

Evaluation of Serum Testosterone Levels and Anogenital Distance in Mouse Pups after *in utero* Exposure to Nitrate

**By
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April, 2014**



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Department of Biology**

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**A Senior Comprehensive Project in Partial Fulfillment of the
Requirements
for a Bachelor of Science Degree from Allegheny College**

*I hereby recognize and pledge to fulfill my responsibilities as defined in the Honor
Code
and to maintain the integrity of both myself and the College community as a whole.*

Pledge

Approval by the Senior Thesis Committee:

Dr. 1st Reader

Dr. 2nd Reader

Abstract: Nitrate is an endocrine disrupting compound consumed in preserved meats and water.

Nitrate can inhibit testosterone production by disrupting the enzyme cytochrome 450sc. This experiment exposed pregnant dams to nitrate via drinking water from gestational day ten until birth. Treatments of 50 mg/L and 200 mg/L of sodium nitrate were administered. The anti-androgenic drug flutamide was used as a positive control. Weight, anogenital distances (AGD), and serum testosterone levels of the offspring were measured as endpoints of sexual development. Trunk blood was harvested and analyzed by ELISA for serum testosterone. Nitrate exposure of the mother did not affect the weights (males: $p=0.5017$, females: $p=0.3063$), the AGDs (males: $p=0.4186$, females: $p=0.9176$), or the serum testosterone (males: $p=0.6773$, females: $p=0.6773$) of three day old pups. The AGDs of the male pups were 0.234 mm (ANOVA, $p = 0.002$) longer than the AGDs of the female pups and the serum testosterone of the male pups was 147.496 pg/ml ($p < 0.0001$) higher than that of the females. It is possible that the placenta served as a protective barrier to nitrate during fetal development. Further research is needed to determine the potential endocrine disrupting effects of nitrate.

Introduction:

I. Endocrine Disrupting Chemicals: Endocrine disrupting chemicals (EDCs) are substances that have the ability to impact the endocrine systems of living organisms. They can act by mimicking hormones and binding to receptors, blocking natural hormones from binding, or affecting the metabolic pathways of hormones (Diamanti-Kandarakis *et al.* 2009). Some EDCs are mass produced by industries, and the list of potential EDCs continues to expand. In recent years the scientific community has begun to identify EDCs and potential modes of action, but due to the rapid growth in types of EDCs and the lack of funding of the scientific community, progress is slow (EPA 1994). The first step toward regulation and policy-making is the recognition of a substance as an EDC.

Unfortunately, the effects of some chemicals are difficult to identify, due to low-dose effects or other variables (Vandenberg *et al.* 2012).

II. Nitrates: Amongst the highly toxic substances such as diethylstilbestrol (DES) and atrazine is a relatively unassuming group of compounds: nitrates. Every year, industries produce 160 million tons of nitrogen, 83 million tons of which is agricultural fertilizer. The widespread use of fertilizer on American farms inevitably results in the contamination of drinking water in private wells that are shallow and vulnerable to pollution (Guillette and Edwards 2005). Furthermore, nitrate and nitrite are both used as preservatives for cured meats (Panesar and Chan 2000) and humans consume approximately 25 million tons of protein nitrogen per year (Jenkinson 2001). In addition, nitrite and nitrate are formed during the nitrification of ammonia by bacteria and absorbed from the soil by plants (Panesar and Chan 2000). In deforested landscapes, however, nitrate is not reabsorbed into plant tissue, and levels in stream water can be nearly fifty times higher than those in undisturbed landscapes (Likens and Bormann 1974). Some estimates for the mean dietary intake of nitrates by humans are as high as 194 mg per day for European countries (Walker 1996). Moreover, there is a growing body of evidence reporting that nitrates can have deleterious effects on the process of steroidogenesis in vertebrates (Panesar 1999; Panesar and Chan 2000; Zrally *et al.* 1997).

III. Steroidogenesis: Steroidogenesis is the process by which steroids are produced in the body using cholesterol as a precursor. For male vertebrates, steroidogenesis begins with the secretion of gonadotropin-releasing hormone from the hypothalamus, which stimulates the release of gonadotropins (particularly luteinizing hormone) from the anterior pituitary gland. When luteinizing hormone reaches the seminiferous tubules in the testes, it binds to receptors on the cell membrane of the Leydig cells and induces the synthesis of cAMP from ATP. From here, cAMP stimulates the activity of protein kinase A, which stimulates the transport of cholesterol into the mitochondria. Once inside the mitochondria, cholesterol is converted to pregnenolone (a testosterone precursor)

by the enzyme P450 side-chain cleavage (p450scc) (Haider 2007). The end result is production of testosterone, which controls the development of male reproductive tissues as well as male-type behaviors (Panesar and Chan 2006). Once testosterone leaves the Leydig cell it exists as one of three subtypes in the body. For humans, only about 2 percent of circulating testosterone is in the free form and biologically active. Sex hormone-binding-globulin (SHBG)-bound testosterone makes up approximately 40-50 percent of total testosterone. SHBG is produced in the liver and helps regulate the amount of free testosterone in the body by rendering it inactive. Albumin-bound testosterone is also inactive, but can be easily converted into free testosterone (Nussey and Whitehead 2001).

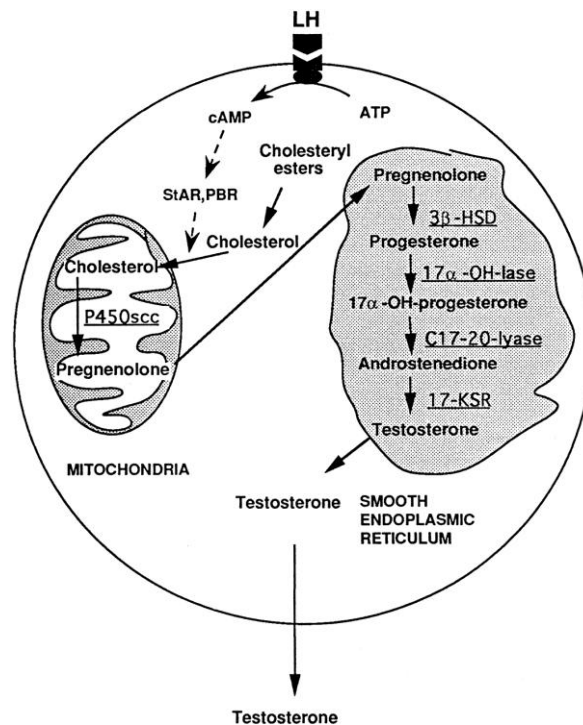


Figure 1. **Testosterone Production by Leydig Cells.** (Zirkin and Chen 2000)

IV. Role of Testosterone during *in utero* Male Reproductive Development: During fetal development the testes secrete androgens to induce the formation of the male phenotype (O'Shaughnessy *et al.*1998). In particular, testosterone produced in the fetal testis is essential for

the maintenance of the Wolffian duct, which differentiates into the epididymis, vas deferens, and seminal vesicles. Further, the development of Sertoli and Leydig cells within the testis depends entirely on local androgens. It is likely that any disturbance of testicular development during early life will have repercussions on the normal development of the reproductive system (Sharpe 2001). The blood-testis barrier, which is formed between the blood vessels and the seminiferous tubules, normally prevents the passage of toxins into the seminiferous tubules. However, in both humans and rodents, this barrier is not formed until juvenile development, leaving fetuses particularly vulnerable to endocrine disrupting chemicals (Bergmann and Dierichs 1983; Latini *et al.* 2006). Hypospadias, cryptorchidism, and decreased anogenital distance in male offspring are common birth defects resulting from prenatal EDC exposure. *In utero* exposure of rats to di(n-butyl) phthalate, vinclozolin, and 2,3,7,8-tetrachlorodibenzo-p-dioxin have all resulted in decreased anogenital distances (AGD) in male offspring. Thus, anogenital distance has been identified by the U.S. Environmental Protection Agency as an endpoint for reproductive toxicity studies in mammals (Hsieh *et al.* 2008).

V. Nitrates as Disruptors of Steroidogenesis: Initial research with nitrates as EDCs led to the realization that nitric oxide (NO) was the inhibiting agent of steroidogenesis (Panesar and Chan 2000). Nitrate and nitrite ions are easily converted to nitric oxide and vice versa by metabolic processes of mammals *in vivo* (Ellis *et al.* 1998); therefore, an increased body burden of nitrate directly correlates to increased levels of the steroid inhibiting agent NO. NO likely inhibits steroidogenesis via its action on the mitochondrial enzyme P450_{scc}, which is essential in converting cholesterol to pregnenolone, the first step in specialized steroidogenesis (Del Punta *et al.* 1996). NO may inhibit cholesterol side-chain cleavage enzyme (cytochrome P450_{scc}) by simply binding to the heme group (Guillette and Edwards 2005).

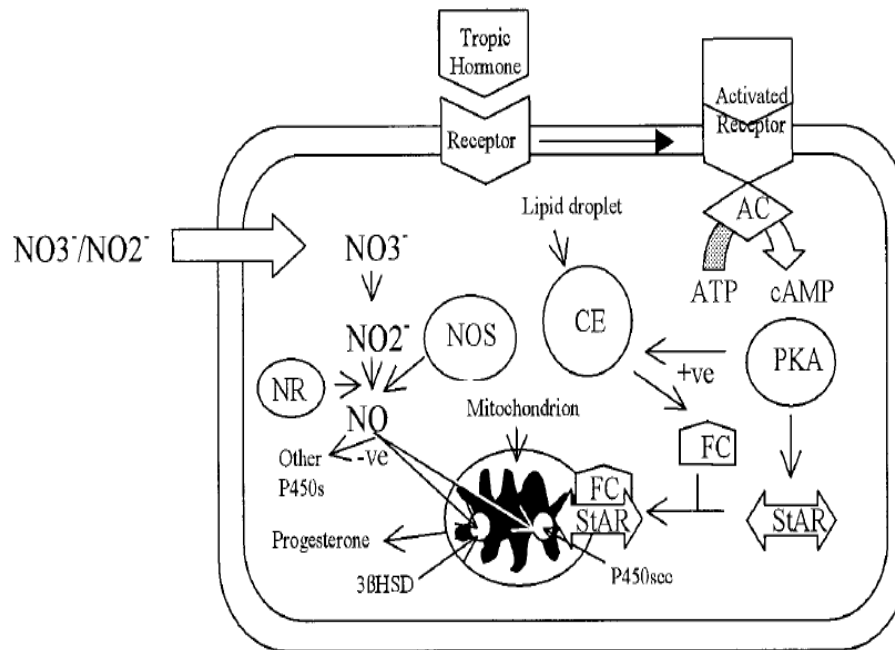


Figure 2. **Inhibitory action of nitrite and nitrate ions on steroid hormone synthesis.** Nitrate and Nitrite are converted to NO, which inhibits the enzyme P450scc in the mitochondria, thus stopping the steroidogenic cycle (Panesar and Chan 2000).

Pharmacological doses (42 mM) of nitrate and nitrite applied to mouse Leydig tumor cells (MLTC-1) *in vitro* dramatically reduced Leydig cell steroidogenesis (Panesar and Chan 2000). Moreover, rats given water containing 50 mg/ L of sodium nitrate or nitrite for 4 weeks have significantly decreased circulating levels of testosterone (Panesar and Chan 2000). Similarly, in the African clawed frog, nitrate exposure at 24.8 or 49.5 mg/L for several days suppressed *in vitro* synthesis of both testosterone and estrogen (Barbeau and Guillette 2007).

Each of the aforementioned studies involved post-natal exposure of vertebrates to nitrate. EDCs, however, often have detrimental effects on developing fetuses *in utero* (Ema *et al.*1998; Sugiura-Ogasawara *et al.*2005; Tewari *et al.*2009). The potential *in utero* effects of nitrate on steroidogenesis is a topic that warrants further research. One study that examined the exposure of

nitrate to male rat fetuses *in utero* revealed no consistent anti-androgenic effects. In this experiment, treatment of the mothers with 900 mg/L sodium nitrate in drinking water during GD7-21 did not affect testosterone levels in male offspring (Hansen *et al.* 2009). The authors suggested that the duration and timing of exposure may be critical for potential effects.

VI. Hypothesis: To determine the effects of nitrate exposure on developmental endpoints in mice, I treated pregnant dams with sodium nitrate in their drinking water from gestational day 10 until birth. My hypothesis was that I would observe a decrease in male anogenital distance as well as a decrease in serum testosterone levels in male offspring. C57/BL strain was used in this study because of their availability in the on-campus colony as well as their easy breeding.

Flutamide is a non-steroidal antiandrogenic drug commonly prescribed to treat prostate cancer. It out-competes testosterone for binding androgen receptors and inhibits responses to male hormones from both the gonads and the adrenals (Brogden and Clissold 1989). *In utero* exposure of rats to 25 and 50 mg/kg/day of flutamide results in a 49 and 50% decrease in AGD of male offspring, respectively (McIntyre *et al.* 2001). Therefore, flutamide was used as a positive control, such that I predicted decreased AGD and decreased testosterone levels in the flutamide-treated mice.

Methods:

Chemicals: Sodium nitrate (99%) and flutamide were each obtained from Sigma-Aldrich (St. Louis, MO). Great Value™ peanut butter was obtained from Walmart (Bentonville, AR).

Animals: Animal care and maintenance was conducted in accordance with the applicable portions of the Animal Welfare Act and the U.S. Department of Health and Human Services' Guide for the Care and Use of Laboratory Animals and approved by the Allegheny College Institutional Animal Care and Use Committee. All C57/BL strain mice were supplied by the colony at Allegheny College. Animals were fed a standardized diet *ad libitum*. Animals were maintained at 22°C on a twelve hour

light:dark cycle (on 0700, off 1900). Cages were cleaned two times a week. Adult female mice (n=8) were paired with males until a weight gain of at least 2.0 grams was observed (defined as successful pregnancy), at which point the males were removed.

Treatment Groups: Four treatment groups of two pregnant mothers were established. (1) The negative control group received normal drinking water. (2) The “low dose” nitrate exposure group received 50 mg/L of sodium nitrate dissolved into their drinking water; (3) the high dose nitrate exposure group received 200 mg/L of sodium nitrate dissolved into their drinking water and (4) the positive control group received 1.0 mg/day of the antiandrogenic drug flutamide mixed into 5.0 mL of peanut butter. All other treatment and control groups also received 5 mL of peanut butter daily. The low dose of 50 mg/L was chosen based on results of Panesar and Chan (2000) and because it is the World Health Organization’s limit of nitrate in drinking water that is safe for human consumption. The high dose of 200 mg/L represents the amount of nitrate present in a deforested landscape or stream water near an agricultural field (Likens and Bormann 1974). Although Likens and Bormann (1974) measured nitrate levels of 114 mg/L, 200 mg/L was chosen to cover a broader range. The flutamide dosages are similar to those chosen by McIntyre *et al.* (2001) and adjusted for the weight of an average mouse (assumed to be 30 grams). Water, both treated and untreated, was refilled *ad libitum*.

Serum Collection: The offspring were sacrificed by decapitation on postnatal day three, following total body mass and anogenital distance determinations. Blood was harvested from the trunk of each animal and centrifuged for 10 minutes to collect the serum fraction. Serum testosterone levels were measured using an ELISA: Testosterone EIA kit from Cayman Chemical (Ann Arbor, Michigan) (Appendix A).

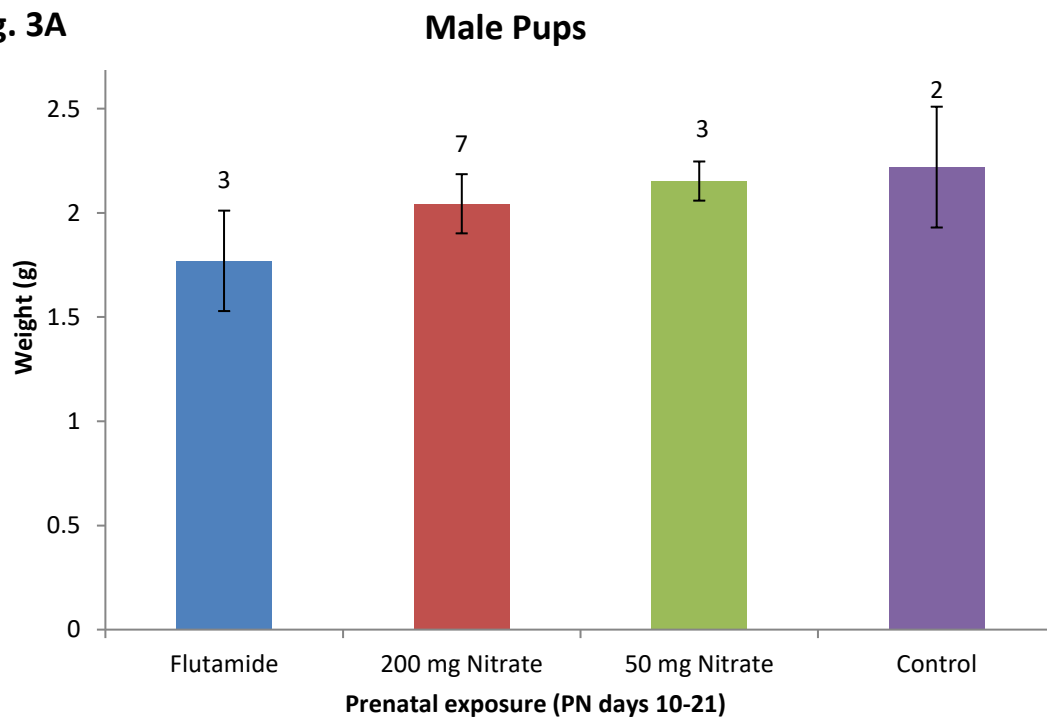
Statistics: Anogenital distance and serum testosterone levels in the pups were analyzed for statistical significance by analysis of variance (ANOVA) and Fisher's post-hoc test was applied where appropriate using StatView statistical package.

Mouse Fatalities: Of the eight female dams, five carried out successful pregnancies. One mouse in the “low dose nitrate” condition gave birth to pre-term pups, which were underdeveloped and not included in subsequent analysis. One positive control mouse had a miscarriage of her pups. One negative control mouse died during parturition and her pups did not survive until post natal day three for tissue harvest.

Results:

Weights of Pups: Nitrate exposure of the mother did not affect the weights of the male pups on post natal day three ($p=0.5017$) (Fig. 3A). Likewise, nitrate exposure of the mother did not affect the weight of the female pups on post natal day three ($p=0.3063$) (Fig. 3B). There was no difference in weight between the sexes.

Fig. 3A



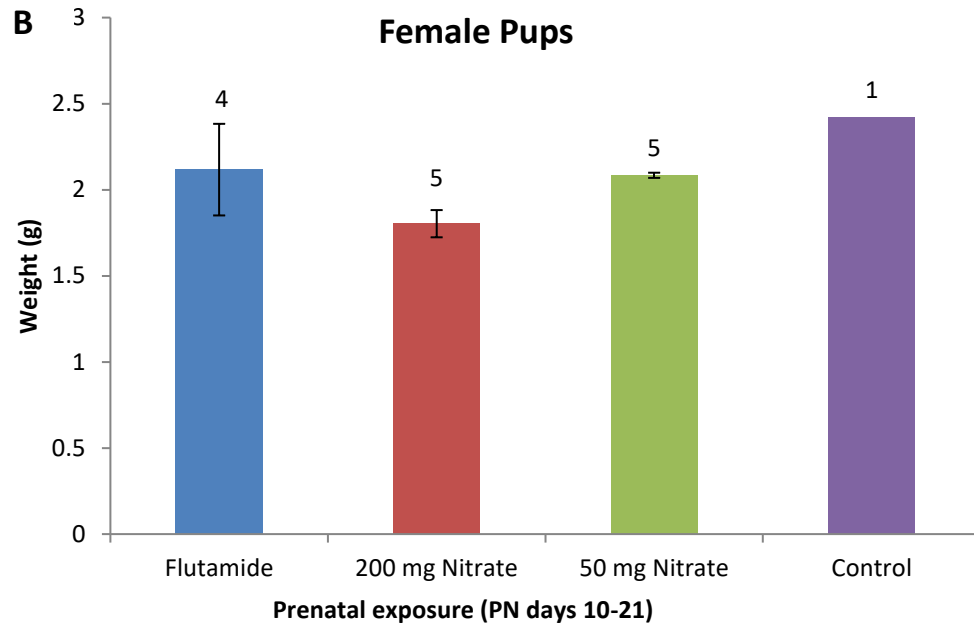
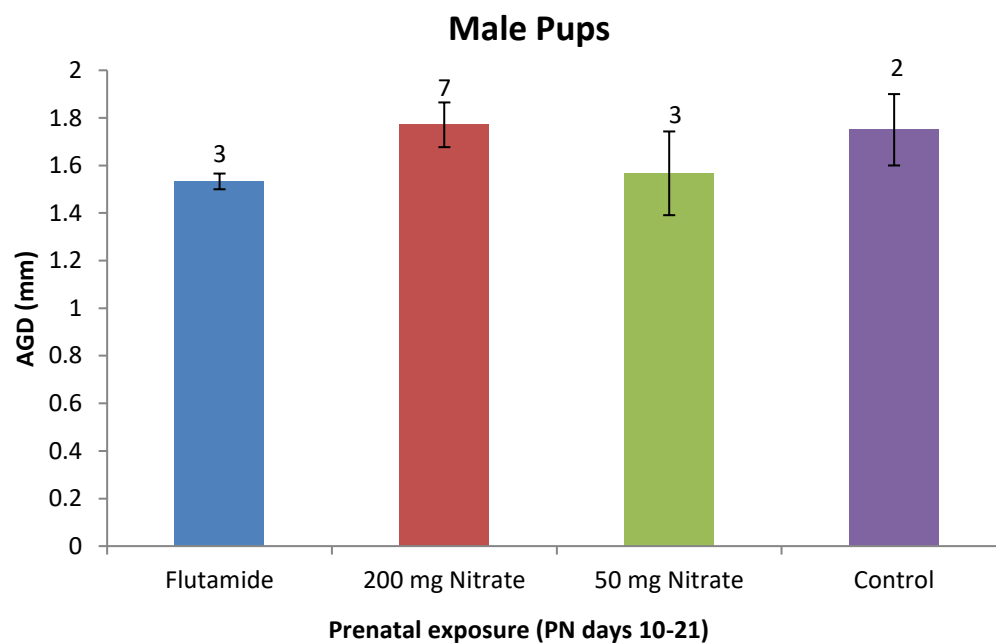


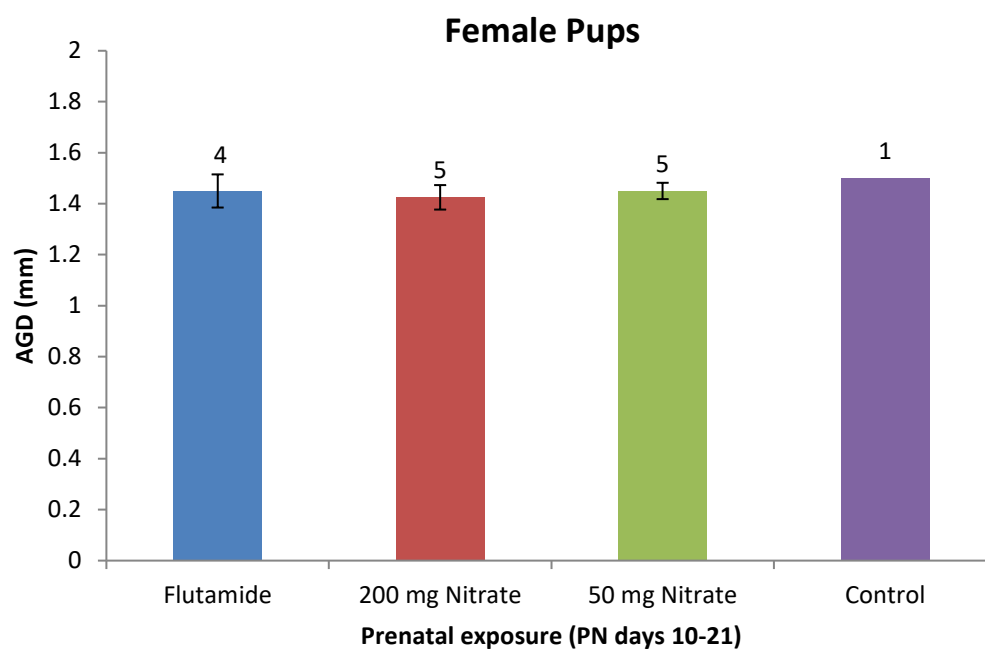
Figure 3. **The effects of pre-natal nitrate exposure on weight of mouse pups at post-natal day three in (A) males and (B) females.** Pregnant dams were exposed to nitrates via their drinking water from gestational day 10 until birth. Flutamide was administered in peanut butter. Error bars represent the standard error of the mean. Sample sizes are shown by each histogram.

Anogenital Distance (AGD): Nitrate exposure did not affect AGD in three day old male pups ($p=0.4186$) (Fig. 4A). The same was true for the female pups ($p=0.9176$) (Fig. 4B). The AGDs of the male pups (regardless of nitrate exposure) were 0.234 mm (ANOVA, $p = 0.002$) longer than the AGDs of the female pups (Fig. 4C).

Fig. 4A



B



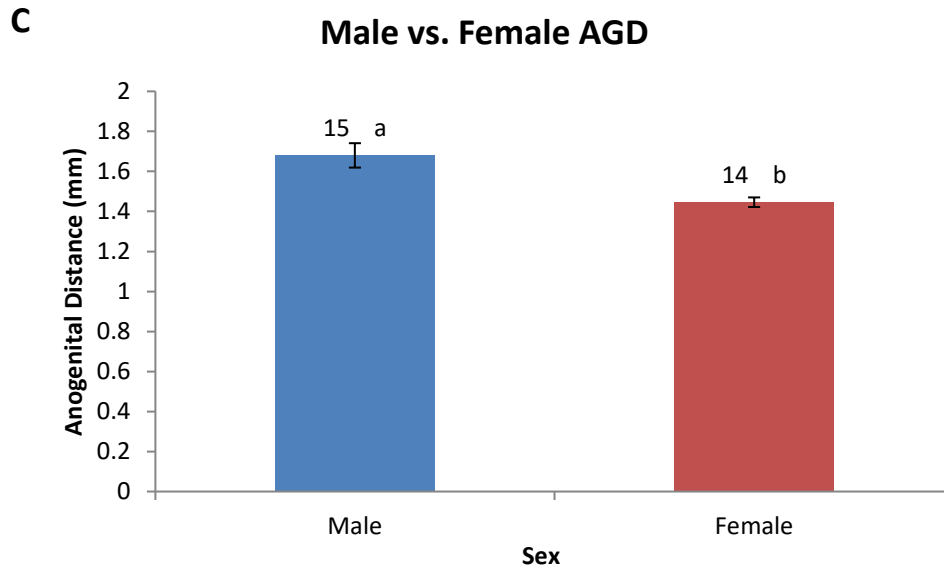
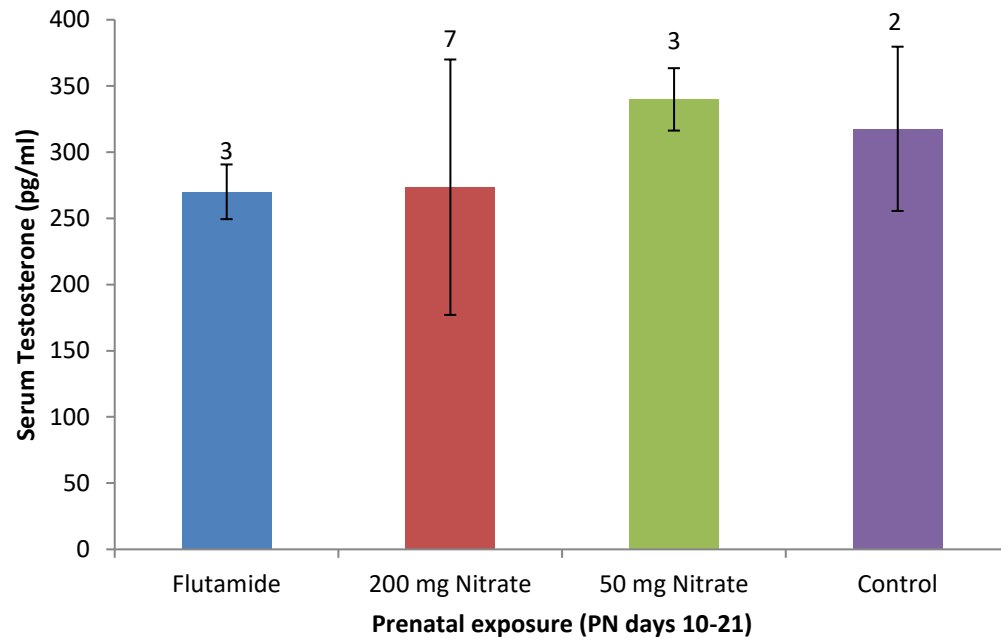


Figure 4. **The effects of pre-natal nitrate exposure on AGD of mouse pups at post-natal day three in (A) males, (B) females, and (C) males vs. females regardless of nitrate exposure.** The anogenital distances were measured with an ocular micrometer on gestational day three. Error bars represent the standard error of the mean. Letters denote significant differences. Sample sizes are shown by each histogram.

Serum Testosterone: For the male pups, nitrate exposure did not affect serum testosterone levels as measured by ELISA ($p=0.6773$) (Fig. 5A). The same was true for the female pups ($p=0.7539$) (Fig. 5B). The serum testosterone of the male pups was 147.496 pg/ml ($p < 0.0001$) higher than that of the females when nitrate exposure was not considered (Fig. 5C).

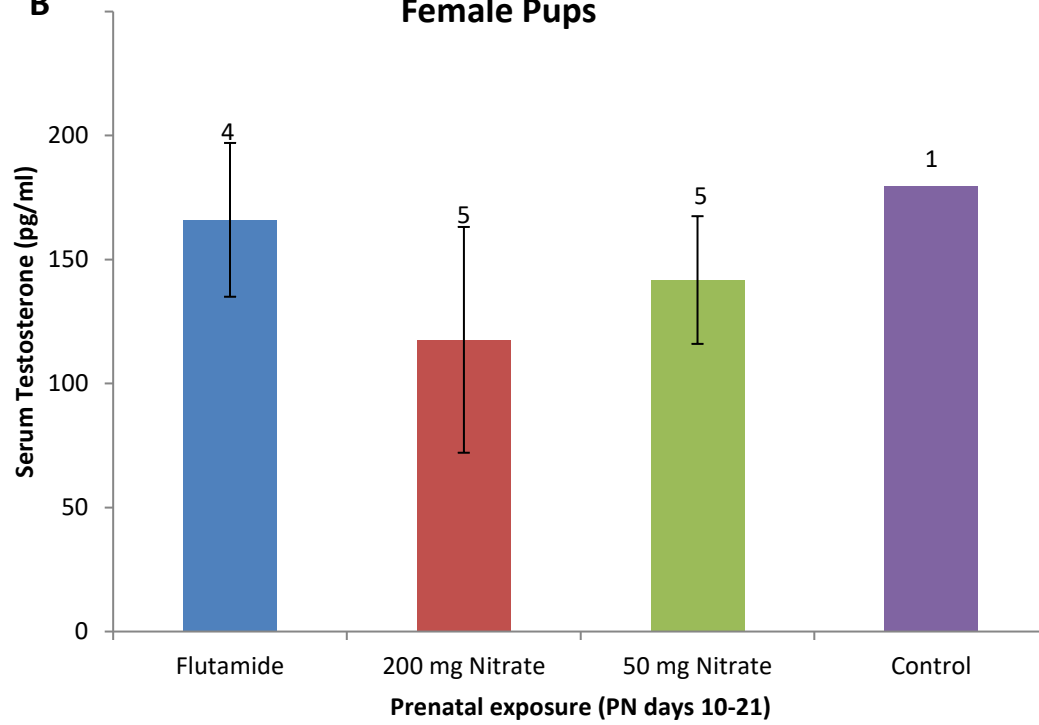
Fig. 5A

Male Pups



B

Female Pups



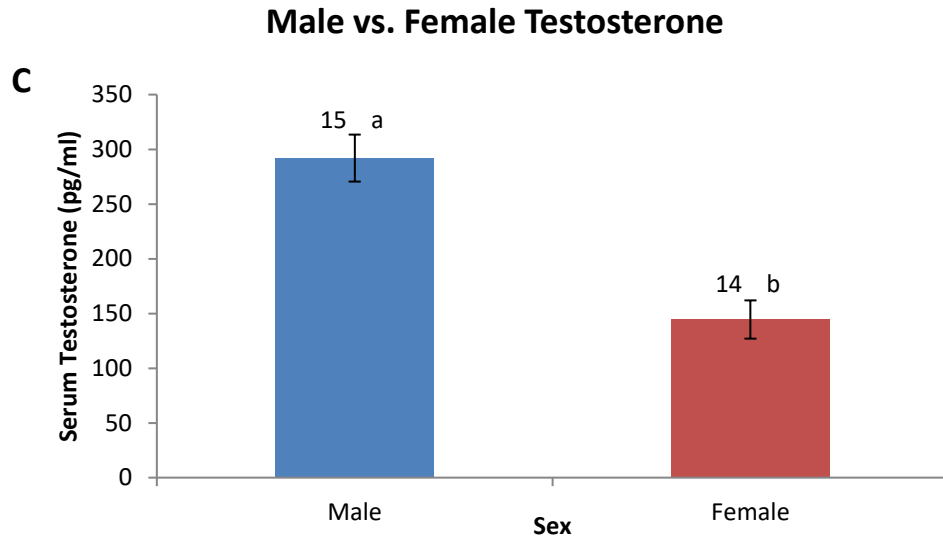


Figure 5. **The effect of pre-natal nitrate exposure on serum testosterone levels in (A) male and (B) female three day old mouse pups. (C) The serum testosterone levels of male and female three day old mouse pups regardless of nitrate exposure.** Mouse serum was collected and analyzed for testosterone via ELISA. Error bars represent the standard error of the mean. Letters denote significant differences. Sample sizes are shown by each histogram.

Discussion: The main objective of this study was to investigate the effects of prenatal exposure of nitrate on steroidogenesis in mouse fetuses. Pregnant dams were given 50 or 200 mg/L of sodium nitrate in their drinking water from GD10-GD21. Serum testosterone levels and AGDs of pups were used to assess the potential anti-androgenic effects. My hypothesis was that nitrate treatment would decrease male anogenital distance as well as decrease serum testosterone levels in male offspring.

Weights: Weight of the pups was not different between treatments and between sexes. This result is consistent with previous findings (Panesar and Chan 2000; Hansen *et al.* 2008). Studies with albino mice find that three day old males weigh 2.8 g while three day old females weigh 2.7 g

(Greenham and Greenham 1977). The weights of pups in the current study were slightly lower, likely due to the difference in strain.

Anogenital Distances: Anogenital distance, an endpoint for endocrine toxicity in animals (Hsieh *et al.* 2008) was not affected by nitrate exposure in this study. This observation is consistent with previous studies (Hansen *et al.* 2009). While studies with certain phthalates *in utero* affect AGD in male offspring (Hsieh *et al.* 2008), the current study suggests that nitrate does not have a similar effect. Sex differences in AGD were observed in this study. Studies with albino mice have shown the AGDs of three day olds to be 2.0 mm for males and 1.7 mm for females (Greenham and Greenham 1977). Although the AGDs in the current study were slightly lower (due to strain), the difference between the sexes is consistent.

Serum Testosterone: The results revealed that 50 and 200 mg/L of sodium nitrate in the drinking water of dams did not affect serum testosterone of three day old pups. These results are consistent with a previous study which showed no treatment effects of nitrate (up to 900mg/l) on hormone levels or testicular weight of male rat fetuses (Hansen *et al.* 2009). Another study showed effects on male rat testosterone production and testes weight after four weeks of exposure to 50 mg/l of sodium nitrite or sodium nitrate (Panesar and Chan 2000). However, in that study male rats were exposed directly to nitrate, whereas in this study nitrate exposure was indirect. Therefore, it remains possible that the placenta provides protection from endocrine disrupting effects, resulting in a decreased blood concentration of nitrate.

In addition, flutamide, a recognized anti-androgenic compound (McIntyre *et al.* 2001) did not affect testosterone production of the pups in this experiment. This result differs from the previous literature. *In utero* exposure of rats (oral gavage) to 25 and 50 mg/kg/day of flutamide results in a 49 and 50% decrease in AGD of male offspring, respectively (McIntyre *et al.* 2001). A

possible explanation is the vehicle of treatment. In the current study, peanut butter was often left in small quantities on the sides of cages, making it difficult to determine if all of the flutamide was consumed.

The aforementioned studies were performed using rats. Despite the similarity between rats and mice, this is another variable to consider. It is possible that differences in body mass could account for discrepancies in results. Furthermore, the study showing antiandrogenic effects of nitrate on adult male rats had an exposure period of 4 weeks. The present study with mice had an exposure period of only 11 days. It is possible that the shorter dosing period can account for the lack of an antiandrogenic effect. Although, *in vitro* studies with mouse Leydig tumor cells (MLTC-1) indicate that both nitrate and nitrite inhibit steroidogenesis (Panesar 1999; Panesar and Chan 2000); *in vitro* studies are often over-simplified and fail to account for the complexity of a living model, which could explain the discrepancy in results.

Differences in serum testosterone between the sexes were observed in the current study, which was to be expected. Studies regarding the serum testosterone levels of neonatal mouse pups are rare. Nonetheless, studies using older animals find serum testosterone levels of 1-4 ng/mL for 25 day old male mice and 0.9-1.1 ng/ml for 17 day old female mice (Barkley and Goldman 1977; vom Saal and Bronson 1980). Though the serum testosterone levels in the present study differ from those in previous studies (due to age differences), the variability between the sexes is consistent.

Considerations/Limitations: Previous studies have used drinking water as a vehicle for nitrate exposure in the rodent model. Although the current study did not measure water intake, previous studies have observed no difference in consumption between control groups and nitrate groups, indicating that water is a viable vehicle (Panesar and Chan 2000; Hansen et al. 2009).

The small sample size of the current study was a major limitation. On a larger scale, the observable differences in serum testosterone and AGD may have been statistically significant. Likewise, the deaths of several mice (mentioned above) resulted in even smaller sample sizes. Further, the method of determining successful pregnancy in this experiment was a major limitation. The weight gain of 2.0g was arbitrary and decided on due to the difficulty of detecting sperm plugs in the female mice. This also made it difficult to decide when to begin treatment, as weight gain of 2.0g does not exactly correspond to GD10. Because fetal development is an especially sensitive period, it is possible that treatment from GD10-GD21 may not have accounted for all critical windows of development with regards to steroidogenesis.

Further research regarding nitrate and steroidogenesis is needed. For instance, studies with adolescent mammals should be performed to observe any effects of nitrate on these crucial developmental periods. The protective blood-testis barrier is forming during this phase of development, leaving juveniles vulnerable to EDCs. In addition, long-term exposure studies should be performed, as increased body burdens of nitrate over time may have deleterious effects.

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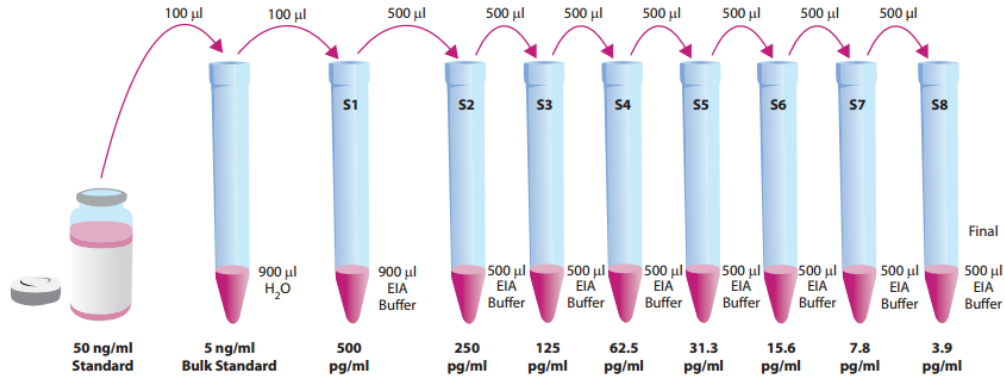
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Appendix A:

Testosterone EIA Procedure: As specified in the manufacturer's instructions.

Testosterone EIA Short Protocol

- EIA Buffer** - Dilute with 90 ml of UltraPure water.
- Wash Buffer** - Dilute the 5 ml vial to 2 L and add 1 ml Polysorbate 20 or dilute the 12.5 ml vial to 5 L and add 2.5 ml Polysorbate 20.
- Tracer** - Reconstitute the 100 dtn vial with 6 ml of EIA Buffer or the 500 dtn vial with 30 ml EIA Buffer.
- Antibody** - Reconstitute the 100 dtn vial with 6 ml of EIA Buffer or the 500 dtn vial with 30 ml EIA Buffer.
- Standard** - Prepare as described in the figure below.



Steps	Reagent	Blank	TA	NSB	B ₀	Std/Sample
1. Add Reagents	EIA Buffer	--	--	100 µl	50 µl	--
	Standard/Sample	--	--	--	--	50 µl
	Tracer	--	--	50 µl	50 µl	50 µl
	Antibody	--	--	--	50 µl	50 µl
2. Incubate	Cover plate and incubate two hours at room temperature (22°C)					
3. Wash	Wash all wells five times					
4. Add Reagents	Tracer	--	5 µl	--	--	--
	Ellman's	200 µl	200 µl	200 µl	200 µl	200 µl
5. Incubate	Cover plate and incubate 60-90 minutes RT with gentle shaking					
6. Read	Read plate at a wavelength between 405-420 nm					