INTEGR. COMP. BIOL., 45:19–27 (2005)

Is Nitrate an Ecologically Relevant Endocrine Disruptor in Vertebrates?[1]

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SYNOPSIS. The last three decades have brought clear recognition that many populations of animals are experiencing severe declines or local and global extinctions. Many examples have become common knowledge to the general public, such as worldwide declines in amphibian populations and extensive loss of coral reefs. The mechanisms underlying these and other changes are poorly understood. However, a growing literature indicates that a wide array of chemical contaminants have the potential to disrupt normal cell-to-cell signaling mechanisms. A global pollutant of most aquatic systems, nitrate has the potential to be an endocrine disrupting contaminant. This paper reviews studies performed on vertebrates demonstrating that nitrate and/or nitrite have the potential to alter endocrine function. Further, a retrospective study of our work on alligators from various lakes in Florida suggests that nitrate could contribute to some of the altered endocrine parameters previously reported in juvenile animals. We propose hypotheses suggesting that nitrate could alter steroidogenesis by 1) conversion to nitrite and nitric oxide in the mitochondria, the site of initial steroid synthesis, 2) altering Cl^- ion concentrations in the cell by substituting for Cl^- in the membrane transport pump or 3) binding to the heme region of various P450 enzymes associated with steroidogenesis and altering enzymatic action. Future studies are needed to examine the endocrine disruptive action of this ubiquitous pollutant. A growing literature indicates that all biologists studying natural systems, whether they choose to or not, must now consider contaminant exposure as a direct influence on their studies. That is, ubiquitous global contamination has the potential to alter the endocrine, nervous and immune systems of all organisms with resulting changes in gene expression and phenotypes.

INTRODUCTION

A central focus of comparative physiology and endocrinology has been the influence of environmental factors on the development and performance of various systems or whole organisms. Over the last century, it has been clearly established that such factors as temperature, pH, salinity, photoperiod and gas tensions affect the endocrinology of vertebrates (Norris, 1997). As part of these studies, we have also become aware of the influence of human activities on the biology of numerous species. The endocrine-like actions of various chemical contaminants have been a recent focus of much research (see Ankley *et al.*, 1997; Guillette and Crain, 2000; McLachlan, 2001). These studies have investigated endocrine disrupting chemicals released from industrial activities, sewage treatment works, refuse dumps, confined animal feed operations and agriculture fields (see Noaksson *et al.*, 2001; Orlando *et al.*, 2004; Soto *et al.*, 2004). In addition, pharmaceuticals and other chemicals with endocrine-like activity have been identified in food products and drinking water (Kolpin *et al.*, 2002; Miyahara *et al.*, 2003).

Endocrine disrupting chemicals affect an organism's physiology through a number of mechanisms. They may mimic naturally occurring steroids, act as hormone receptor agonists or antagonists or alter the enzymes responsible for hormone synthesis and degradation (for reviews, see Crain *et al.*, 2000; Gray *et al.*, 2001; Rooney and Guillette, 2000). Using these definitions, synthetic chemicals such as pesticides, plasticizers or industrial chemicals and naturally occurring heavy metals and plant or fungal compounds have been defined as endocrine disrupting contaminants (EDCs). However, absent from this discussion have been the possible endocrine altering roles of ions, such as nitrates and nitrites, that occur at high levels as environmental pollutants (Sampat, 2000).

Each year, it is estimated that the human population consumes approximately 25 million tons of protein nitrogen, a figure expected to climb to 40–45 million tons by 2050 (Jenkinson, 2001). Currently, humans fix 160 million tons of nitrogen per year, of which 83 million tons is used as agricultural fertilizer. The extensive use of fertilizers has dramatically increased the combined nitrogen entering freshwater and estuarine habitats. In addition, an increasing number of reports have identified agricultural nonpoint source pollution as the leading source of water quality impacts to freshwater systems, including freshwater aquifers (Cassman *et al.*, 2002; Sampat, 2000). The USA has the third largest land area under irrigation for food production in the world with 43% of its annual groundwater use going to irrigation (Sampat, 2000). The impact of agricultural practices on groundwater quality is of particular concern because a majority of the population on American farms, both human and livestock, receive their drinking water supply from private wells. Many of these wells are shallow and are vulnerable to water pollution, especially from nitrate (Cassman *et al.*, 2002; Mitchell and Harding, 1996; Sampat, 2000). Waste from animal production and fossil fuels also contribute significantly to environmental nitrogen

[1] From the Symposium *EcoPhysiology and Conservation: The Contribution of Endocrinology and Immunology* presented at the Annual Meeting of the Society for Integrative and Comparative Biology, 5–9 January 2004, at New Orleans, Louisiana.

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loading. In the United States, nitrate contamination is the major reason public water supplies have been closed. The current public health standard for safe drinking water in the USA requires that nitrate not exceed concentrations of 10 mg/L (ppm) as nitrate-N or 45 mg/L (ppm) as nitrate (10 ppm nitrate-N ≈ 45 ppm nitrate)[3]. When nitrate in a water supply reaches or exceeds these drinking water standards, very costly measures must be taken.

Extensive, and continuing research has been performed on the ecological impacts of nitrogen loading. A large literature exists examining the influences of nitrate and nitrite loading on physiological performance of the vascular and digestive systems of terrestrial vertebrates exposed through food or drinking water, but relatively few studies examine the physiology of vertebrates living in eutrophic aquatic systems. In large part, this lack of study has been due to the belief that inorganic nitrate and nitrite ions affect plant and bacterial life but have little direct physiological impact on multicellular animals, especially vertebrates. A growing number of studies suggest that nitrate and nitrite ions act on many systems and could serve as direct precursors for the production of nitric oxide, a potent physiological regulator in vertebrates. This commentary proposes a hypothesis relating direct disruption of the reproductive endocrinology of wildlife with exposure to nitrate rich, eutrophic freshwater environments. Thus, nitrates could pose a direct threat to the conservation and restoration of vertebrate populations and the ecosystems they depend on for survival.

REPRODUCTIVE-ENDOCRINE ALTERATIONS WITH NITRATES

Nitrogenous compounds have become a major global pollutant in freshwater and estuarine ecosystems. Two compounds with known physiological influence are nitrate and nitrite (Jensen, 2003; Levallois and Phaneuf, 1994; Zraly *et al.*, 1997). Nitrate and nitrite have been reported to be toxic in humans and animals for decades (Avery, 1999). As early as 1945, methemoglobinemia (Blue Baby syndrome) was associated with drinking nitrate-contaminated well water on farms from the Midwest USA. Methemoglobin is formed during nitrate-induced oxidation of hemoglobin. This prevents normal oxygen binding and leads to hypoxia. Methemoglobinemia, as well as additional concerns, continue today with increasing nitrate contamination of ground water (Avery, 1999; Porter *et al.*, 1999). Nitrogen-laden rainwater discharges have also been associated with algal blooms, fish kills and anoxic conditions in many freshwater systems. Although public drinking water from modern utilities falls below the 10 mg/L (ppm) NO_3-N limit, as required by law, contamination of rural water supplies, aquifers, rivers, ponds and farm wells continues to occur (Porter *et al.*, 1999; Sampat, 2000). For example, in one study, 18% of Iowa's private drinking water wells were contaminated with more than 10 mg/L NO_3-N (Kross *et al.*, 1993). In Florida, a number of springs discharging directly from the aquifer have levels >20 mg/L of detectable NO_3-N (Katz *et al.*, 1999).

Mortality and developmental changes with nitrate

The effects of nitrate on various species range from gross toxicity to more subtle changes in physiology and development. For example, mortality of larval cutthroat trout, Chinook salmon, and rainbow trout occurred at nitrate concentrations ranging from 2.3–7.6 mg/L NO_3-N (Kincheloe *et al.*, 1979). Survival of Chorus frog and Leopard frog tadpoles was significantly decreased after exposure to 10 mg/L NO_3-N (Hecnar, 1995). In contrast, the 96-hour LC_{50} (median lethal concentration) for fathead minnow larvae was 1,341 mg/L NO_3-N, whereas it is 462 mg/L NO_3-N for adult *Daphnia magna* (Scott and Crunkilton, 2000).

Metabolic effects of nitrate have been observed in several frog species. Toad tadpoles (*Bufo bufo*) exposed to 11 or 23 mg/L NO_3-N began and completed metamorphosis earlier and grew faster than controls. Exposed toads also tended to have unusual swimming patterns and deformities, including missing or deformed forelimbs and toes (Xu and Oldham, 1997). Cascades frogs (*Rana cascadae*) exposed to 3.5 mg/L NO_2-N metamorphosed more slowly than controls, but emerged from the water at the same time as controls, even though they were less developed (Marco and Blaustein, 1999). These examples suggest that sensitivity to nitrate varies greatly among species and is stage of development dependent. Further, these data suggest that at least one endocrine axis involving the thyroid could be influenced, as metamorphosis and growth in tadpoles is significantly influenced by thyroid hormones.

Thyroid alterations with nitrates

A number of studies have observed alterations in various endocrine parameters associated with dietary or experimental exposure to nitrates. In the early 1950s, iodine uptake in rats was shown to be depressed

[3] ***A note on the reporting of nitrate concentrations***—Nitrate (NO_3) and nitrite (NO_2) concentrations in water are reported using various approaches. Traditionally, water quality for aquacultural settings, surface water, and agricultural runoff has been reported as NO_3-N, or nitrate-nitrogen. This measure actually represents the amount of nitrate and nitrite in the sample, given that the two molecules usually exist in some sort of equilibrium. Generally 95–97% of the NO_3-N will be nitrate, and the remaining 2–5% will be nitrite, although this depends on other factors such as microbial action and fish population density. Some experimental studies report concentrations of NO_2-N because nitrite was specifically dosed. Nitrate-nitrogen (or nitrite-N) represents the concentration of nitrogen molecules only, based on molecular weight. Therefore, the concentration of nitrate (NO_3), for example, is approximately 4.4 × the value reported as NO_3-N because the NO_3 measurement must include the weight of the oxygen. Aquatic nitrogen concentration may also be reported as total nitrogen (TN). Total nitrogen consists of dissolved and particulate organic and inorganic nitrogen, not including N_2 gas. NO_3-N and TN are reported in parts per million (ppm), mg per liter (mg/L) or millimolar (mM) units. In contrast, physiological studies report NOX concentrations, which represent nitrogen associated with nitrate, nitrite, and nitric oxide. Depending on the literature examined, any of the above reporting conventions can be used.

by nitrate, with subsequent alterations in thyroid gland morphology and function (Wyngaarden *et al.*, 1952, 1953). Altered uptake of iodine has been demonstrated in humans (Ellis *et al.*, 1998), domesticated mammals (Pisarikova *et al.*, 1996; Zraly *et al.*, 1997) and fish (Lahti *et al.*, 1985). Lahti *et al.* (1985) also demonstrated that this occurred in multiple fish species and was not limited to iodine uptake by the thyroid—other tissues were affected as well. This effect occurred at ecologically relevant, although elevated, concentrations of nitrate in water (77 mg/L NO_3-N). Adult bulls receiving oral potassium nitrate (100 g/day), also exhibited altered iodine uptake with depressed thyroid gland activity. Indeed, decreased thyroxine concentrations were observed during the period of administration (Zraly *et al.*, 1997). Further, thyrotropin levels were non-detectable for up to 35 days post administration suggesting an influence on the pituitary axis as well. Nitrate (4 g/animal/day in feed) was also goitrogenic in sheep, and their offspring if exposure occurred during pregnancy (Kursa *et al.*, 2000). The goitrogenic effect of nitrate could be suppressed with dietary supplements of iodine. In combination with various herbicides, nitrate also affected thyroxine concentrations in rodents (Porter *et al.*, 1999).

Androgen alterations with nitrates

The studies described above suggest that nitrates can be disruptive of the thyroid axis. Additional studies using rodents or *in vitro* cell culture (mouse Leydig tumor cells—MLTC-1) indicate possible endocrine disruptive actions for dietary nitrates. Inorganic nitrate has been demonstrated to inhibit gonadotropin-induced androgen synthesis from mammalian testicular Leydig cells *in vitro* and androgen synthesis *in vivo* (Panesar, 1999; Panesar and Chan, 2000). These studies followed an observation that nitrate/nitroglycerin therapy in humans influenced circulating androgen concentrations, as well as blood pressure. The *in vitro* study reported a dramatic reduction in Leydig cell steroidogenesis at pharmacological doses of nitrate and nitrite and decreased responsiveness of Leydig cells to gonadotropin stimulation (Panesar, 1999). Drinking water experiments with nitrate (50 mg/L $NaNO_3$) reported that exposed male rodents had significantly decreased circulating T concentrations (Panesar and Chan, 2000). These researchers proposed several mechanisms, including one involving nitrate to nitric oxide conversion (see discussion below).

Oral administration of nitrates to bulls (100–250 g/day/animal) also depressed the function of the Leydig cell during and particularly after the administration period (Zraly *et al.*, 1997). In particular, the Leydig cells of the treated bulls appeared less responsive to gonadotropin stimulation, as reported above for rats *in vitro.* Treated bulls also had increased acid phosphatase activity and reduced fructose concentration in their semen. Further, these bulls showed reduced sperm motility, increased secondary sperm abnormalities, and degenerative lesions in the spermatocyte and spermatid germ layers of the testis (Zraly *et al.*, 1997). These effects are similar to those reported in various human populations worldwide (Toppari *et al.*, 1996).

Nitrates and endocrine disruption in wildlife populations

What is the relevance of the above medical, laboratory or farm based studies? Nitrate and nitrite levels have increased in many aquatic systems worldwide due to human activity. The regulation of nitrogen pollution has become one of the priorities for ground and drinking water managers (Knox and Canter, 1996) and should be a major concern for conservation and ecosystem restoration plans as well. Over the last decade, we have become increasingly aware that environmental contaminants act through multiple mechanisms to alter endocrine functioning in vertebrate and invertebrate species (Guillette and Crain, 2000). One of the most common observations is an alteration in steroidogenesis, in particular androgen synthesis and function in male vertebrates (see Rodgers-Gray *et al.*, 2000). For example, fish obtained downstream from sewage treatment plants not only display elevated yolk protein concentrations in their blood—an estrogenic response—but also exhibit reduced plasma androgen concentrations (Folmar *et al.*, 1996). Our laboratory has also reported alterations in the synthesis, plasma concentrations and hepatic metabolism of androgens in alligators living in eutrophic lakes with exposure to pesticides (Guillette *et al.*, 2000; Gunderson *et al.*, 2001). These data and the many other studies from laboratories worldwide require that we examine factors that are ''antiandrogenic,'' irrespective of mechanism or class of compound (Gray *et al.*, 2001).

Alligators, endocrine disruption and eutrophic lakes

Contaminants can induce or suppress normal endocrine responses. Alligators living in several Florida lakes have endocrine alterations that are worse if the animals remain in a polluted lake *versus* being held in clean water after hatching (Guillette *et al.*, 2000; Rooney, 1998). Examinations of the reproductive and endocrine systems of hatchling and juvenile male alligators from Lake Apopka have demonstrated elevations in plasma estradiol-17β (E_2), reductions in the androgens testosterone and 5α-dihydrotestosterone (T and DHT) and altered thyroxine concentrations. In addition, we have observed morphological abnormalities of the testis, phallus and thyroid (Guillette *et al.*, 1999*b*; Pickford *et al.*, 2000). The alterations in plasma hormone concentrations also occur in females but females display a more complex pattern (Guillette *et al.*, 2000). During the first year of life, female alligators exhibit elevated plasma concentrations of E_2 that then drop to concentrations at or below those reported in reference populations during their juvenile years (Guillette *et al.*, 1999*b*). Altered endocrine parameters in juvenile alligators also occur in other Florida wetlands not associated with significant pesticide spills or point source contamination, such as Lake Okeechobee

TABLE 1. *Regression analyses between mean plasma sex steroid concentrations in juvenile alligators and mean lake water concentration of various nitrogenous pollutants/components in seven Florida lakes.**

Hormone	Sex	Total N	NO_3	NH_4	Organic N
Testosterone	Male	−0.46 (0.09)	−0.34 (0.16)	0.08 (0.59)	−0.24 (0.26)
	Female	**−0.56 (0.03)**	−0.47 (0.09)	−0.19 (0.38)	−0.11 (0.46)
	Both	−0.483 (0.08)	−0.33 (0.18)	0.005 (0.91)	0.06 (0.65)
Estradiol	Male	**0.59 (0.04)**	0.02 (0.74)	0.17 (0.41)	0.31 (0.19)
	Female	0.23 (0.28)	**0.62 (0.03)**	0.005 (0.88)	0.32 (0.18)
	Both	0.21 (0.16)	0.005 (0.98)	0.14 (0.54)	0.08 (0.59)

* The seven Florida lakes examined, include; lakes Woodruff, Apopka, Orange, Monroe, Jesup, Okeechobee, Griffin. Data are presented as r^2 (*P* value). Information on lakes can be found in Guillette *et al.* (1999*b*). Bold = significant.

and Lake Griffin, FL, USA (Guillette *et al.*, 1999*b*). Importantly, we have previously examined the relationship between plasma organochlorine (OCs) pollutants and steroid hormone concentrations in juvenile alligators and found no relationship (Guillette *et al.*, 1999*a*). Plasma hormone concentrations in juvenile alligators from Orange Lake (low OCs, high total nitrogen) and Lake Apopka (high OCs, high total nitrogen) are similar whereas they differ significantly from concentrations found in alligators from Lake Woodruff (low OCs and total nitrogen). This does not mean that pesticides and other pollutants do not influence the endocrinology of alligators. We have shown that developmental exposure to pesticides alters steroidogenesis in the neonatal gonad at concentrations similar to levels occurring in eggs (Crain *et al.*, 1997). In addition, recent gonadotropin challenge studies have documented significant depression in the testicular steroidogenic response of males obtained from Lake Apopka mothers, but raised in captivity under identical conditions with males originating from a reference lake (Edwards and Guillette, unpublished data). These data demonstrate clear organizational abnormalities. Organization in this context refers to the ordering of tissues and signaling patterns during development (see Guillette *et al.*, 1995). Embryonic organization determines the subsequent capacity of cells and tissues to respond to the variety of stimuli encountered during an organism's life. Response to stimuli is referred to as activation. We hypothesize that the abnormalities seen in the juvenile alligators represent both organizational changes due to embryonic exposure to pesticides coupled with activational modifications due to other pollutants, including nitrate, in the lakes.

A first test—a retrospective analysis

Retrospective studies are seldom used in physiological ecology, but readily used in epidemiological and public health studies because they detect patterns that help direct prospective studies that can identify causal agents. A retrospective analysis of sex steroid concentrations in juvenile alligators from eutrophic Florida lakes suggests that some component of the nitrogenous pollutants could contribute to the problems reported previously.

In the spring of 1995, our group examined the endocrinology of juvenile alligators from seven lakes in Florida (see Guillette *et al.*, 1999*b*). We observed significant differences in plasma androgens and estrogens, as well as altered morphological parameters such as penis size, among the populations of alligators inhabiting the study lakes. At that time, we obtained basic lake water parameters such as water temperature and pH but no data on total nitrogen or nitrate/nitrite concentrations. During the spring of 1995, and other seasons as well, various water quality measurements were documented by other researchers for the seven lakes we studied (data were obtained from the US EPA STORET http://oaspub.epa.gov/storpubl/legacy and St. John's Water Management District). Using the water quality data available for samples obtained in the middle of each lake during the spring of 1995 (the same period in which the alligator samples were obtained), we performed a regression analysis (Table 1) to determine if a relationship existed between mean total nitrogen, nitrate, organic nitrogen, pH or ammonia levels in lake water and mean plasma concentrations of testosterone in juvenile alligators. Water samples collected at the center of lakes are considered a better measurement of nitrogen availability, as values obtained at the marshy periphery of lakes can be dramatically altered due to decaying vegetation and photosynthetic activity.

Results of the regression analysis indicate that mean total nitrogen in lake water was inversely correlated with plasma testosterone (T) concentration in juvenile male (Fig. 1A) and female alligators (Table 1). In males, the relationship does not appear to be linear, but a 2 factor polynomial regression generated a highly significant inverse relationship between plasma T concentration and total nitrogen ($R^2 = 0.81$; $P = 0.037$) and nitrate ($R^2 = 0.9$; $P = 0.01$). For males and females, much of the relationship was driven by the values for Lake Woodruff, the only oligotrophic lake. If Lake Woodruff is removed from the analysis, a negative, linear relationship was seen between plasma T concentrations and total nitrogen concentration ($r^2 = 0.51$; $P = 0.05$) for the eutrophic lakes (Fig. 1B–data shown for males only; results for females are similar). These data suggest that some component of the total nitrogen could influence the regulation of plasma T concentrations. Organic N and ammonium concentrations do not seem to explain the relationship, but nitrate concentrations are suggestive (Table 1).

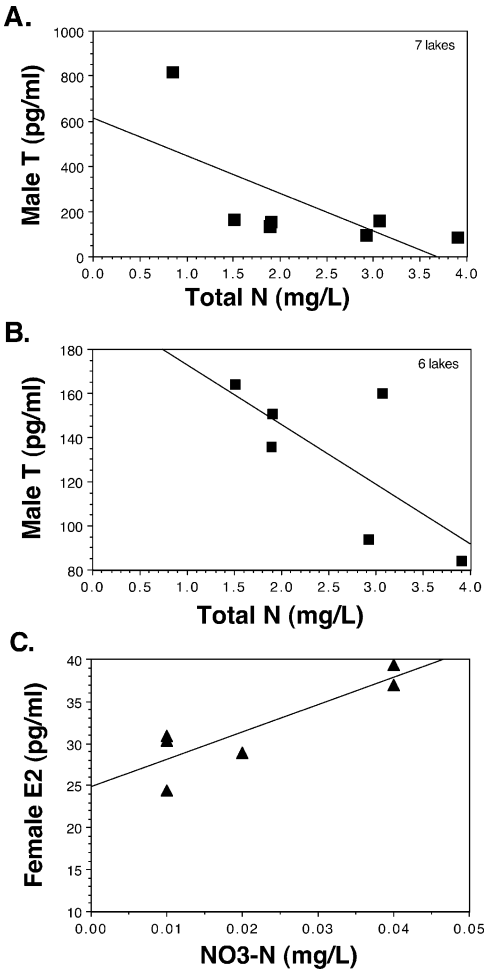

FIG. 1. A. Relationship between lake water total nitrogen and plasma testosterone concentration in juvenile male alligators from seven Florida lakes. B. Relationship between lake water total nitrogen and plasma testosterone concentration in juvenile male alligators from six Florida lakes. The lake removed for panel B is Lake Woodruff, the most oligotrophic lake. C. Relationship between lake water NO_3-N and plasma estradiol concentration in juvenile female alligators from six eutrophic Florida lakes (Apopka, Orange, Monroe, Jesup, Okeechobee, Griffin). Information on the seven lakes (Woodruff, Apopka, Orange, Monroe, Jesup, Okeechobee, Griffin) can be found in Guillette *et al.* (1999*b*).

Converse to results with testosterone, plasma E_2 concentrations exhibited a positive relationship with total nitrogen concentration in males but was weak for females (Table 1). However, a significant positive relationship between mean plasma E_2 and lake water nitrate concentrations was observed among females, even if data from Lake Woodruff were removed (Table 1; Fig. 1C).

As mentioned above, these data suggest the hypothesis that a component of the total nitrogen content of the lake water, or a factor that covaries with total nitrogen content, influences the regulation of androgen and estrogen concentrations in the plasma of juvenile alligators. This analysis was performed to help inform further study and should not be taken as positive support of the hypothesis but must be viewed with caution, as 1) it represents a retrospective study using means and not integrating variation in pollution and hormone levels in each population, and 2) the range over which the nitrate and hormones vary is relatively small. However, it is important to note that these relatively small changes in average hormone concentration in juvenile alligators have been seen repeatedly and were related to significant changes in the morphology of the penis and gonad as well as changes in liver function with regard to enzymes associated with steroid hormone metabolism (Guillette *et al.*, 1999*b*; Gunderson *et al.*, 2001). These data do not provide evidence of a specific mechanism, nor even provide specific associative evidence given that different teams collected the data sets. However, these data indicate that the hypothesis that nitrates can alter steroid regulation warrants further study. Prospective studies examining lake water concentrations matched with plasma and urine nitrate concentrations from specific animals are needed so that variation within and between populations can be included in an analysis. The current analysis among populations indicates that 45% of the variance or more in plasma sex steroids can be explained by either total nitrogen or nitrate concentration in lake water. Within a population, serum OC concentration explains less than 15% of variance in plasma sex steroid concentrations in juveniles (Guillette *et al.*, 1999*a*). It should be noted that similar statistical analyses comparing plasma T or E_2 concentrations in juvenile alligators to other lake water quality factors such as dissolved oxygen, total phosphate, pH or temperature showed no relationships.

NITRATES AND STEROIDOGENESIS: POSSIBLE MECHANISMS

Nitrate and nitrite ions are highly soluble in water and form water-soluble salts with many cations, like potassium and sodium. Dietary intake of nitrate results in ready absorption by the proximal small intestine (Walker, 1996). Transport across the membrane is apparently controlled by a nitrate/H^+ cotransporter (Chow *et al.*, 1997). This cotransporter system could have special implications for some of the endocrine actions reported below. Dietary nitrate and nitrite are used by oral and gastric bacteria to synthesize nitric oxide, implicated in gut physiology and immune functions (Benjamin *et al.*, 1994; Walker, 1996). Nitrate is readily found in the plasma, can be found in sweat, rarely occurs in the feces, and is primarily excreted by the mammalian kidney in the urine as nitrate or urea (Ellis *et al.*, 1998). Plasma and urine concentrations increase with dietary intake of nitrate (see Ellis *et al.*, 1998).

In 1995, it was reported that vertebrate mitochondria are capable of NO synthesis via 'non-enzymatic'–non nitric oxide synthase (NOS)–activity (Zweier *et al.*, 1995). Since those initial studies, a growing literature supports the observation that mitochondria obtained from vertebrates under stressful conditions can produce NO using nitrite as a precursor (Cadenas *et al.*,

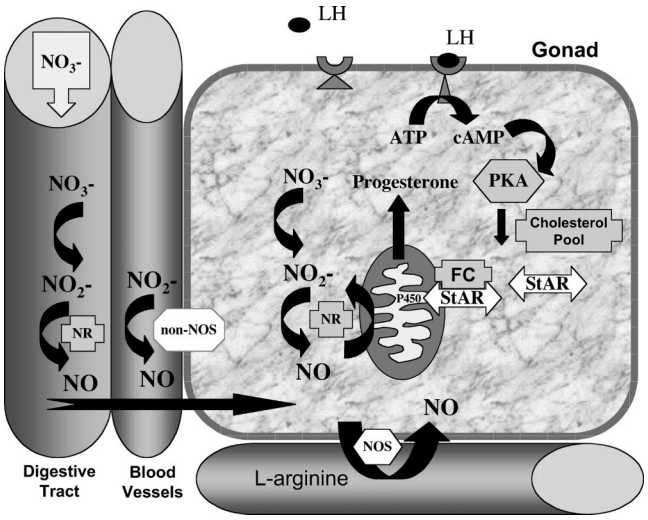

FIG. 2. Schematic hypothesis for the role of nitrate (NO_3-) and nitrite (NO_2-) in nitric oxide (NO) generation in gonadal tissue. NO_3- and NO_2- enter via the gut and are transported to the gonad via the vascular system. Gut bacteria have nitrite reductase (NR) activity and can generate NO from NO_2- locally. It has also been suggested that NR activity is present in mitochondria and endoplasmic reticulum, although several studies indicate that NO_2- can be converted to NO without enzymatic action. NO is readily generated from L-arginine by nitric oxide synthase (NOS). NO has been shown to inhibit gonadotropin (LH) induced steroidogenesis via its actions on the Steroid Acute Regulatory protein (StAR) or the enzyme $P450_{scc}$ (see text). This figure is modified from Panesar and Chan, 2000.

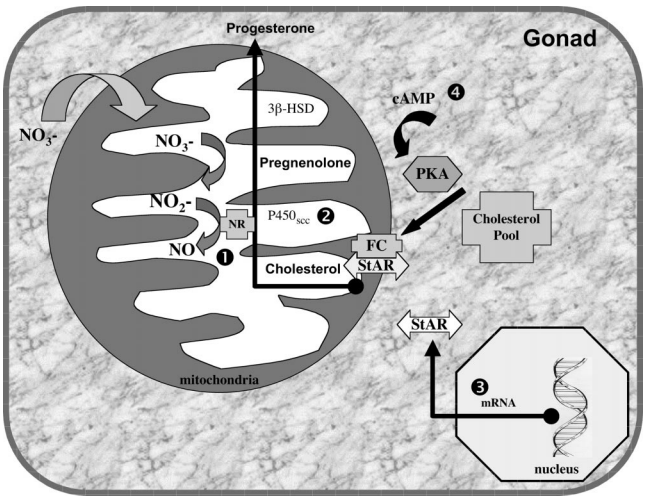

FIG. 3. Schematic representation of actions in the mitochondria of steroidogenic cells in the gonad. Protein kinase A is activated by cAMP, freeing cholesterol from the cytoplasmic pool. Free cholesterol (FC) is transported across the mitochondrial membrane by Steroid Acute Regulatory Protein (StAR). This cholesterol serves as the precursor for steroidogenesis and the enzymes $P450_{scc}$ and 3β-HSD produce pregnenolone and progesterone that is released back into the cytoplasm. Progesterone serves as a precursor for the production of androgens and estrogens. Nitrate (NO_3-) has the potential, either by direct action or via the production of nitric oxide (NO) ❶, to alter P450 enzyme activity ❷, alter gene expression and thus transcription or translation of StAR ❸ or alter cAMP production ❹ by altering cytoplasmic ion concentrations, such as modifications in chloride ion concentration.

2000; Kozlov *et al.*, 1999; Lepore, 2000). Nitric oxide is a potent cellular signal, used in a wide variety of regulatory physiological pathways. The basis for the non-NOS synthesis of NO is still under intense study and debate exists as to whether this phenomenon occurs in healthy tissue. It has been suggested that other enzymes can generate NO from nitrate (see Meyer, 1995; Nohl *et al.*, 2001; Nohl *et al.*, 2000). Zweier and colleagues have shown that a change in pH (7.4 to 5.5) alone is capable of generating NO from nitrite in biological tissues (Samouilov *et al.*, 1998; Zweier *et al.*, 1999). In short, nitrites could be used *in vivo* to generate NO and other reactive oxides that would be disruptive to cellular function (Fig. 2). Multiple mechanisms could be involved depending on the tissue, physiological state of the organism and environment. Well established actions of NO have been observed on steroidogenesis in humans, laboratory species and cell cultures (for examples, see Del Punta *et al.*, 1996; Kostic *et al.*, 1998; Van Voorhis *et al.*, 1994; Weitzberg and Lundberg, 1998). Both stimulatory and repressive roles of NO have been described.

Alternative hypotheses could also explain the observations related above. For example, alterations in chloride ion concentration in steroidogenic cells could alter hormone synthesis as 1) nitrate is known to substitute for chloride in the chloride-bicarbonate membrane transporter system and 2) chloride ion concentrations are known to influence steroidogenesis (depletion augments steroidogenesis [Cooke *et al.*, 1999; Panesar, 1999; Ramnath *et al.*, 1997]). This latter observation could explain the positive relationship between lake water nitrate and estradiol observed in juvenile alligators. However, it does not explain the depression in testosterone.

The molecular and cellular activities driving steroidogenesis have been studied intensely. These include the activity of several factors regulating steroid synthesis, such as the steroidogenic acute regulatory (StAR) protein and the enzyme $P450_{scc}$ (Fig. 3). StAR regulates entry of cholesterol (the basic steroid hormone precursor) into the mitochondria, prior to steroidogenesis (Stocco and Clark, 1996). $P450_{scc}$ modifies cholesterol in the mitochondria prior to the formation of pregnenolone, which is converted to progesterone and subsequently all other steroid hormones (Fig. 3). Several reports indicate that pesticides can alter transcript levels of StAR and $P450_{scc}$ as well as modify the cellular activity of these proteins (Stocco and Clark, 1996; Walsh and Stocco, 2000; Walsh *et al.*, 2000). NO has also been shown to alter StAR expression and activity. Furthermore, we have observed that steroidogenesis was depressed in the MA-10 human Leydig cell line following exposure to nitrate and nitrite exposure (Stocco and Guillette, unpublished data). In addition, nitrate, nitrite and NO could influence the action of cytochrome $P450_{scc}$ and other P450 enzymes, (essential for steroidogenesis), by binding to the heme group that characterizes all enzymes of the

P450 superfamily (Danielson, 2002). When NO binds to the heme group it usually inhibits enzymatic action (White *et al.*, 1987). These data suggest that alterations of enzymatic activities of various P450s in the liver and gonad could also be the basis for our observations. That is, it is plausible to hypothesize that observations of altered plasma steroids in contaminant-exposed juvenile and adult alligators are, in part, a response to nitrogenous pollutants. In summary, we suggest that nitrate/nitrite exposure can act directly on steroidogenesis, especially those steps occurring in the mitochondria as well as steroid biotransformation activities in the liver. The relationships presented here are illustrated in Figures 2 and 3.

Future studies need to examine the interaction between nitrate pollution of aquatic systems and the physiology of the organisms living in those systems. It is very likely that future studies will document direct effects of these pollutants on the endocrine systems of organisms. Given the central role of the endocrine system in reproduction, behavior, immune function, growth and metabolism, it is essential that we begin broad scale studies. Nitrogen pollution will continue to grow during this century as human populations grow.

ECOTOXICOLOGY, CONSERVATION AND THE FUTURE

Over the last decade, it has become increasingly obvious that the outcomes and conclusions from studies of wild populations, whether vertebrates or invertebrates, can be influenced dramatically by the contaminant exposure history of the animals under study. These studies, some of which have been presented above, document the sublethal effects of contaminants on the cellular signaling mechanisms of animals (McLachlan, 2001). They suggest that researchers examining the biology of any and all species should investigate the contaminant history of the ecosystem they work in. In short, all biologists studying natural systems, whether they choose to or not, must now consider contaminant exposure as a direct influence on their studies. That is, ubiquitous global contamination has the potential to alter the endocrine, nervous and immune systems of all organisms with resulting changes in gene expression and phenotypes. Contaminants act as one major factor by which the environment alters the expression of the genotype and thus the phenotype (Fox, 1995; Iguchi *et al.*, 2001). These changes are associated with altered reproductive potential, developmental pathways, behavior and survival. Conservation biology, with its goal of preserving and restoring populations and ecosystems, must actively embrace this new world view, and incorporate an understanding of the multiple mechanisms by which contaminants alter the biology of organisms, populations and ecosystems. To do less, would be to exclude a major factor influencing the potential success of such programs.

As we move forward with our studies, we must continue to appreciate that laboratory or even microcosm studies do not represent the temporal and spatial complexity in contaminant exposure seen by the organisms we study. It has become increasing obvious that future studies must examine the organizational as well as activational roles of pollutants (see Guillette *et al.*, 1995). The use of the organization/activation concept, originally derived from the study of neuroendocrinology and behavior, to understand the possible influences of chemical contaminants on the biology of organisms, is just one example of the future need for truly integrative biology. Toxicology is a science of integration, using concepts and theory from the fields of chemistry, biochemistry, physiology, genetics, population biology and probability mathematics, to name but a few. Modern ecotoxicology must embrace our growing understanding of the complexity and variation associated with the biology of organisms, from genomic to ecosystem levels of organization. To do less, would guarantee that we continue to underestimate the impact of chemical pollution on the world's ecosystems.

ACKNOWLEDGMENTS

We thank a number of colleagues for valuable discussions concerning nitrates, steroidogenesis and endocrine disruption. They include Tamatha Barbeau, Doug Stocco, John McLachlan and Taisen Iguchi. The analysis of nitrate effects in wildlife and preparation of this manuscript was made possible by a grant from the Homeland Foundation to LJG.

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