroleum refineries may constitute important sources of nitrogen pollution. A considerable proportion of this fixed nitrogen is eventually returned to the earth's surface as NO$_3^-$.

Biological nitrogen fixation, i.e. its reduction to NH$_3$, can be accomplished by only a limited number of organisms. Symbiotic nitrogen fixation takes place in the root nodules of legumes such as soya bean, clover, and alfalfa, which contain bacteria of the *Rhizobium* species. There are also symbiotic processes with plants other than legumes involving, for example, some cyanobacteria, catalyzed by a complex enzyme, nitrogenase. Plants can assimilate only a part of the NO$_3^-$ present in soils; some leaches into ground water and rivers and may reach estuaries and oceans, the rest is subjected to denitrification, another natural biochemical process that degrades NO$_3^-$ to nitrogen or nitrous oxide (dinitrogen oxide) which are released into the atmosphere. Denitrification takes place in the soil and also at the interface between water and sediment in oceans, rivers, lagoons, and lakes. NO$_3^-$ from natural fixation and artificial fertilizers are ultimately used for the synthesis of biological molecules, particularly proteins. Plants and animal waste and dead
tissues return fixed nitrogen to the soils, where part of it is recycled and part returned to the atmosphere, thus completing the nitrogen cycle. Nitrogen fixation on a world basis may exceed denitrification by about 10% (Koop, Booth et al. 2001; Kim, Kim et al. 2008).

**Animal waste.**

Another major source of NO$_3^-$ is farm animal wastes which contain large amounts of nitrogenous materials that may be converted into NO$_3^-$. The problem is more pronounced where farming is intensive, a common practice in the U.S. for both livestock and poultry. For example, a 450 kg steer excretes about 43 kg of nitrogen per year, and therefore a 3200 head feedlot would produce 1400 tons annually on a relatively small area, an amount equivalent to the waste generated by about 260,000 people. Such feedlots become insurmountable sources of nitrogen runoff. Only 10% of these wastes are returned to cultivated land (Barles 2007). Animal husbandry, even when carried out on pastures or with the return of the animal wastes to cultivated land, may still impose problems. Adriano et al. (1971) found that wastes from a maximum of 7-8 cows could efficiently be used per hectare of farmland or pasture and that higher application rates might raise NO$_3^-$ levels above 10 mg/L in underground waters.

**Nitrogen released via decomposition**

As mentioned, living organisms possess a large mass of assimilated nitrogen. This localized organic concentration is “locked up” in the tissue and cells until the organism dies, at which time the nitrogen is released back into the environment by aerobic decomposition. This
process is known as eutrophication. When large organisms die, decomposition demands large amounts of oxygen that may be very taxing of the system. This is the very reason why oxygen redox potential levels drop significantly when decaying matter is present.

Because food inputs are nothing more then introduced organic masses, they go through a similar process of releasing nitrogenous byproducts into the environment. Foods that are consumed will pass through the organism and will be processed to assimilate the nutrients the animal needs, and the rest as is discarded as excrement. Although the organism will incorporate a considerable quantity of nitrogen, their excrement is still rich in nitrogenous compounds, which are quickly released into the water through decomposition. Foods that go unconsumed will eventually die (if not already dead) and decay, contributing previously assimilated nitrogen back into the environment via decomposition.

The decomposition process produces large quantities of NH$_3$ through the process of ammonification (Fig 6). Heterotrophic microbes utilize the organic compounds of decomposing matter as their carbon source. NH$_3$ is the byproduct of this consumption. Ammonia, in its neutral state, exists as ammonium ions (NH$_4^+$), which has several divergent pathways from that point forth. Plants and algae can assimilate NH$_3$ and NH$_4^+$ directly for the biosynthesis and the remaining bulk of decomposed byproducts are utilized by bacteria in a process called nitrification.
Nitrification is the oxidation (affixation of oxygen) of \( \text{NH}_4^+ \) by chemolithotrophic bacteria species. During this process, specific species of nitrifying bacteria strip the \( \text{NH}_4^+ \) of its hydrogen molecules as an energy source. Oxygen molecules are then affixed to the stripped nitrogen, forming the oxide \( \text{NO}_2^- \). Another group of bacteria utilize the enzyme \( \text{NO}_2^- \text{ oxidase} \) that is then responsible for converting \( \text{NO}_2^- \) into \( \text{NO}_3^- \).

The biochemical progression of nitrification is:

\[
\text{NH}_3(aq) + \text{H}_2\text{O}(l) \rightarrow \text{NH}_4^+ (aq) + \text{OH}^- (aq)
\]

\[
\text{NH}_4^+ + \text{O}_2 + 2e^- + 2\text{H}^+ \rightarrow \text{NH}_2\text{OH} \ + \text{H}_2\text{O}
\]

\[
\text{NH}_2\text{OH} + \text{H}_2\text{O} + \frac{1}{2} \text{O}_2 \rightarrow \text{NO}_2^- + 2\text{H}_2\text{O} + \text{H}^+
\]

\[
\text{NO}_2^- + \frac{1}{2} \text{O}_2 \rightarrow \text{NO}_3^-
\]

Briefly, the processes described thus far progresses in the following manner:
As the term suggests, the nitrogen cycle is an unbroken sequence of pathways for nitrogen. It is never "concluded", as there is no end to the nitrogen cycle. Instead, nitrogen is constantly recycled from one form to another.

Assimilative reduction is the process whereby NO₃⁻ is reduced to organic nitrogen for the construction of organic matter. Assimilation may occur in either the presence or absence of oxygen, with only enough NO₃⁻ reduced to fulfill the organism's requirements. Photosynthetic plants, algae, and zooxanthellae (the symbiotic dinoflagellates found in photosynthetic corals, anemones, and some sponges) assimilate NH₃, NO₂⁻, and/or NO₃⁻ for biosynthesis of proteins, amino acids, and nucleic acids. Of these, NO₃⁻ is the most utilized compound because it is very low in toxicity, and is readily acceptable. When utilizing NO₃⁻, these organisms perform the following reduction:

\[
\text{NO}_3^- \rightarrow \text{NO}_2 \rightarrow \text{NH}_2\text{OH} \rightarrow \text{NH}_3 \rightarrow \text{R-NH}_2 \text{ (organic N)}
\]

NO₂⁻ is the least utilized compound for biosynthesis since it is the most toxic. In the presence of acid, NO₂⁻ forms nitric acid, a known and dangerous mutagen. In advanced organisms such as fish, NO₂⁻ will bind with red blood cells and hinder their capability of transporting O₂, asphyxiating the organism.

Whereas the assimilative pathway is generally aerobic and only uses enough nitrogenous compounds to meet an organism's requirements (i.e. no excess is produced), the dissimilative pathway is generally anaerobic and produces copious amounts of excess byproducts. Denitrification is the key dissimilative pathway for NO₃⁻ reduction.
Denitrification is the terminology used to describe the conversion of nitrogen oxides (NO$_2^-$ and NO$_3^-$) back into gaseous nitrogen (N$_2$, N$_2$O, or NO). Denitrification results in nitrogen being lost from the local environment (e.g. water) to the atmosphere. This process, as most processes are in the nitrogen cycle, is accomplished primarily by bacteria species. However, unlike nitrification, denitrification is an anaerobic process, meaning it occurs in the absence of oxygen. Denitrifying bacteria metabolize nitrogenous compounds (with the assistance of the molybdenum-containing enzyme, NO$_3^-$ reductase) in the reverse way that nitrifying bacteria does: they turn oxides back into nitrogen gas or nitrous oxides for energy generation. These gases then volatize and return back into the atmosphere.

Because the enzyme NO$_3^-$ reductase is synthesized only when O$_2$ is repressed, anoxic conditions are required for most denitrifying bacteria. This is why the denitrification process predominantly occurs in deeper substrates and in areas of stagnant flow where oxygen levels are depressed, and in deep sand beds. As water slowly diffuses deeper, aerobic organisms strip all available oxygen for respiration. In the deep, oxygen-deprived layers, denitrifying anaerobes are given the opportunity to convert nitrogen compounds into nitrogenous gases.

The net loss of nitrogen to the atmosphere will be regained via introduced foods and water.

Bacteria are necessary and ubiquitous, found everywhere even in the air. If NH$_3$ present in water, the bacteria that utilize NH$_3$ (i.e. *Nitrosomonas*) establish colonies in the substrate of the biological filter, provided there is an adequate supply of oxygen and surface area for the bacteria to adhere to.
With a spike of NH₃, *Nitrosomonas* bacteria have "food" and will convert it to NO₂⁻. This leads to a spike in NO₂⁻ levels and a second set of bacteria, *Nitrobacter*, are able to form colonies in the biological filter and the substrate where the conversion of NO₂⁻ to NO₃⁻ takes place.

NO₂⁻ is an oxidized form of nitrogen and is formed when NH₃ is oxidized by nitrifying bacteria (*Nitrosomonas*). NO₂⁻ is toxic to fish, only slightly less than NH₃, and leads to a condition known as "brown blood" due to the brown color of the gills of the affected fish (Bernet, Schmidt et al. 2001). NO₂⁻ has the ability of entering the fish’s blood stream and it oxidizes the hemoglobin into metHb, which is not as efficient at transporting oxygen. Therefore fish react as if there is not enough oxygen in the water and they may die within hours or days.
Manuscripts

“Validation of an ELISA for measurement of fecal cortisol in fishes” was submitted to *Aquaculture Research* on Sep 17, 2007 and accepted on Mar 24, 2008.

“Noninvasive assessment of nitrate-induced stress in *Cyprinus carpio* by fecal cortisol measurement” was submitted to *General and Comparative Endocrinology* on Mar 13, 2008 and is still pending a response from the editors.
Validation of an ELISA for measurement of fecal cortisol in fishes.

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Abstract

Quantification of glucocorticoid (GC) metabolites in feces has become an established method for the noninvasive assessment of adrenocortical activity. These hormones are frequently determined in plasma samples as parameters of adrenal activity and response to stress. GCs are metabolized and excreted with both intact hormone and their metabolites present in feces. Therefore, the concentration of GCs (or their metabolites) can be measured in excreta. Fecal samples also present the advantages of easy collection, no stress to the animal and elimination of the issue of potentially misleading acute GC spikes. The aim of this study was to determine if an enzyme-linked immunosorbent assay (ELISA) for cortisol was appropriate for monitoring adrenocortical activity in fecal casts of fishes. Performance of the cortisol ELISA was validated by comparison to high performance liquid chromatography (HPLC), which is an established method for measuring free glucocorticoids and glucocorticoid metabolites in feces. Parallelism and sample extraction efficiency were compared for the two methods. Pearson correlation across samples for these methods was 0.996. Results demonstrated that the ELISA was an efficient, sensitive and reliable method for cortisol measurement in fecal extracts, which should permit integration of noninvasive stress monitoring into studies of fish behavior and physiology.

Key Words: cortisol; stress; ELISA; HPLC
1. Introduction

Endocrine activity in an extensive range of species, including fishes, can be significantly altered under stressful circumstances contributed by both natural and anthropogenic factors (Carballo et al., 2005). Fishes subjected to various stressors exhibit primary stress responses similar to mammals, including the release of cortisol from interrenal tissue (equivalent of the mammalian adrenal cortex) (Axelrod and Reisine, 1984). Under chronic stress conditions (e.g., environmental deterioration), long-term mild to moderate cortisol hypersecretion may occur (van den Heuvel et al., 2005). Specific physiological consequences of chronic stress include inhibition of innate immunity (Watanuki et al., 2002), growth (Jentoft et al., 2005) and reproduction (Berg et al., 2004). Quantification of fecal glucocorticoid (GC) metabolites has become a useful method for the noninvasive assessment of adrenocortical activity in temperamental, dangerous or poorly accessible species (Turner et al., 2003, Stead, 2001, Wasser, 2000). While GCs, and especially cortisol, have frequently been determined in plasma as an indicator of stress, blood sampling is itself invasive and stressful. In addition, fecal samples amortize hormone levels across time, eliminating the issue of potentially misleading acute hormonal spikes associated with sampling plasma (Turner et al., 2003). GCs are metabolized and excreted with both intact hormones and their metabolites present in feces (Touma et al., 2003). The main metabolites of glucocorticoids are 17-β-hydroxycorticoids, with a smaller amount (5-10%) metabolized to 17-ketosteroids, and approximately 10% is excreted as free hormone (Chelini et al., 2006). These proportions are relatively stable, making measurement of free hormone a direct and reliable endpoint. The aim of this study was to determine whether an enzyme-linked immunosorbent assay (ELISA)
for cortisol could be applied to measurement of fecal cortisol in fishes. Cortisol monitoring of fishes in both aquaculture and natural settings is a potentially valuable tool. Performance of the cortisol ELISA was validated by comparison to high performance liquid chromatography (HPLC), which has been used for measuring free glucocorticoids and glucocorticoid metabolites in feces of numerous mammalian species (Wasser et al., 2000) and more recently in fishes (Turner et al., 2003). Tests for parallelism and extraction efficiency were used to compare the cortisol ELISA and HPLC in fecal casts of parrotfishes.

2. Materials and Methods

2.1 Animal model

The fish fecal samples used in this study were obtained from adult male and female stoplight and queen parrotfishes (*Sparisoma viride* and *Scarus vetula*, respectively) located within reefs of Great Lamesur Bay, Saint John, US Virgin Islands. These samples were part of a separate study involving fecal collections across several years from fishes inhabiting this bay (Turner et al., 2003). Samples were collected via SCUBA and snorkeling at the same time of day to minimize possible influences of diurnal cortisol concentrations. Since sufficient material was not obtainable from a single fecal cast for the HPLC portion of the study (Turner et al., 2003), each cortisol value was obtained from a pool of samples from 6 different individuals.
2.2 Extraction and Reconstitution

2.2.1 Extraction

Desiccated fecal casts for the ELISA (0.2 g) or HPLC (0.5 g) were mixed in 12 mL spectrophotometeric-grade dichloromethane (MeCl₂, Curtin Matheson Scientific, Houston, TX) and shaken for 60 minutes on a motorized shaker (Burrel, Pittsburg, PA) at 25° C and then centrifuged at 3000 rpm for 10 minutes. Ten mL of the liquid layer was combined with 1 mL 1.0 M sodium hydroxide in a glass scintillation vial, shaken, allowed to separate and frozen on dry ice. Eight mL of the MeCl₂ layer was withdrawn, aliquoted (4 mL each) into two 12x75 mm borosilicate glass tubes, evaporated to dryness and stored at -40° C until reconstituted. Dry samples were reconstituted in ethanol and enzyme conjugate (for ELISA) or acetonitrile and distilled water (AcN, for HPLC) for assay (fig. 1). Ethanol was used in the ELISA due to the denaturing effect of AcN on proteins and its propensity to dissolve the plastic microtitre plates.

2.2.2 Reconstitution for ELISA

Extracted cortisol samples (from 0.2 g dessicated fecal material) were mixed with 50 μL ethanol (EtOH) and vortexed for 2 min. Fifty μL increments of aqueous enzyme conjugate were added and vortexed for an additional 2 min. each to give a final volume of 500 μL (see section 2.3). Fifty μL of each sample (in duplicate) was added to the ELISA plate.
2.2.3. Reconstitution for HPLC

Extracted cortisol samples (from 0.5 g dessicated fecal material) were mixed with 100 µL acetonitrile (ACN) and vortexed for 2 min. One hundred µL increments of dH₂O were added and vortexed for an additional 2 min. each to give a final volume of 1000 µL (see section 2.4). A 250 µL aliquot of each sample was loaded onto the chromatography column.

2.3 Cortisol ELISA

A sandwich-type ELISA, modified from methods described by Munro and Lasley (1988), was adapted for determination of fish fecal cortisol. The assay employed a cortisol-horseradish peroxidase ligand and antiserum (Antibody R4866; C.J. Munro, University of California at Davis) and commercial cortisol standards (hydrocortisone reference standard; Sigma-Aldrich Inc., St. Louis, MO). The ELISA was completed in 96-well microtiter plates (Nunc-Immuno, Maxisorp Surface; Fisher Scientific, Pittsburgh, PA) layered 24 hrs beforehand with cortisol 100 antiserum (50 µl/well; diluted 1:8500 in bicarbonate coating buffer; 0.05M Na₂CO₃/NaHCO₃, pH 9.6). Plates were sealed tightly with waterproof plate sealer covers and incubated overnight at 4° C. On the day of the assay, the enzyme conjugate (cortisol-3-CMO:HRP) was diluted to 1:20,000 in phosphate buffer (0.1 M PBS containing 0.1% BSA, pH 7.0). Non-bound antibody was removed from the wells of the microtiter plates with wash solution (0.15 M NaCl containing 0.05% v:v Tween 20), blotted and allowed to dry at room temperature. The polyclonal antiserum was raised in rabbits against cortisol-3-carboxymethyloxime, linked to bovine serum albumin and cross-reacts with
cortisol (100%), prednisolone (9.9%), prednisone (6.3%), cortisone (5%) and <1% with androstenedione, androsterone, corticosterone, desoxycorticosterone, 11-desoxycortisol, 21-desoxycortisone, and testosterone (Munro and Lasley, 1988). Fifty µL of phosphate buffer was pipetted across the entire plate followed by 50 µL of cortisol-3-CMO:HRP conjugate solution containing cortisol standards or a reconstituted extracted fecal sample. Stock cortisol standards stored in ethanol were dried under air and reconstituted in 50 µL ethanol and 450 µL diluted enzyme conjugate to give a standard range of 5 to 1000 pg/µL. The dried fecal samples were reconstituted similarly and 50 µL of each sample were pipetted in duplicate for the ELISA. Plates were covered tightly with plate sealer covers and the competitive reaction allowed to proceed for 2 hours. To separate free from bound hormone, the plates were emptied, rinsed 3 times with wash solution, blotted and allowed to dry at room temperature. One hundred µL of freshly prepared substrate solution (0.05 M citrate, 1.6 mM hydrogen peroxide, 0.4mM 2,2-azino-di-3-ethylbenzthiazoline sulfonic acid diammonium salt (ABTS, pH 4.0) was then added to all wells and color was allowed to develop to determine the amount of conjugate (cortisol:HRP) bound to the solid-phase antibody. The color change was stopped after approximately 60 minutes by the addition of 100 µL stop solution (0.15 M hydrofluoric acid containing 0.006 M NaOH and 0.001 M EDTA, pH 3.3). Absorbance was measured at 405 nm with a Bio-Rad Model 550 automatic microtiter plate spectrophotometer (Hercules, CA, USA) and the data were transferred to an interfaced computer for analysis (Gateway model E4300, Irvine, CA). Cortisol concentrations are expressed as nanograms per gram of dry feces (ng/g).
2.4 HPLC

Although details of this procedure for fecal cortisol have been previously reported (Turner et al., 2003), a brief description is provided for the present context. Free cortisol in fish fecals was determined by reverse-phase high performance liquid chromatography (HPLC, Dionex, Sunny vale, CA) utilizing a standard 3.9 x 300 mm C-18 column (Waters, Milford, MA) and a variable wavelength UV detector (Dionex) set at 240 nm. Prior to sample analysis, a water blank was run until the column was free of major peaks and a reference standard containing cortisol (hydrocortisone reference standard; Sigma-Aldrich Inc., St. Louis, MO, 50 µL in 10% acetonitrile (ACN)/90% water) was run to verify retention times. The flow rate was 1 mL/min and the elution gradient changed from 10% ACN/90% water to 90% ACN/10% water over a period of 25 minutes (ensuring complete separation of sample compounds) and returned to the initial concentration over another 5 minutes. Additionally, the run was continued for 15 minutes at the initial concentration to re-acclimate the column. The standard curve was developed by HPLC runs of duplicate samples of 7 known cortisol concentrations (ranging from 5 pg cortisol/µL to 1000 pg cortisol/µL).

3. Results

3.1. HPLC

Cortisol was readily detectable and measured by the HPLC analysis and the elution time was 11.91±0.02 minutes. The lower limit for cortisol detection in the HPLC portion of this study
was 1.8 ng in a 250 µL sample loaded onto the chromatographic column. Hormone values are reported as ng cortisol/g dry feces. HPLC standards ranged from 5 to 1000 pg/µL in 7 incremental amounts (fig.1). The HPLC-assayed cortisol values ranged from 1118.5 ng per g dry fecal for the 1999 sample to 169.5 ng cortisol per g dry fecal material for the 2003 sample.

3.2 ELISA

A representative ELISA standard curve is shown in figure 2. ELISA-derived cortisol values (ng/g dry fecal) were based on the same concentration as for HPLC with $R^2 = 0.9899$ for the calculated curve (fig. 2). The lower limit using ELISA was 1.0 ng in the usual 50 µL sample placed in each microplate. ELISA-assayed cortisol values ranged from 1314.2 ng/g dry fecal for the 1999 sample to 210.7 ng/g dry fecal for the 2003 sample.

3.3 ELISA-HPLC Comparison

Fecal cortisol assayed by both HPLC and ELISA across the sampling years 1999 to 2004 yielded nearly identical patterns of cortisol levels (fig. 3). A Pearson correlation coefficient performed on these data was 0.996.

4. Discussion
To date there have been no reports of use of an ELISA for measurement of cortisol in fish feces, as radio immunoassay (RIA) and HPLC are the most commonly used methods. The present study has shown that fish cortisol can be measured in fecal material by ELISA and this assay exhibits equal reliability and much greater efficiency and sensitivity than HPLC. On a per-mL basis, the ELISA showed 9-fold greater sensitivity than HPLC, which reduces fecal sample weight required for reliable assay (HPLC = 0.5 g, ELISA = 0.2 g). From both per-sample assay time and personnel cost perspectives, the ELISA is more practical and less expensive than HPLC analysis. The time required for a single fecal analysis via HPLC is approximately 45 minutes, whereas an ELISA plate can accommodate up to 96 samples (48 in duplicate) in less than 4 hours. Finally, the equipment cost for ELISA is less than 5% of that for HPLC. We conclude that the fecal-cortisol ELISA validated in this study shows high potential as a sensitive, reliable and cost-effective tool for noninvasive stress assessment in fishes.

5. Acknowledgements

We wish to thank Dr. V. Basrur (Medical University of Ohio) and Dr. M. Kim (University of Toledo) for assistance with the HPLC portion of the study and J. Clark for lab assistance in the ELISA portion.
6. References


Noninvasive assessment of nitrate-induced stress in *Cyprinus carpio* by fecal cortisol measurement.

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Abstract

Cortisol is frequently measured as a parameter of stress. Since cortisol metabolism includes some excretion of intact hormone in feces, fecal cortisol can serve as an index of stress. This study determined the effects of nitrate (NO$_3^-$), a common aquatic pollutant and possible stressor, on fecal cortisol levels in koi (Cyprinus carpio). NO$_3^-$ was presented in two formats: 1) bolus increase followed by dilution to assess cortisol response and recovery and 2) bolus increase followed by further incremental increases to assess cortisol response limit. Fish were maintained group-wise (n=7) in treatment and control aquaria, and NO$_3^-$ (as NaNO$_3$) was added to the water beginning at 0.4 or 0.5 g/L. Fecal samples were collected daily and alternative-day samples were extracted and assayed for cortisol via an ELISA. NO$_3^-$ effects were compared for baseline (12 days) vs. treatment (12 days) and control group (n=7) vs. treatment group (n=7) conditions. Exposure to NO$_3^-$ (0.4 g/L) increased cortisol excretion from 49.5±1.3 ng/g dry feces (pretreatment) to 337.7±24.0 ng/g dry feces. Additional NO$_3^-$ increments did not further increase cortisol, suggesting that the initial exposure produced maximal response. In a separate repeated-measures experiment a 74% NO$_3^-$ decrease by dilution after initial exposure to 0.5 g/L NO$_3^-$ was associated with a cortisol decrease from 523.0 to 27.5 ng/g dry feces in 24 hours. Restimulation by 1.0 g NO$_3^-$/L re-elevated cortisol to 748.9 ng/g dry feces. This study demonstrates that fecal cortisol measurement in fish via an ELISA can be useful as an indicator of NO$_3^-$-induced stress in the aquatic environment.
1. Introduction

Aquatic habitats are in decline worldwide from a combination of natural and anthropomorphic stressors (Viarengo et al., 2007) and fish associated with these habitats are likely to experience increased stress, which can impair aspects of their immune function (Salo et al., 2007), growth (Hamlin et al., 2007) and reproduction (Kurtz et al., 2007). Entire fish populations inhabiting a given site and exposed to repetitive or chronic stressors, such as nitrate (NO$_3^-$), may be compromised (Chew et al., 2006). The expulsion of untreated sewage into aquatic environments represents a significant source of pollutants (Salo et al., 2007). NO$_3^-$ can also be released into the environment via sedimentation (commercial dumping) and agricultural runoff associated with developed coastlines (Yeom et al., 2007). In a separate arena, there is interest in fish stress associated with aquaculture because of the need to minimize stress of crowding and poor water quality in the production of commercially valuable fish in artificial environments (Cuesta et al., 2003). Ecotoxins are injurious substances of natural biological origin, and NO$_3^-$ in excess is one of these (Camargo et al., 2005). Decomposition demands large amounts of oxygen and produces large quantities of ammonia (NH$_3$) and NO$_3^-$ (de Croux et al., 2004). Municipal and industrial wastes are concentrated sources of nitrogen compounds that are, to a large extent, released directly into surface waters (Shimura et al., 2004). Even if treated, this waste will represent a heavy water pollution load since secondary treatment removes less than half of the nitrogen (Jittawattanarat et al., 2007). Ammonium ions in the effluent of septic tanks may be rapidly converted to NO$_3^-$, which may penetrate some distance from the tank. Sludge from treatment plants and septic tanks have also to be disposed of and represent another significant source of nitrogen pollution (Liu et al., 2007). This study examines the effects of elevated aquatic NO$_3^-$
on the stress hormone cortisol in butterfly koi (*Cyprinus carpio*). We have employed an ELISA modified to measure cortisol levels in feces as an index of stress. Measurement of fecal glucocorticoids is well established for mammals (Wallner et al., 1999; Wasser et al., 2000) and has been reported for birds (Dehnhard et al., 2003), fish (Turner et al., 2003) and sharks (Karsten and Turner, 2003).

2. Methods

2.1. Animal model

*C. carpio* was chosen as a conservative target for assessing NO$_3^-$ as a stressor, since this species is tolerant to compromised water quality and is also of interest due to its commercial value. All fish were obtained from a commercial supplier (T. Carter, Alliance, OH) and maintained in aquaria at a fish density of $< 3.6$ g/L. Water was maintained at pH 6.5 to 7.0 and 24° C. Commercial food pellets (Hartz Mountain, Secaucus, N.J.) were provided daily at approximately 2% fish body weight.

2.2. Experimental design

For this report, 3 experiments were conducted: (1) a preliminary test to determine the concentration of NO$_3^-$ lethal to 50% of a population sample (LC$_{50}$) as a basis for choosing NO$_3^-$ treatment doses, (2) a NO$_3^-$ stress-and-recovery test to assess responsiveness of cortisol feedback regulation and (3) a NO$_3^-$ challenge test employing incremental NO$_3^-$ increases to assess the limit of cortisol response. A repeated measures (baseline vs. treatment) design was employed in experiments 2 and 3. A control group was also tested coincidentally with the experimental group to account for possible non-NO$_3^-$ water quality issues across time.
2.3. Protocols

In the preliminary LC$_{50}$ experiment, juvenile goldfish (*Carassius auratus*, 6 groups of n=5) were acclimated to their environment (2L aquarium per group) for 7 days. Juveniles (<10 cm length) were used, since immature fish are more sensitive than mature fish to NO$_3^-$ intoxication and respond more vigorously. Previous studies showed that NO$_3^-$ above 100 mg/L could be stressful to cyprinids (Tilak, et al. 2007). We therefore chose this initial dose, with stepped increases to 15 fold to determine the concentration lethal to 50% of our sample population. Lethality was assessed across a 144 hr exposure.

For experiment 2 (stress and recovery), juvenile *C. carpio* (n=6) were maintained in 38 L aquaria and allowed to acclimate to the environment for 7 days. After 8 additional (Baseline) days, with NO$_3^-$ levels maintained below 10 mg/L, NaNO$_3$ was added to the aquarium at a dose of 500 mg/L, dictated from the LC$_{50}$ test. This concentration was maintained for 48 hrs and then reduced by water dilution by 40% daily for 2 days. Thereafter (day 20) NO$_3^-$ levels were increased rapidly to 1.0 g/L and were maintained for 72 hrs.

For experiment 3, mature *C. carpio* were maintained in rectangular 96 L aquaria (n = 7 fish per group, 2 groups) and allowed to acclimate to their environment for 12 days. NaNO$_3$ was added in a step-wise fashion beginning with an initial concentration of 0.4 g NO$_3^-$/L water on day 13, then increased to 0.8 g/L on day 17, to 1.2 g/L on day 20 and finally to 1.6 g/L on day 23.
In experiments 2 and 3, overnight fecal samples were collected daily and were obtained generally intact using a plastic grate covering the entire aquarium floor approximately 6 cm from the bottom. The grate allowed fecal casts to pass through to the bottom, preventing the fish from dispersing the casts with their fins. Since we used overnight samples, NO$_3^-$ administered on a given day evidenced its effects in fecals from the next day. Fecal casts were stored at -40 °C and later assayed for cortisol content via ELISA. Fish were monitored in all experiments for observational signs of NO$_3^-$ toxicity (i.e. lethargy, rapid breathing, color changes and appetite loss).

### 2.4. Cortisol Measurement

Each daily sample from a given aquarium was pooled across all fish in that aquarium and was desiccated overnight at 40° C using a microscope slide warmer. Dried samples were weighed to 0.2 g, added to 12 mL MeCl$_2$ in a glass scintillation vial and shaken for 60 minutes at room temperature. Samples were then centrifuged at 3000 rpm for 10 min. The MeCl$_2$ layer was placed in a new glass scintillation vial and mixed with 1 mL 1 M NaOH. Four mL of the MeCl$_2$ layer were then transferred to each of two new glass vials and evaporated to dryness in a heated centrifuge (3000 rpm, at 40° C) for approximately 60 minutes.

Extracted cortisol samples (from 0.2 g desiccated fecal material) were mixed with 50 µL ethanol (EtOH) and vortexed for 2 min. Fifty µL increments of aqueous enzyme conjugate were added and vortexed for an additional 2 min each to give a final volume of 500 µL for the ELISA. A 50 µL aliquot of each sample (in duplicate) was added to the ELISA plate.
A sandwich-type ELISA, modified from methods described by Munro and Lasley (1988), was adapted for determination of fish fecal cortisol. The assay employed a cortisol-horseradish peroxidase ligand and antiserum (Antibody R4866; C.J. Munro, University of California, Davis) and commercial cortisol standards (Sigma-Aldrich Inc., St. Louis, MO). The ELISA was completed in 96-well microtiter plates (Nunc-Immuno, Fisher Scientific, Pittsburgh, PA) layered 24 hrs beforehand with cortisol antiserum (50 μl/well; diluted 1:8500 in bicarbonate coating buffer; 0.05M Na₂CO₃/NaHCO₃, pH 9.6). The polyclonal antiserum was raised in rabbits against cortisol-3-carboxymethyloxime, linked to bovine serum albumin and showed cross-reaction with cortisol (100%), prednisolone (9.9%), prednisone (6.3%), cortisone (5%) and <1% with androstenedione, androsterone, corticosterone, desoxycorticosterone, 11-desoxycortisol, 21-desoxycortisone, and testosterone (Munro and Lasley, 1988). A standard ELISA protocol was followed, employing cortisol-3-CMO:HRP conjugate solution containing cortisol standards or a reconstituted extracted fecal sample. Cortisol standards ranged from 5 to 1000 pg/µL Absorbance was measured at 405 nm with a Bio-Rad Model 550 microtiter plate spectrophotometer (Hercules, CA, USA) with data-processing (Gateway model E4300, Irvine, CA). Cortisol concentrations were expressed as nanograms per gram of dry fecal matter.

3. Statistical Analysis

Cortisol values are presented as mean ± standard error. The repeated-measures data from experiments 2 and 3 (NO₃⁻ effect on fecal cortisol levels) were analyzed using one-way repeated-measures ANOVA followed by Tukey HSD post hoc test. Comparisons between the
untreated control group and pre-treatment experimental group were analyzed by the Student t-test (Zar, 1984). Significant differences were accepted for \( p < 0.05 \). LC\(_{50}\) data were not statistically evaluated.

4. Results

4.1 LC\(_{50}\) Determination

All fish survived all doses for 24 hrs and at least 40% of fish in all dose groups expired by 6 days. No fish survived 1.5 g/L for 6 days. The LC\(_{50}\) occurred between 250 and 500 mg NaNO\(_3\)/L aquarium water within 48 - 72 hrs (table 1). As expected, NO\(_3^-\) dose and % fish survival were inversely related. All fish showed some signs of stress within 24 hours of NO\(_3^-\) treatment and prior to expiration, including decreased movement, aphagia and respiratory distress. Higher NO\(_3^-\) concentrations were associated with more pronounced physical signs of stress.

4.2. Stress/recovery

Based on the LC\(_{50}\) data, a 22-day experiment was conducted in juvenile *C. carpio* to assess the cortisol response associated with NO\(_3^-\) addition and its subsequent reduction in aquarium water. Relative to Baseline cortisol levels (43.3±4.6 ng/g dry feces), fish showed a 12.1-fold increase to 523.0 ng/g in response to 0.5 g/L NO\(_3^-\) (Fig 1). Subsequent reduction in NO\(_3^-\) levels through water changes of 40% daily over two consecutive days (i.e., from 0.5 g/L to 0.1 g/L) resulted in return to near basal levels. A second increase in NO\(_3^-\) (to 1.0 g/L) yielded fecal cortisol levels of 748.9 ng/g. Treatment with each amount of NO\(_3^-\) was limited to 2 days, since treatment caused lethargy, loss of color, increased respiration and aphagia to
varying degrees in all fish within this period. Cortisol levels across Treatment (0.5 and 1.0 g/L NO₃⁻) averaged 636.1±70.3 ng/g dry fecal, and this level was 16-fold and significantly (p<0.001) greater than Baseline.

4.3. Incremental stress

In this 24-day study (Fig 2), there was no difference between cortisol levels of the untreated control group and pre-treatment experimental group (65.1±12.4 and 49.5±16.3, respectively). Fecal cortisol was increased from pretreatment levels for all NO₃⁻ treatments (p<0.001) with initial NO₃⁻ exposure (0.4g/L) yielding a 6.8-fold cortisol increase to 337.7±24.0 ng/g dry feces. ANOVA revealed no differences in cortisol among the variously NO₃⁻-dosed groups. Fish were sacrificed on day 24 while NO₃⁻ concentration was at 1.6 g/L, and fish at that time had exhibited physical manifestations of stress previously described.

5. Discussion

NO₃⁻ in soil and in surface and groundwater result from natural decomposition by microorganisms of organic matter and are also introduced into the aquatic environment through anthropological sources such as fertilizer, industrial byproducts and untreated or partially processed sewage. NO₃⁻ is a by-product of nitrite (NO₂⁻) conjugation during the latter stages of the nitrogen cycle and will be present to some degree in all aquatic environments. Fish excrete the ammonium ion (NH₄⁺) as waste from their gills and kidneys, and levels can increase further due to decaying plant material (Yu et al., 2004). Both NO₂⁻ and NO₃⁻ ions can be formed from NH₄⁺ in a two-step biological oxidation (nitrification) process (Mancinelli, 1996).
Although NO$_3^-$ is not directly lethal in the way NH$_4^+$ or NO$_2^-$ are, sustained high levels of NO$_3^-$ are stressful to fish (Spokas et al., 2006), especially in concentrated aquaculture environments with limited water exchange. The amount of NO$_3^-$ that elicits a cortisol response varies across species (Camargo et al., 2005). The resulting stress makes fish more prone to disease and inhibits their ability to reproduce (Brauner and Wood, 2002). Chronically elevated NO$_3^-$ levels are especially harmful to fry and young fish, affecting their growth and metabolism (Brauner and Wood, 2002), since sufficiently elevated NO$_3^-$ can exceed the capability of homeostatic mechanisms to return cortisol levels to normal. Furthermore, conditions associated with chronically elevated aquatic NO$_3^-$ often cause decreased oxygen levels, which can further stress fish through hypoxia (Jensen, 2003).

Elevated NO$_3^-$ in the present study was a significant stressor for koi as evidenced by increased fecal cortisol, loss of appetite, color change, hyperventilation and lethargy. In experiment 2 the dramatic cortisol response to elevated NO$_3^-$ and the return to near-basal cortisol levels after NO$_3^-$ dilution demonstrated that the homeostatic regulation of cortisol remained functional after NO$_3^-$ exposure. It also indicates that even partial reduction in aquatic NO$_3^-$ levels can facilitate return to normal feedback regulation of cortisol. The 2-day cortisol response to 1.0 ng/L NO$_3^-$ was numerically greater than the 2-day response to 0.5 g/L NO$_3^-$, but significance could not be determined with an ‘n’ of 2. It was not possible to continue treatments beyond 2 days due to observed physical compromise in the fish.
In experiment 3, the absolute levels of cortisol increased with each NO$_3^-$ addition, but these increases were not significant. This may be due to small sample sizes. On the other hand, it appears that the initial NO$_3^-$ exposure yielded near-maximal cortisol response, i.e., further NO$_3^-$ increases (up to 4-fold) produced little additional cortisol.

Since the NO$_3^-$ levels were highly elevated and only for several days, this study did not address the more likely natural condition of moderate, long-term elevation. Nonetheless, it suggests that NO$_3^-$ intoxication of aquatic wildlife warrants more comprehensive examination. While fecal cortisol measurement has been previously reported for marine fish (Turner et al., 2003), the present study is the first assessment of fecal cortisol in freshwater fish and demonstrates that this methodology can be useful as a noninvasive indicator of NO$_3^-$-induced stress.

6. References


Table 1. LC$_{50}$ (as % of fish surviving) for NaNO$_3$ in aquarium water of juvenile \textit{Carassius auratus}, n=5/group.

<table>
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<th>NaNO$_3$ [mg/L]</th>
<th>24</th>
<th>48</th>
<th>72</th>
<th>96</th>
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<td>100</td>
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Figure 1.
Figure 2
Fig 1.  Cortisol response of *C. carpio* to increased and subsequent reduction of NO$_3^-$ in aquarium water. NO$_3^-$ concentrations were 0.5, 0.2 and 1.0 g/L, consecutively. Each daily sample collection was from overnight samples. Treatment group n=6, Control group n=6.

Fig 2.  Relationship between NO$_3^-$ treatment and fecal cortisol levels in *C. carpio*. Pretreatment vs. all treatment levels of cortisol were significant (p<0.0001). There was no difference among individual nitrate-treated groups or between untreated control and pretreatment conditions. Each daily sample collection was from overnight samples. Treatment group n=7, Untreated control group n=7.
DISCUSSION

PART 1: ELISA for Cortisol

To date there have been no reports of use of an ELISA for measurement of cortisol in freshwater fish feces, as radio immunoassay (RIA) and HPLC are the most commonly used methods. The ELISA study presented in this thesis showed that fish cortisol can be measured in fecal material by ELISA and this assay exhibits equal reliability and much greater efficiency and sensitivity than HPLC. On a per-ml basis, the ELISA showed 9-fold greater sensitivity than HPLC, which reduces fecal sample weight required for reliable assay (HPLC = 0.5 g, ELISA = 0.2 g). From both per-sample assay time and personnel cost perspectives, the ELISA is more practical and less expensive than HPLC analysis. The time required for a single fecal analysis via HPLC is approximately 45 mins, whereas an ELISA plate can accommodate up to 96 samples (48 in duplicate) in less than 4 hrs. Finally, the equipment cost for ELISA is less than 5% of that for HPLC. The fecal cortisol ELISA validated in this study shows high potential as a sensitive, reliable and cost-effective tool for noninvasive stress assessment in fishes.

Comparison of HPLC, RIA and ELISA

HPLC: This method is highly specific, precise and sensitive. Additionally, multiple analyses can be done. The drawbacks are that an extraction step is required, this method is slow due to single serial analysis of samples, the column degenerates with time and complex analyses require considerable processing. HPLC is a useful technique because thermolabile compounds can also be analyzed.
Radio-immuno assay (RIA): The method most commonly used for measuring antibody responses to vaccines and for evaluating the immunogenicity of their individual polysaccharide components has been radio-immunoassay. This method is sensitive, reasonably precise but requires the use of radionucleotides. Cross reactivity with other closely reacted drugs is a potential problem with this technique. Besides it is not possible to find out the optically active isomer. The hazards of using radioactive material are a considerable limitation of this method.

Enzyme-linked immunosorbent assay (ELISA): This technique offers some advantages over RIA in that no radioactive tracer is required and there is no need to separate the bound from the unbound fractions. However the potential for cross-reactivity still exits. To test the reproducibility of the cortisol ELISA measurements were made in duplicates and samples were often assayed several times. The ELISA method as described here is about as sensitive for measuring cortisol as the RIA that has been most commonly used so far. ELISA allows, however, for easy measurement of antibodies of different Ig classes and thus makes possible a detailed characterization of the total antibody response. In general, the most common cause of discrepancies between ELISA and RIA results is believed to be that the ELISA technique is more sensitive to antibody affinity.

Caution in using indicators of stress

There are various prospective applications of the stress response and monitoring via the ELISA described in this thesis. For example, experimental biologists need to know the baseline from which to evaluate whatever response they are studying. Some aspects of the
impact of environmental perturbations can be assessed through the response of aquatic organisms such as fish.

It is important to know whether fish under intensive aquaculture are in a stressed state. Thus the features of the physiological and cellular stress responses can serve this purpose. However, it is central that the baseline or any variable be established for species, season, and any other factor that can influence the measured variable. For example, plasma cortisol values can vary by species, stock, rearing history (wild or cultured), nutritional state, conditioning to stress, developmental stage, and environmental factors such as water pH, temperature and salinity. The maximum plasma cortisol values in fish can vary by 2 orders of magnitude (Kuhn, Corneillie et al. 1986). The lowest of these values have been reported in the chondrosteans (eg. sturgeon, 13 ng/ml, paddlefish 60 ng/ml) and the highest in teleosts such as the chub and stripped bass (1,500 – 2,000 ng/ml; (Barton, March et al. 2002). The resting plasma cortisol values differ according to time of day and by season. Heat shock proteins may present a potentially convenient biomarker for several reasons. They may be measured when the blood volume is very small. They might be used some indicator of cellular when other indicators of stress are not available or well established; for example for shellfish and crustaceans. They can be measured quite easily and tissue-localization is possible. HSP70 has been shown not to respond to handling in fish (Vijayan, Pereira et al. 1997). However, conditions that may exist in intensive aquaculture, such as anaesthesia, hypoxia, hyperoxia, capture, crowding, food deprivation, and cold shock have all be shown not to affect HSP70 in Atlantic salmon (Pan, Zarate et al. 2002). Therefore, it is critical to establish the baseline for the species and condition in which the assessment is being made. A
changing baseline can have significant implications in determining whether a state of stress exists. Stress can exist when the measured variable does not change, or have patterns that are not obvious without this baseline reference.

PART 2: Nitrate Stress

Toxic effects of nitrogenous compounds

In humans and other terrestrial animals, NO$_2^-$ and NO$_3^-$ research is primarily focused on intake of nitrogenous compounds via food and drinking water and the ability of NO$_3^-$ (which can be reduced to NO$_2^-$ in the gastrointestinal tract) to form metHb and potential mutagenic and carcinogenic N-nitroso compounds (Brunato, Garziera et al. 2003). Aquatic animals are more at risk for nitrogenous intoxication, as NO$_2^-$ and NO$_3^-$ can be readily absorbed through the chloride channels across the gill epithelium of fishes and rapidly equilibrate within body fluids (Jensen 2003). The fish gill is a multi-use organ that provides for aquatic gas
exchange, plays central functions in osmotic and ionic regulation, acid-base regulation, and excretion of nitrogenous wastes. Thus, regardless of all fish groups having functional kidneys, the gill epithelium is the site of many processes that are mediated by renal epithelia in terrestrial vertebrates (Evans, Piermarini et al. 2005). In a recent experiment, fish were exposed to lethal concentrations of nitrogen to study the changes in hematological parameters and the rate of oxygen utilization (Fig 7). During the time of exposure to nitrogenous compounds, a broad decline in the hemoglobin content was observed. MetHb content increased during NO$_2^-$ exposure while the hemoglobin levels decreased significantly. It was also observed that the rate of oxygen consumption decreased in time with the increase of toxicant concentration and length of the exposure (Tilak, Veeraiah et al. 2007).

Nitrogenous intoxication has multiple physiological effects in aquatic animals. Its uptake is at the expense of chloride, leading to chloride depletion. Nitrite also activates efflux of potassium ions (K$^+$) from skeletal muscle and erythrocytes, disturbing intracellular and extracellular K$^+$ concentrations (Bogdanova, Sherstobitov et al. 1998). Nitrogen transfer across the erythrocyte membrane leads to oxidation of hemoglobin to metHb, compromising blood O$_2$ transport. Hyperventilation is also observed, and eventually tissue O$_2$ starvation is echoed in elevated lactate concentrations. Heart rate increases rapidly, before any significant elevations in metHb or extracellular potassium occur. This suggests nitrogen-induced vasodilatation (possibly via nitric oxide generated from nitrite) is opposed by increased cardiac output to re-establish blood pressure. Nitrogenous compounds can form nitric oxide and thereby interfere with processes regulated by this local hormone. Steroid hormone synthesis may be inhibited, while changes in ammonia and urea levels and excretion rates reflect an influence of nitrite on nitrogen metabolism. (Jensen 2003)
Increased mucus production, occurs due to ammonia and urea as the primary forms of nitrogen excretion in teleost fish (Kajimura, Croke et al. 2004) However, the sum of ammonia plus urea is less than that of total nitrogen, indicating that 'unknown' end products are important in nitrogen metabolism. Other physiological manifestations that occur due to nitrogen intoxication include: loss of appetite, lethargy, chronic stress, reluctance to breed, delayed wound healing and general ill health.

Although NO$_3^-$ is not directly lethal as NH$_4^+$ or NO$_2^-$ are, sustained high NO$_3^-$ levels are stressful to fish (Spokas et al., 2006), especially in concentrated aquaculture environments with limited water exchange. The amount of NO$_3^-$ that elicits a cortisol response varies across species (Camargo et al., 2005). The resulting stress makes fish more prone to disease and inhibits their ability to reproduce (Brauner and Wood, 2002). Chronically elevated NO$_3^-$ levels are especially harmful to fry and young fish, affecting their growth and metabolism (Brauner and Wood, 2002), since sufficiently elevated NO$_3^-$ can exceed the capability of homeostatic mechanisms to return cortisol levels to normal. Furthermore, conditions associated with chronically elevated aquatic NO$_3^-$ often cause decreased oxygen levels, which can further stress fish through hypoxia (Jensen, 2003). Elevated NO$_3^-$ studied in the present thesis was a significant stressor for koi as evidenced by increased fecal cortisol, loss of appetite, color change, hyperventilation and lethargy.

In the stress/recovery experiment, the dramatic cortisol response to elevated NO$_3^-$ and the return to near-basal cortisol levels after NO$_3^-$ dilution demonstrated that the homeostatic regulation of cortisol remained functional after NO$_3^-$ exposure. It also indicates that even partial reduction in aquatic NO$_3^-$ levels can facilitate return to normal feedback regulation of
cortisol. The 2-day cortisol response to 1.0 ng/L NO$_3^-$ was numerically greater than the 2-day response to 0.5 g/L NO$_3^-$, but significance could not be determined with a sample size of 2. It was not possible to continue treatments beyond 2 days due to observed physical compromise in the fish. A greater sample size would be beneficial to obtain results to further indicate the noxious effect of NO$_3^-$ as individual fish may be monitored as opposed to a group and determination of NO$_3^-$ toxicity may be determined for sex- and age-specific physiological perturbations.

In the incremental stress experiment, the absolute levels of cortisol amplified with each additional NO$_3^-$ treatment, but these increases were not statistically significant, perhaps due to small sample sizes. Alternatively, the primary NO$_3^-$ exposure gave a near-maximal cortisol response, where additional NO$_3^-$ increases (up to 4-fold) generated modest additional cortisol secretion. Since the NO$_3^-$ levels were highly elevated and the duration for only several days, this study did not deal with the more probable naturally occurring condition of moderate, long-term elevation. Even so, it implies that NO$_3^-$ intoxication of aquatic wildlife justifies a more comprehensive examination. While fecal cortisol measurement has been previously reported for marine fish (Turner et al., 2003), the present study is the first assessment of fecal cortisol in freshwater fish and demonstrates that this methodology can be useful as a noninvasive indicator of NO$_3^-$-induced stress.

NO$_3^-$ toxicity has also been studied histologically in Medaka fish, *(Oryzias latipes*, (Shimura, Ma et al. 2004). To investigate the effects of short-term exposure to NO$_3^-$, juvenile Medaka were exposed to NaNO$_3$ at concentrations of 100 and 125 mg NO$_3$-N/L for 96 hours. At the conclusion of the experimental exposure, survival rates were found to be 30% and 10%, for
the 100 and 125 mg NO$_3$-N/L concentrations, respectively. Histological examination of the organs indicated a disruption of cell alignment, a common characteristic in the gills, liver and kidney. A long-term exposure experiment was also carried out, whereby Medaka were exposed to NaNO$_3$ (100 and 125 mg) for three months from egg stage. Eggs treated with NaNO$_3$ hatched within 10 days after fertilization. At the end of the experimental exposure, survival rate for the 100 and 125 mg conditions were 40% and 30%, respectively. In the juveniles subjected to long-term NO$_3^-$ exposure, hepatic cell fibrosis and scoliosis were detected. These experiments imply that the high mortality resulting from short-term acute exposure to NO$_3^-$ is caused by general dysfunction throughout the whole body. The chronic toxic effects attributed to NO$_3^-$, following long-term exposure, were likely to have resulted from nutrient deficiency caused by hepatic dysfunction.

**Stress and behavior**

One of the first indications of a stressed state in fish is a behavioral response, as behavioral and physiological responses are closely interrelated. The physiological stress responses serve to preserve, express, and probably limit the behavioral response. A characteristic behavioral response to chemically-induced stress appears to be aphagia (Ioannidou, Paraskevopoulos et al. 2003). Stressful conditions can disrupt many other aspects of feeding behavior in fish, including food searching, finding, and capturing prey (Baatrup 1991). Furthermore, several different types of stress, including environmental, low pH, high ammonia, low dissolved oxygen and pollutants, crowding, and physical handling challenges, have all been documented to hinder food consumption in fish (McGeer, Szebedinszky et al. 2000; Overli, Korzan et al. 2004; Volkoff, Canosa et al. 2005). Activities such as food acquisition, predator avoidance, prey capture, migration, and habitat preference are critical to the survival
of the organism and thus the population, and are commonly used as indicators of environmental stressors (Gregory and Wood 1999). Modifications of such behaviors may take minutes to weeks to return to pre-stress states, depending on the nature and extent of the stressor (Bernier, Bedard et al. 2004; Bernier 2006). Behaviors that are most vital to the survival of the organism tend to return to pre-stress conditions in the briefest time (Yeoh et al. 1996). Suitable behavioral responses to the perception of a stressor will diminish the possible magnitude of that stressor, and will raise chances of survival for that individual. Yeoh et al. (1996) point out that the appropriate response may be “avoidance, or other behavioral change that mitigates exposure to the stressor.” As this quote states, the adaptive behavioral response to a stressor may reduce the energetic requirements on the physiological systems that must respond to it. Modifications in behavior brought about by stressors may reflect deleterious changes in how an animal senses and responds to its environment if evasion or behavioral alleviation is not possible.

**PART 3: Consideration for future Studies**

**Stress and Innate Immunity**

A relationship between environmental contamination and disease has long been recognized and this association stems partially from the impairment of the innate immune response (Kahn, Maran et al. 2007). The innate immune system is an assortment of defense mechanisms that guard an organism against infection without depending upon previous exposure to any microorganism in particular (Bols, Brubacher et al. 2001), which include specific barriers to infection such as skin, stomach acid, mucus, and enzymes in tears and saliva that occur as part of the natural biologic makeup. Phagocyte respiratory burst appears
to be particularly sensitive to toxicants (Bols, Brubacher et al. 2001). Immunotoxic effects of sewage effluents on numerous fish species have been detailed (Karrow, Bols et al. 2001; Salo, Hebert et al. 2007). A study to examine the immunotoxicity of treated sewage effluents from Montreal, Canada, on juvenile female rainbow trout after series of immunological assays was used to evaluate the effects of exposure for 1 and 4 weeks to 1, 3, 10 and 20% sewage effluent. This study showed that phagocytic ingestion of fluorescent latex beads by head kidney macrophages and granulocytes was suppressed following 1-week of exposure, with the highest exposure concentration being correlated to the most oppressed rate of endocytosis. Phagocytic activity returned to pre-treatment levels after 4 weeks of exposure (Salo, Hebert et al. 2007). Also in O. mykiss, to mimic a stress response by aquatic pollutants, treatment with cortisol at 1000 and 100 ng/ml aquaria water showed that cell numbers and $^{3}$H-thymidine incorporation by cell cultures of monocyte/macrophage cell lines were decreased in comparison to non-treated control cell lines (Pagniello, Bols et al. 2002). Various studies have observed the effect of stress responses on leucocytes, where both lymphocyte proliferation and the concentration of circulating lymphocytes have been reduced, thereby jeopardizing the innate immune system (Hamoutene, Payne et al. 2008; Perez-Casanova, Rise et al. 2008). In an investigation into the physiological response of Atlantic cod to stress, plasma cortisol and glucose levels were measured and the results suggest that immune function is influenced by complex interactions between elevated circulating cortisol levels and stress (Hamoutene, Payne et al. 2008; Perez-Casanova, Rise et al. 2008).
Using fish models to study interactions between the immune and endocrine systems has distinctive advantages in that the head kidney merges corticosteroid synthesis with immune characteristics such as hematopoiesis and the production of antibodies (Weyts, Flik et al. 1998). Many immune cell types are currently being investigated for use as possible markers for environmental pollution. Several studies found that amplified cortisol production has other detrimental effects on immune function, such as reduced antibody titers (Engelbrecht Nielsen and Buchmann 1997) and redistribution of leukocytes (Maule and Schreck 1990). Weyts et al., (1998) showed that cortisol-induced apoptosis is also responsible for the removal of circulating, activated T and B cells following an immune response in the common carp.

**Stress and reproductive interference**

In most female fish, amphibians, reptiles and birds one essential function of ovarian-produced estrogen is to evoke the expression of the Vtg gene in the liver (a tissue specific gene) and in some species (i.e. carp) in the ovaries as well (Berg et al., 2004). Vtg is the phosphoprotein precursor of egg yolk proteins and its synthesis in the liver is estrogen dependent and is then transported in the bloodstream to the ovary during oogenesis/oocyte maturation. Vtg normally cannot be detected (or is at very low concentrations) in the bloodstream of

Figure 8. Proposed relationship between NO$_3$-exposure and compromised reproduction. M = male, F = female
males because they produce no or very little estrogen. The male fish liver Vtg and Zr genes can, however, be activated by estrogen (Fig 8). Both Zr and Vtg showed a dose-dependent increase with increasing 17-β estradiol (E2) concentrations (Berg et al., 2004). Co-injection of cortisol, with both high and low doses of E2, resulted in a significant upregulation of Zr protein with no Zr induction with cortisol alone suggesting that cortisol acts to potentiate the effect of E2 (Berg et al., 2004). The appearance of Vtg in the plasma of adult male or juvenile fish is widely accepted as evidence of exposure to estrogenic chemicals and other endocrine disrupting compounds (Mori, Matsumoto et al. 1998). An important characteristic of Vtg induction in male and juvenile fish is the specificity of the response to estrogens or estrogen mimics (Game, Gagnon et al. 2006). Although Vtg has been purified from plasma and detected by Western blotting, the protein is unstable and difficult to handle due to its role as a precursor for shorter peptide fragments (lipovetellin and phosvitin) and is very sensitive to proteolytic breakdown. Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) amplification of Vtg and Zr mRNAs transcripts and quantitation of corresponding protein levels has been validated for identifying biomarkers (Garcia-Reyero et al., 2004) and therefore can be an attractive method of elucidating the genetic modifications that nitrogenous compounds can induce in fish.

**Stress and steroidogenesis dysregulation**

NO$_3^-$ can cross the gills, sometimes against a concentration gradient, by substituting for Cl$^-$ in the chloride-bicarbonate exchange mechanism that normally regulates the osmotic and respiratory functions of the gill (Goss, Perry et al. 1998). The ability of the gill epithelium of freshwater fish to accumulate Cl$^-$ suggests that NO$_3^-$ and NO$_2^-$ can also accumulate.
Therefore, the circulating $\text{NO}_3^-$ concentration can exceed that of the surrounding water, and thus, $\text{NO}_3^-$ may also influence vertebrate reproduction by affecting steroid hormone balance by NO regulation (Fig 9). Essentially, NO appears to alter steroid hormone synthesis by inhibiting several steroidogenic enzymes or other major factors in the steroidogenic pathway including steroidogenic acute regulatory protein (StAR), and the enzymes P450-sidechain cleavage (Cyp11a1), 3-hydroxysteroid dehydrogenase (3-HSD) and aromatase (Berg 2004, Fig 10). Exposure of male fish to natural and/or synthetic estrogens, as well as to estrogen mimics, has resulted in feminization/sex-reversal, inhibition of testicular growth, spermatogenesis inhibition, decreased capacity to fertilize eggs, reduced male sex hormone (testosterone and/or 11-ketotestosterone) production, and altered reproductive behavior (Brian, Harris et al. 2005). $\text{NO}_3^-$ plays a significant role in predicting egg dry weight, where increases in ambient $\text{NO}_3^-$ concentrations lead to reduced weights (Hiramatsu, Hara et al. 2002). There is also a strong relationship between increased $\text{NO}_3^-$ and reduced reproductive activity among mature females (Edwards and Guillette 2007). These novel biomarkers are well suited to determine effect of nitrogenous compound on sex reversal and reproductive dysregulation in the aquatic environment.

Fig 9. Hypothesis for the role of $\text{NO}_3^-$ and $\text{NO}_2^-$ in NO generation in gonadal tissue. (Berg, 2004)
Stress and metabolism

The 70-kDa stress protein family (HSP70) plays vital roles in a assortment of physiological development, including protein chaperoning, defense against apoptosis, steroidogenesis, and general cellular stress responses, and has also been used as a biochemical marker of environmental stress, such as toxicant exposure (Yoo and Janz 2003). Upregulated HSP70 has thus far been detected in liver, gill, and ovarian tissues in response to pollutant challenges. NO\textsubscript{3} can influence NO synthesis as well as cellular ion concentrations and enzyme actions thereby bringing about expression of HSP70. One plausible mechanism involves alterations in thyroid function. Environmentally relevant concentrations of NO\textsubscript{3} have been shown to reduce thyroid function, feeding behavior, and growth rate in a variety of vertebrates including fish (Saeij, van Muiswinkel et al. 2003). The response of gene expression to chemical exposure is rapid and sensitive to low chemical concentrations but not necessarily specific to a given stressor. Amplification in the expression of HSP mRNAs was measured in a constitutively expressed form as well as in a stress inducible form (Lee, Lee et al. 2006). The importance of thyroid function during development and growth suggests that embryos, fetuses and juveniles could
be more susceptible than adults to the disruptive effects of NO$_3^-$ exposure and that NO$_2^-$, or its metabolites (NO$_2^-$ and NO) can influence some aspects of growth and metabolism (Evans 2002). Tissue specific differences and seasonal variation in expression of HSP70, as well as alterations in circulating testosterone levels has been observed in female fish chronically exposed to metals. For these reasons, future studies into the expression of HSP70 may offer valuable insight to the effects of nitrogenous pollution in aquatic environments.

**Conclusion**

Measurement of cortisol in fish fecal casts, in both aquaculture and natural settings, is a potentially valuable tool as it is both noninvasive and highly correlated to more commonly invasive methods, such as plasma monitoring. Performance of the cortisol ELISA developed in this study was validated by comparison to high performance liquid chromatography (HPLC), which has been used for measuring free glucocorticoids and glucocorticoid metabolites in feces of numerous mammalian species and has recently been incorporated into studies of fish physiology and behavior. Tests for parallelism and extraction efficiency showed that the ELISA and HPLC resulted in almost identical values. This is the first report of use of an ELISA for measurement of cortisol in fish feces, as RIA and HPLC are the most commonly used methods. The study has also shown that fish cortisol can be measured in fecal material by ELISA and this assay exhibits equal reliability and much greater efficiency and sensitivity than HPLC. From both per-sample assay time and personnel cost perspectives, the ELISA is more practical and less expensive than HPLC analysis, showing
that this cortisol ELISA has high potential as a sensitive, reliable and cost-effective tool for noninvasive stress assessment in fishes.

In the second part of the study, elevated NO$_3^-$ was shown to be a significant stressor for koi as evidenced by increased fecal cortisol, loss of appetite, color change, hyperventilation and lethargy. The dramatic cortisol response to incremental NO$_3^-$ and the return to near-basal cortisol levels after NO$_3^-$ dilution demonstrated that the homeostatic regulation of cortisol remained functional after NO$_3^-$ exposure. It also indicates that even partial reduction in aquatic NO$_3^-$ levels can facilitate return to normal feedback regulation of cortisol. The absolute levels of cortisol increased with each NO$_3^-$ addition, but these increases were not significant. This study also suggests that NO$_3^-$ intoxication of aquatic wildlife warrants more complete examination. While fecal cortisol measurement has been previously reported for marine fish, the present study is the first assessment of fecal cortisol in freshwater fish and demonstrates that this methodology can be useful as a noninvasive indicator of NO$_3^-$-induced stress.
References


