Summer 2016

A Test for Heritable Variation in A Wild Population in Response to Endocrine Disrupting Events

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A thesis presented to the Graduate Faculty
of the College of William and Mary in Candidacy for the Degree of
Master of Science

Biology Department

The College of William and Mary
January 2017

APPROVAL PAGE

This Thesis is submitted in partial fulfillment of
the requirements for the degree of

Master of Science

Emily van den Blink

Approved by the Committee, May, 2015

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Protocol number(s) 10183

Date(s) of approval 3/14/2016

ABSTRACT PAGE

Endocrine disrupting chemicals (EDCs) are a pervasive threat to the health of both human and wildlife populations. EDCs bind to hormone receptors and mimic or block their action, causing disrupted growth, metabolism and reproduction. Fertility affected by EDCs might be compensated if some individuals are genetically resistant. Genetic resistance in a variable population might cause hormonal feedback pathways to be more resilient to disruption. The effects of EDCs on fertility will be studied using a naturally variable population. A wild population of neonatal male mice, *Peromyscus leucopus*, was treated with testosterone propionate during a critical developmental period. Genetic variation was measured by collecting testis and seminal vesicle mass. These results were used to determine the potential change in fertility of the mice. Results indicate that there is no difference detected in individuals in their response to EDCs, providing no evidence for an inherited difference between selection lines to these two chemicals. This suggests that heritable variation in response to short photoperiod in this population does not necessarily cause variation in fertility in response to other environmental variables, such as EDCs.

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ACKNOWLEDGEMENTS

This writer wishes to express her appreciation to Professor Heideman, under whose guidance this investigation was conducted, for his patience, guidance and criticism throughout the investigation. Thanks to Gabby Smith for help with all phases of planning and implementing experiments. Thanks to Anna Ayre, Megan Thackaberry, Carly Lin, Caroline Mooney, Steph McDonnell, Lauren Canestrini who worked on this project. Funding was provided by NIH R15 – HD068962. The author is also indebted to Professors Bradley and Saha for their careful reading and criticism of the manuscript.

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Background

Part I. Endocrine disruptors

Endocrine disruptors, both man-made and naturally occurring, are found in nearly all environments. These compounds can have widespread health impacts in human and animal populations (Diamanti-Kandarakis et al. 2009). Exactly how pervasive and detrimental these compounds are is the subject of studies focusing on obesity, cognitive development, cancer, and reproductive problems (reviewed by Vandenberg et al. 2013). Most studies focus on exposure in either prenatal or neonatal individuals, or adults (Jones, Shimell, and Watson 2011). This study includes tests at both age classes, assessing effects of potential endocrine disruptors on fertility in a naturally variable adult population of white-footed mice, *Peromyscus leucopus*.

Endocrine disruptors that include phytoestrogens, polychlorinated biphenyls (PCBs), Di(2-ethylhexyl) phthalate (DEHP), dichlorodiphenyltrichloroethane (DDT), bisphenol-A (BPA), and various other pesticides and herbicides have been shown to cause a wide variety of reproductive issues, often in a sex-specific manner. The herbicide Atrazine has been shown to completely feminize adult male frogs (*Xenopus laevus)*, producing fertile females (Hayes et al. 2010). Exposure to Atrazine could lead to a decline in the number of frogs within a particular population, which could ramify to affect other trophic levels in the frogs' habitat. A 1976 study on ringed seals in the Baltic Sea found dramatic effects on reproductive health following exposure to PCBs (Helle, Olsson, and Jensen 1976). Exposed female seals had pathological

uterine changes that caused reproductive failure and a subsequent population decline. When compared to the control population, PCB exposure was found to be the only significant difference. Phytoestrogens, present in soybeans, flax, licorice, thyme and hops are commonly consumed by wild herbivores, and humans (Hughes 1988). These compounds can act as both estrogenic and antiestrogenic compounds, causing both deleterious and therapeutic effects (Brzezinski and Debi 1999). They act as weak estrogens or antiestrogens by interfering with the luteinizing hormone (LH) feedback loop in the hypothalamic-pituitary-ovary axis in females (Woclawek-Potocka et al. 2013). Domestic animals, such as sheep and cows, that have been fed a highly estrogenic diet (e.g. clover) have been shown to have induced temporary or permanent infertility (Adams 1995). Behavioral effects of phytoestrogens have been detected in red colobus monkeys, in which increased fecal estrogen and cortisol levels have been linked to increased aggression and copulation (Wasserman et al. 2012).

The wide range of activity of phytoestrogens may be due, in part, to variation in individual sensitivity at the level of estrogen receptor, cofactor, response element or ligand (Krishnan, Heath, and Bryant 2000). Individual sensitivity may cause differences in how the molecules found in these plants target specific tissues and trigger the transcription of disruptive gene products or alter enzyme function. I have found no studies on individual variation in response to phytoestrogens.

Bisphenol-A (BPA) is a pervasive estrogen disruptor found in plastic bottles, dental sealants, receipts and canned food. Figure 1 highlights in blue the

portions of BPA and estradiol that interacts with estrogen-related receptors. BPA has a high affinity for the nuclear receptor Estrogen-related-receptor gamma (ERR-γ) and a relatively low affinity for estrogen receptor alpha (ER-α□□and estrogen receptor beta (ER-β) [(Blair et al. 2000). ERR-γ is associated with regulation of transcription of estrogen sensitive genes (Ariazi, Clark, and Mertz 2002). BPA has been shown to block estrogen at low doses and amplify estrogen action at high doses, although the exact mechanism for estrogen disruption is not completely understood (McCaffrey et al. 2013).

BPA may act as an endocrine disruptor for fertility via different pathways. Studies in humans have shown that BPA inhibits testis development and decreases sperm production, contributes to underdevelopment of the brain and kidneys in embryos and fetuses, and is associated with miscarriages (Hunt et al. 2009). Gestational exposure of BPA on murine (mouse) models has caused increased ovarian weights and higher incidence of oocyte damage, as well as a lower weight of testes and seminal vesicles (Wolstenholme, Rissman, and Connelly 2011). Further research is needed to assess the critical doses and exact mechanisms through with BPA acts on the body.

Part II. Heritable variation in humans and animals

The hypothalamic-pituitary-gonadal (HPG) axis is made up of gonadotropin releasing hormone (GnRH) neurons releasing GnRH, the pituitary releasing luteinizing hormone (LH) and follicle stimulating hormone (FSH), and the gonads producing estrogen and testosterone (Rivier and Rivest 1991). In addition to GnRH neurons, kisspeptin neurons have steroid receptors that

receive input from circulating hormone levels (or hormone mimics) to alter GnRH secretion (Han et al. 2005). GnRH cells are also controlled by estrogens that can act directly via membrane receptors or ER-□ receptors (Clarke 2011). If there are sustained high levels of sex steroids in the body, a negative feedback is exerted on the GnRH pathway (Handa et al. 1994). These pathways give a possible explanation for the mechanistic action of BPA in the male mouse. Because BPA is an estrogen mimic that may act as an agonist or an antagonist, it may give a dose-dependent positive or negative feedback signal to GnRH neurons.

Photoperiod regulation of fertility is controlled by the interaction between melatonin and the hypothalamic-pituitary-gonadal (HPG) axis (Prendergast, Kriegsfeld, and Nelson 2001; Paul D. Heideman and Pittman 2009). Melatonin is responsible, among other things, for regulating circadian rhythms in response to light cycles (Altun and Ugur-Altun 2007). Female hamsters that were deprived of light had decreased levels of luteinizing hormone and prolactin in the pituitary gland and were infertile (Reiter, Rudeen, and Vaughan 1976). Administration of melatonin was enough to restore fertility in the blinded hamsters. Melatonin acts on the HPG axis by binding to gonadotropin inhibitory hormone receptors to inhibit gonadal development and maintenance (Chowdhury et al. 2010). Endocrine disrupting chemicals have a broad range of mechanisms of action that may or may not overlap with photoperiod regulation mechanisms (Fig 2a).

How a mouse responds to BPA can also vary between populations and individuals within populations. Typical populations are expected to have some degree of naturally occurring variation in fertility (Heideman et al. 2010). It may

be important to take into account heritable variation among individuals in a group when studying the effect of BPA on fertility. Previous toxicology studies have typically used single strains of laboratory animals (Festing 2010), but a recent study on the effects of lead found that different strains of mice had a wide variety of responses to exposure (Schneider et al. 2014). These differences were caused by genetic diversity among the strains, leading to the conclusion that using a single strain of mice is not sufficient to determine the risk of a compound (Schneider et al. 2014). Instead, a population with natural variation in neuroendocrine regulation of fertility may give a more accurate picture of the effects of toxins and endogenous hormones on the reproductive system.

Fertility can be affected by individual variation working in concert with external, inhibiting factors (fig 2a). Figure 2b shows an average mouse with an average number of GnRH neurons under ideal conditions of food, light, and disease/parasite load (fig 2b). With nothing inhibiting the release of GnRH, it reaches threshold and delivers the adequate frequency and amplitude needed for fertility. That same mouse under moderately inhibitory conditions (fig 2c) will have a decreased level of GnRH secreted. In this model, the reduction is not enough to impact fertility. Only the combination of external factors and a genetic reduction in the number of GnRH neurons (fig 2d) will have sufficient impact on GnRH pulsatile release to cause the amplitude and/or frequency to be too low, and, according to our model, cause infertility. Thus, interactions that cause infertility may be missed by tests of single factors.

Our model *P. leucopus*

This study of the effects BPA on fertility used the naturally reproductively variable white-footed mouse (*Peromyscus leucopus)* (Heideman et al. 1999) as a model. Several species of *Peromyscus* are found across North America, with each population having varying seasonal breeding patterns (Heideman and Bronson 1991). The *Peromyscus leucopus* used in this study were selection lines from a wild-source population caught in a forest on the campus of The College of William and Mary in 1995 (Heideman et al. 1999). The offspring of wild-caught mice were raised in short –day photoperiods (L8:D16) and, at the age of 70 days, the reproductive status of males and females was determined with testis length and width and, in females, ovarian and uterine diameter. Mice that were classified responsive (R) had reproductive organs that were less than half the size of the nonresponsive (NR) mice during the short-day photoperiods. R males were bred with R females and NR males were bred with NR females for multiple generations to create two selection lines within the colony (for more details see Heideman and Pittman, 2009).

Because the effects of BPA in a human population are a major public health concern, a vertebrate mammalian model is preferable as a study system. While there are limitations that arise when using a mouse model due to physiologic differences between mice and humans, the availability of wild populations and ease of husbandry make these models useful to understand some of the mechanisms underlying the effect of endocrine disruptors on fertility.

Objectives Experiment 1 - BPA in Adult, Genetically Variable Males

To better understand the effects of endocrine disruptors on fertility, BPA was studied *in vivo*. Because BPA mimics estrogen and high levels of estrogen provide a negative feedback control on GnRH, I hypothesized that the GnRH feedback mechanism should be disrupted at high doses of BPA. This feedback loop inhibits release of luteinizing hormone, which is required for spermatogenesis. After the BPA dosing period, counts of immunoreactive (IR) gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus of our model, *Peromyscus leucopus* can test one prediction from this hypothesis. If a lower number of GnRH neurons is counted in dosed mice, it could mean that either there are fewer active neurons or there are more recently fired neurons depleted in GnRH. A pilot study by Dr. Julian Pittman showed lower levels of GnRH immunoreactivity in dosed NR mice. In this experiment, I compared the dosage groups to determine relative differences in neurons.

The two hypotheses are as follows: Hypothesis 1: Individuals that have genetically greater sensitivity to inhibition in SD have an HPG that is less resilient to environmental perturbation (i.e. endocrine disruptors). If so, then I predict the NR and R selection lines may differ in changes to the number of IR-GnRH neurons when dosed with BPA. Alternative hypothesis: In our selection lines that have genetically different sensitivity to inhibition in SD, that sensitivity is specific to inputs (photoperiod). Other environmental inputs will have effects independent from the effects of SD. If so, then I predict the NR and R selection lines will not

differ in the number of IR-GnRH neurons in response to BPA. Because variation exists in all populations of animals, I used one selection line of *P. leucopus* that has shown to be reproductively responsive to changes in photoperiod (R line) and compare those subjects to our nonresponsive line (NR line).

Methods Experiment 1 - BPA in Adult Males

To test the effects of BPA on fertility, mice from both selection lines of *Peromyscus leucopus* were orally dosed with BPA on cereal. Dosing with cereal decreases stress on the test subjects and researchers and replicates the major known method of exposure to BPA in humans. A study size of 7 mice per treatment group in each line, using males aged 70 +/- 14 days, were dosed via six pieces Froot Loops™ cereal at 4pm daily, in addition to ad lib food and water. This treatment was based on a pilot study run in the summer of 2014 assessing whether the mice would eat the dosed cereal. Three dosage treatments, 0mg/L, 50mg/L and 250mg/L, were fed to mice for a period of 21 days. The 50mg/L dosage is based upon previous toxicology studies in laboratory mice that determined the minimal level of toxic exposure to elicit a response (Pottenger et al. 2000). The 250mg/L dosage is approximately ¼ the dose of BPA the mice will tolerate before exhibiting gross symptoms of pathology (Takahashi and Oishi 2000).

After the dosing period of 21 days, the mice were sacrificed using Isoflurane for anesthesia and euthanasia. Lauren Canestrini, a senior honors thesis candidate, performed a terminal retro-orbital bleed under isoflurane

anesthesia (30%induction, 2% maintenance) to collect blood for a luteinizing hormone (LH) assay. Post-euthanasia, weights on testes and seminal vesicles were collected. Luteinizing hormone levels were measured at the University of Virginia School of Medicine using an ELISA assay. For details, see Heideman, et al. (2010).

In order to collect the brain for neuron counting, the mice were maintained under isoflurane anesthetic and perfused with Zamboni's fixative following an established perfusion protocol (Gage, Kipke, and Shain 2012). Brains were left for 24 hours in Zamboni's fixative, followed by 24 hours in 15% sucrose solution, and finally stored in a 30% sucrose solution at -5 degrees Celsius for up to 3 months. The fixed brains were cryosectioned and every fourth slice was stained for GnRH neurons using the ICC protocol described by Avigdor, et al (2005). Counts of IR-GnRH neurons were by Emily van den Blink and Gabrielle Smith following protocols for accurate and consistent counting of neurons (Avigdor, Sullivan, and Heideman 2005). Briefly, counts were made in each mouse from four sections that typically have the highest abundance of GnRH neurons (Heideman et al. 2007), with a subset recounted blind by another individual as a check for accuracy and consistency.

Results Experiment 1 – BPA in Adult Males

There were no significant differences in the plasma level of LH between the responsive and nonresponsive lines ($F = 2.6292$, $P > 0.05$) or between dosages ($F = 1.5114$, $P > 0.05$) (Fig. 3) N = 7/treatment group. Testes mass was collected

post-mortem. There was a significant difference in testes mass between the responsive and nonresponsive lines ($F = 49.0893$, $P < 0.05$), but no difference between dosages ($F = 3.4206$, $P > 0.05$), nor was there an interaction between line and dose ($F = 0.3286$, $P > 0.05$) (Fig. 4). Mass of the seminal vesicles was collected post-mortem and weighed after stripping the seminal vesicles of fluid. There was a significant difference between the responsive and nonresponsive lines ($F = 27.1941$, $P < 0.05$), but no difference between dosages ($F = 1.4513$, $P > 0.05$), nor interaction between line crossed with dose ($F = 0.7620$, $P > 0.05$) (Fig. 5) N = 7/treatment group. IR-GnRH neuron counts showed no significant difference between lines ($F = 0.1960$, $P > 0.05$), between doses ($F = 1.1114$, $P > 0.05$) or between line crossed with dose ($F = 0.0899$, $P > 0.05$) (Fig 6) N = 7/treatment group.

A BPA sandwich ELISA (MyBioSource Mouse Bisphenol A ELISA Kit, Catalog # MBS2600653; detection range 3.12 – 200 ng/ml of BPA) was performed after the trial to determine how much BPA was in the serum 24 hours after the dosing. No detectable BPA was found after 20-24 hours.

Discussion Experiment 1 – BPA in Adult Males

Overall, the results of the BPA dosing experiment showed no measured effects. We expected in a short-day photoperiod that the responsive line, compared to the nonresponsive line, would have significantly lower levels of LH (Avigdor, Sullivan, and Heideman 2005). LH can be used as a proxy for circulating testosterone levels because there is a proven relationship between levels of circulating testosterone and frequency of LH pulses (Coquelin and

Desjardins 1982). Instead, there was no difference in LH levels due to line or dose (Fig. 3). This may be due to a small sample size combined with substantial individual variation in LH pulses. Testes and seminal vesicle mass were shown to have a significant difference between the responsive and nonresponsive lines (Figs. 4 & 5), which confirms the difference in photoperiod sensitivity between the lines (Heideman et al. 1999).

There was no significant effect based on dose of BPA (Figs. 3-6). One possibility is poor absorption of dietary BPA. The assay performed to test the amount of BPA in the blood after 20-24 hours showed undetectable BPA. While it is possible that nearly all absorbed BPA had been cleared, this is unlikely due to longer clearance rates reported in similar studies (Takahashi and Oishi 2000). Based on all of these results, we can neither support nor reject our hypothesis: that individuals that have genetically greater sensitivity to inhibition in SD have an HPG that is less resilient to environmental perturbation.

Route of administration may play an important role in the outcome of BPA studies. Previous studies looking at the effects of BPA have traditionally used gavage or injection (intraperitoneal or subcutaneous) to administer BPA. When these traditional routes of administration were compared in adult female rats, oral gavage showed a much lower bioavailability of BPA compared to either type of injection(Pottenger et al. 2000). A multi-generational dietary dosing study using similar concentrations of BPA found no change in the structure or function of reproductive organs in rats; this study concluded that BPA was not a reproductive toxicant in this context (Tyl et al. 2002). This is important because

the primary method of human exposure to BPA is through food and beverage contamination (Vandenberg et al. 2007). The main difference between animal studies and observational human studies seems to be that animals are commonly exposed in a single, high dose by injection or gavage, while humans may be subjected to a persistent, low dose via ingestion.

Although there is a dearth of studies looking at long-term, low dose administration of BPA in adult animals, there are several studies that examine the long-term effects of BPA exposure during critical developmental periods in neonatal test subjects (Pottenger et al. 2000). Mice and rats that had been dosed 1-5 days after birth had significant changes in the adult reproductive tract (Newbold, Jefferson, and Padilla-Banks 2007). It is important to note that in studies of neonates, there was no difference between plasma concentration of BPA when administered by subcutaneous injection or oral gavage (Taylor, Welshons, and vom Saal 2008). Neonatal exposure of BPA in both females (Newbold, Jefferson, and Padilla-Banks 2007) and males (Salian, Doshi, and Vanage 2009) has been linked to decreased fertility. Studies focusing on prenatal and neonatal exposure to toxins may prove to be more relevant to human health than studies of adults.

In this study, the protocol of a 21-day dietary dosing regime of BPA may match potential exposure of humans. Based on previous dosing studies, we predicted a decrease in male fertility in both the responsive and nonresponsive lines (Al-Hiyasat, Darmani, and Elbetieha 2002). Not only was there no difference between the two lines when dose was taken into account, there was

no difference between the control vehicle and two doses of BPA (Figs. 3-6). Based on this, as well as the BPA assay demonstrating no detectable BPA after 20-24 hours in blood plasma, we cannot be certain that we tested our hypothesis. We cannot conclude that we have evidence for or against the hypothesis that individuals that have genetically greater sensitivity to inhibition in SD have an HPG that is less resilient to environmental perturbation

Future studies on BPA and fertility are needed to reach clear conclusions of the effects of BPA exposure on genetically variable populations. New experiments will need to tease out method of administration, dose, duration of dose, and sex and life stage of test animal. It is likely that prenatal and neonatal exposures have the biggest impact on fertility. Tests using exposure during development may be able to show a difference between a responsive mouse and a nonresponsive mouse in terms of their sensitivity to an environmental stressor.

Objectives Experiment 2 – Testosterone in Neonatal Males

Prenatal or neonatal exposure to endocrine disrupting events can cause long-lasting physiological and behavioral effects (Jones, Shimell, and Watson 2011). Previous toxicology studies have shown that dosing during key developmental time points of neonatal brain development causes irreversible neurotoxic effects (Viberg et al. 2003). To study developmental exposure, in this experiment testosterone propionate (TP) was used as a model chemical to represent synthetic androgens. Studies in rats have shown that TP enhances short-day induced inhibition of testicular growth (Heideman, Deibler, and York

1998).

Here, I tested the hypothesis that our selection lines (responsive and nonresponsive) differ in the sensitivity of their HPG axis to endocrine disruption. If the hypothesis is correct, then I predict that our responsive line will be less resilient to neonatal exposure of an endocrine disruptor than our nonresponsive line.

Methods Experiment 2 – TP in Neonatal Males in Long Photoperiod

To test the effects of testosterone propionate (TP; Sigma Aldrich) on neonatal development, male pups aged 3 +/- 1 days received one subcutaneous injection of TP. Only one injection should be necessary because TP has a half-life of 4 days, compared to 100 minutes for testosterone. The dosage, 0.1mg of TP dissolved in 0.05 ml of corn oil, was based on previous studies on cane mice and F344 rats (Bronson and Heideman 1992; Heideman, Deibler, and York 1998). Neonatal rats received a dose of 0.1mg of TP in 0.1ml of corn oil (Paul D. Heideman, Deibler, and York 1998) and cane mice received a dose of 0.5mg of TP in 0.05ml of corn oil (Bronson and Heideman 1992). These doses were found to have an effect on photoperiod sensitivity in rats (Heideman, Deibler, and York 1998), but not in cane mice, which are not photoperiodically sensitive (Bronson and Heideman 1992). *P. leucopus* pups average around 25-50% the mass of neonatal F344 rats, and the dosage was adjusted to match differences in body weight.

All males in separate control litters were injected with 0.05ml corn oil

vehicle using the same method as the experimental pups. This resulted in a total of four treatment groups (NR-Control, NR-TP, R-Control, R-TP). Pups were returned to their mother following injection and weaned around 21 days. Mice were born and raised in a long-day photoperiod (16h light, 8 hour dark). At 70 days +/- 3 days the mice were euthanized using an overdose of isoflurane and testes and seminal vesicle masses were collected. 4-8 litters per treatment group, with 6-8 mice total pups per treatment group, were collected.

Results Experiment 2 – TP in Neonatal Males in Long Photoperiod

Testes mass at day 70 +/- 3 days was measured immediately after euthanasia (Fig. 7; N = 3-4 litters/treatment group). There was a significant difference between the responsive and nonresponsive lines ($F = 49.2849$, $P < 0.05$). There was also a significant difference between the control dose and the TP treatment ($F = 6.7214$, $P < 0.05$), but no significant effect of line interacting with treatment.

Similar results were found for stripped (fluid removed) seminal vesicle mass (Fig 8; N = 3-4 litters/treatment group). There was a significant difference between lines ($F = 22.3026$, $P < 0.05$) and between doses ($F = 7.1864$, $P < 0.05$), but no significant effect of line crossed with dose ($F = 2.5615$, $P > 0.05$).

Methods Experiment 2a – TP in Neonatal Males in Short Photoperiod

The methods in experiment 2 were used in an experiment on mice raised in a short-day photoperiod (8 hour light, 16h dark). TP was dissolved in corn oil

(0.1mg/0.05ml) in a heat block at 40 degrees Celsius, a TP preparation that was different from experiment 2, as the mixture was vortexed 6-7 times, but not left on a shaker overnight. This experiment was conducted to test if the administration of an exogenous hormone (TP) compounds the inhibitory effects of short photoperiod on fertility in one or both strains.

Results Experiment 2a - TP in Neonatal Males in Short Photoperiod

Testes mass at day 70 +/- 3 days was measured immediately after euthanasia (Fig. 9; N = 7-8 litters/treatment group). There was a significant difference between the responsive and nonresponsive lines ($F = 48.4095$, $P < 0.05$). There was no significant difference between the control dose and the TP treatment ($F = 3.0860$, $P > 0.05$) and no significant difference for line and treatment ($F = 0.1521$, $P > 0.05$).

Similar results were found in the stripped (fluid removed) seminal vesicle mass (Fig. 10; N = 7-8 litters/treatment group). There was a significant difference between lines ($F = 16.2502$, $P < 0.05$), but not between doses ($F = 0.0169$, $P > 0.05$) or in line and dose ($F = 0.0305$, $P > 0.05$).

Methods Experiment 2b – Higher Dose TP in Neonatal Males in Short Photoperiods

The same methods from experiments 2 and 2a were used on mice raised in a short-day photoperiod (8h light, 16h dark) with double the dose of testosterone propionate (0.2mg TP/0.05ml corn oil). Powdered crystalline TP was dissolved in

10 microliters of 100% ethanol and mixed with the corn oil. This solution was placed on a shaker at room temperature (21 degrees Celsius) overnight to dissolve. As in experiment 2a, this study was used to test whether a higher dose of an exogenous hormone (TP) compounds the effects of photoperiod on fertility.

Results Experiment 2b - Higher Dose TP in Neonatal Males in Short Photoperiods

Testes mass at day 70 +/- 3 days was measured immediately after euthanasia (Fig. 11; N = 6-8 litters/treatment group). There was a significant difference between the responsive and nonresponsive lines ($F = 77.3077$, $P < 0.05$). There was no significant effect of TP treatment ($F = 2.3252$, $P > 0.05$) or of TP crossed with line ($F = 0.0566$, $P > 0.05$).

Similar results were found for stripped (fluid removed) seminal vesicle mass (Fig 12; N = 6-8 litters/treatment group). There was a significant difference between the responsive and nonresponsive lines ($F = 24.9924$ $P < 0.05$), but no difference between dose ($F =0.0643$, $P < 0.05$) or line crossed with TP ($F = 0.0051$, $P < 0.05$).

Discussion Experiment 2, 2a, 2b – TP in Neonatal Males

Experiment 2, which looked at the effect of TP under a long-day photoperiod, demonstrated the efficacy of TP at suppressing fertility in both the responsive and nonresponsive lines. There was a significant difference in testes and seminal vesicle weight between the responsive and nonresponsive lines,

confirming the variation between selection lines. There was also a difference between the control dose of the corn oil vehicle and the dose of 0.1mg TP in 0.05ml corn oil. There was no effect on selection line crossed with dose, suggesting that our hypothesis that the responsive line would be more sensitive to suppression by TP than the nonresponsive line, is not supported.

Experiment 2a, which looked at the effect of TP under a short-day photoperiod, showed only a difference between the responsive and nonresponsive lines. There was no difference between the control dose and the TP dose, nor was there a difference in line crossed with dose. These results suggest that our hypothesis is not supported, but the lack of response in TP dosed animals might be due to one of two factors. The first explanation is that the suppressive effect of short photoperiod is strong enough to override any additional suppressive effect of an endogenous hormone like TP. The second could be that because of an unintended change in the TP dose preparation protocol, the TP was not adequately dissolved in solution and the mice never received the dose. To assess the second possibility, we conducted experiment 2b, and even with the higher dose, TP did not enhance reproductive inhibition due to short photoperiod. This is consistent with our alternative hypothesis,that the selection lines differ genetically in sensitivity to short photoperiod, but not in sensitivity to neonatal treatment with TP.

Methods Experiment 2c – TP Dosing Control

This experiment was used as a control to test for pharmacological effects of the

aliquots of testosterone propionate for experiments 2a and 2b. Male mice, born and raised in SD photoperiod, were injected twice at age 25 +/- 3 days and age 30 +/- 3 days with aliquot 2a, 2b, or corn oil. 5 mice from each treatment group were injected with the original dose (0.1mg TP/0.05ml corn oil for 2a and 0.2mg TP/0.05ml corn oil for 2b) and another 5 mice per treatment group were injected with 0.5mg/0.25ml (2a) and 1mg/0.25ml (2b) in order to create a dose response curve. Mice were euthanized at day 38 +/- 3 days using an overdose of isoflurane. Testes and seminal vesicle mass were collected.

Results Experiment 2c – TP Dosing Control

Testes mass at day 38 +/- 3 days was measured immediately after euthanasia (Fig. 14; N = 7-15/treatment group), F 3.155= P > 0.05. There was a significant difference between the oil treatment and (2b) TP. There was no significant difference between (2a) TP and (2b) TP and no significant difference between (2a) TP and oil

Seminal vesicle mass at day 38 +/- 3 days was measured immediately after euthanasia (Fig. 13; N = 7-15 litters/treatment group), F = 5.5186 P < 0.05. There was a significant difference between the responsive and nonresponsive lines. There was no significant effect of dose or of dose crossed with line.

Discussion Experiment 2c – TP Dosing Control

The results of the testosterone propionate (TP) dosing control experiment suggest that the original dose used in experiment 2a was not an effective dose of

TP. Most significantly, there was no difference in testes or seminal vesicle mass between the oil treatment and the original dose of TP. This suggests that the original dose of TP was ineffective, either due to the amount of TP in the dose (0.1mg/ml) or due to improper mixing techniques.

The results of doses of the solution of TP used in experiment 2b (0.2mg/ml) were significantly different from results from the oil treatment. This result validates the effectiveness of the 0.2mg/ml dose and confirms that the results in experiment 2b involved a response to a pharmacologically relevant treatment with TP.

Objectives: Experiment 3 – Estradiol Benzoate in Neonatal Females

Developmental exposure to EDCs may affect female animals differently due to differences in physiology and timing of development. Alpha-fetoprotein in female mammals binds estrogen and prevents exposure of the developing brain to estrogen. Without this protein, estrogens masculinize and defeminize the female brain. A large dose of an exogenous estrogenic compound may saturate alpha-fetoproteins and allow these chemicals to reach the developing brain(Patisaul and Polston 2008). These brain changes can result in inappropriate mating behavior that ultimately decreases the fitness of the affected female. To study this, *P. leucopus* females were dosed with estradiol benzoate (EB) during the neonatal period. In mice, females dosed with EB at 3 days old exhibited increased aggressiveness as adults (Bronson and Desjardins

1968).

Methods Experiment 3 – Estradiol Benzoate in Neonatal Females

3 day old female *Peromyscus leucopus* were injected subcutaneously with 0.4mg estradiol benzoate in 0.05ml corn oil (Bronson and Desjardins 1968). Control females received a s.c. injection of 0.05ml corn oil alone. All mice were raised under LD conditions with unlimited access to food.

At age 50 days, females were paired with males that had been proven to be successful breeders. Mice were paired for five nights. The morning after each night, females were checked by vaginal lavage with saline for presence or absence of motile spermatozoa. After the five nights, females were sacrificed using isoflurane and ovary and uterine tissue was collected and weighed.

Results and Discussion Experiment 3 – Estradiol Benzoate in Neonatal Females

There were significant differences found in the uterine weights of female *P. leucopus* based on line (responsive or nonresponsive) ($F = 10.0910$, $P < 0.05$), but not based on dose of estradiol benzoate (0.4 mg/0.05ml corn oil or 0 mg/0.05ml corn oil) ($F = 0.4141$, $P > 0.05$). The same results were obtained for ovarian weights ($F = 2.7471$, $P > 0.05$; $F = 0.3081$, $P > 0.05$). We had predicted a significant reduction in fertility in females from the responsive line dosed with 0.4mg EB, a smaller reduction in fertility in females from the responsive line

dosed with a control and the non-responsive line dosed with 0.4mg EB, and no reduction in fertility in the non-responsive line dosed with control (Figs. 15-16) N = 5-8 litters/treatment group. Based on these results, our hypothesis was not supported. There is no evidence that responsive females are more sensitive to suppression by EB than nonresponsive females.

Endocrine disrupting chemicals can have effects on the development of female reproductive physiology (Diamanti-Kandarakis et al. 2009). Examples of decreased fertility or reproductive tract abnormalities have been seen in invertebrates, reptiles, birds, mammals and humans that have been exposed to exogenous chemicals during both development and adulthood (Sharara, Seifer, and Flaws 1998). Diethylstilbestrol, a synthetic estrogen that was prescribed to pregnant women to reduce the risk of loss of the fetus, was found to increase the risk of reproductive tract abnormalities and cancers in both sons and daughters that were exposed in utero (Giusti, Iwamoto, and Hatch 1995). Neonatal exposure to genistein, a phytoestrogen, was shown to cause disruption to ovarian function, estrous cycling, and overall fertility in mice (Jefferson, Padilla-Banks, and Newbold 2007).

Studying the effect of estrogenic compounds in prenatal and neonatal female rodents is complicated by the presence of alpha-fetoprotein. Alpha-fetoprotein is a glycoprotein that binds estradiol in rodent fetuses to prevent masculinization of the developing female brain (Bakker et al. 2006). Compounds with high affinity to alpha-fetoprotein, including estradiol benzoate, are inactivated at low and moderate doses, but compounds that are weakly bound

may still be able to affect the developing brain (Vandenberg et al. 2012). This difficulty can make it difficult to assess if a specific compound and dose will be disruptive in females.

This study was unable to demonstrate any effect of estradiol benzoate on the responsive and nonresponsive lines. We cannot be certain we tested our original hypothesis: that responsive female mice will be more sensitive to an environmental stressor such as estradiol benzoate during a critical developmental period than nonresponsive female mice. This does not mean that a different compound, dose, dosing schedules or method would have similar negative results. Difficulties associated with studying endogenous chemicals and hormones in female subjects due to alpha-fetoprotein in developing rodents and shifting hormonal states due to estrous and menstrual cycles in adults will require more complicated experimental design.

Summary

Endocrine disrupting compounds have the potential to act differentially on mammals from varying genetic backgrounds. Our results suggest that genetic variation in reproductive sensitivity to winter-like photoperiods may not cause differences in response to an entirely different environmental factor, endocrine disruption using the chemicals tested in these experiments. Genetic differences in susceptibility to EDCs may exist separately from genetic variation in susceptibility to the effects of short photoperiod. If so, then our artificial selection lines could not reveal or detect such variation in sensitivity to EDCs.

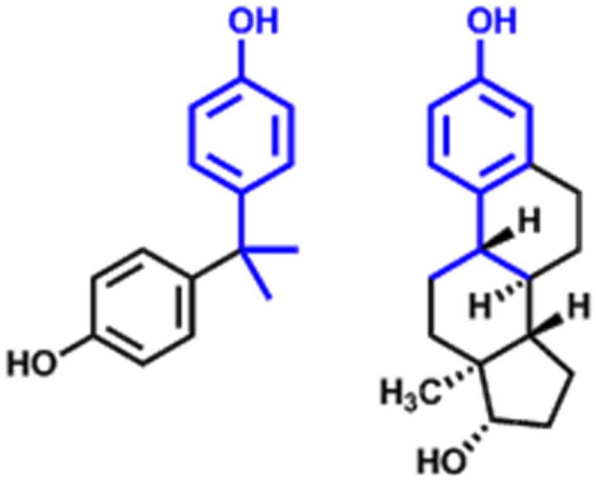
Future Directions

This study cannot rule out our hypothesis that variation in sensitivity to short photoperiod also results in variation in response to EDCs and other environmental factors that might alter fertility. Different timing, EDCs, and doses might be necessary. In each case, a pilot dose response study should establish an effective dose, followed by a comparison of the R and NR selection lines. The study of the effects of endocrine disrupting chemicals on fertility and reproduction can be divided into prenatal, neonatal, and adult studies. Various classes of chemicals taken from known EDCs (Vandenberg et al. 2013) should be tested at all three of these stages to determine the developmental risk. These studies can be done using an organ as a proxy for fertility, such as testes and ovaries as used in our study, or can also be accomplished through other measurements. Fertility can also be measured by breeding exposed and non-exposed animals to look at success of fertilization and maturation of offspring. Hormonal assays can also be performed to assess the effects of EDCs on luteinizing hormone, estrogen, or testosterone at various stages in both males and females.

In studies specifically measuring the individual variation in response to exposure, genetic assays may give some indication of key areas in the genome that may offer more resiliency in some individuals. For example, sequencing studies of the R and NR lines might reveal differences in the number of plasma proteins that transport EDCs, in sequence or expression of receptors that bind

EDCs, or of enzymes that metabolize EDCs. Any of these could underlie genetic variation in response to EDCs.

New selection lines of mice could be made by trapping mice from an area that has been contaminated with EDCs. The mice would then have testes measured with calipers and ovaries and uteri measured via laparoscopy (Paul D. Heideman et al. 1999) to select for mice that have a reproductive response to chemical exposure. These EDC selection lines would provide a direct test for heritable variation in response to endocrine disruptors.



Bisphenol-A (BPA) **Estradiol**

http://www.scienceminusdetails.com/2011/11/shape-science-or-dr-licorice-explains.html

Figure 1. Structure of BPA compared to estradiol. The regions highlighted in blue interact most strongly with estrogen receptors.

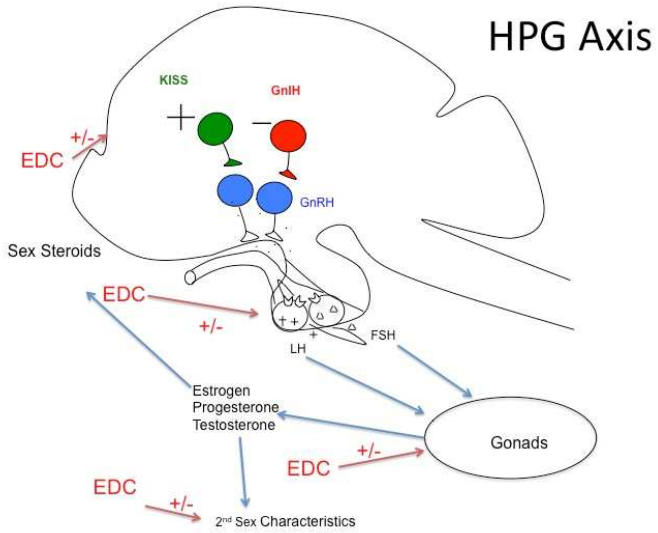


Figure 2a. Endocrine disrupting chemicals (EDCs) can have agonistic or antagonistic effects on multiple levels of the hypothalamic-pituitary-gonadal (HPG) axis. These effects can happen at the level of the neuron (kisspeptin-KISS; gonadotropin inhibiting hormone-GnIH; gonadotropin releasing hormone-GnRH), hormone (lutenizing hormone-LH; follicle-stimulating hormone-FSH), or secondary sex characteristics.

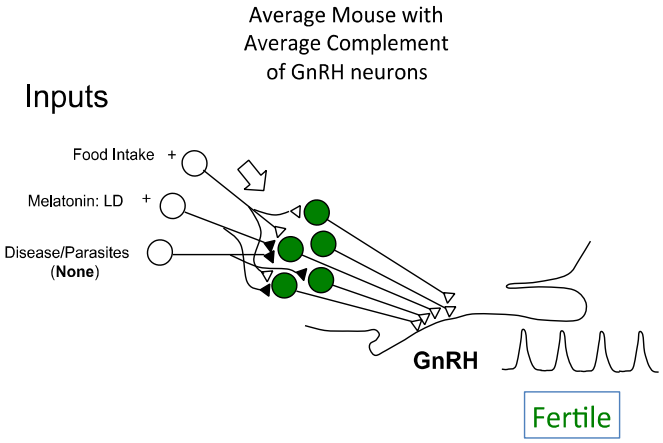


Figure 2b. Representation of an average number of gonadotropin releasing hormone (GnRH) neurons in an average mouse. With adequate food, melatonin (long day-LD photoperiod), and an absence of disease/parasites the output of GnRH pulses will result in fertility

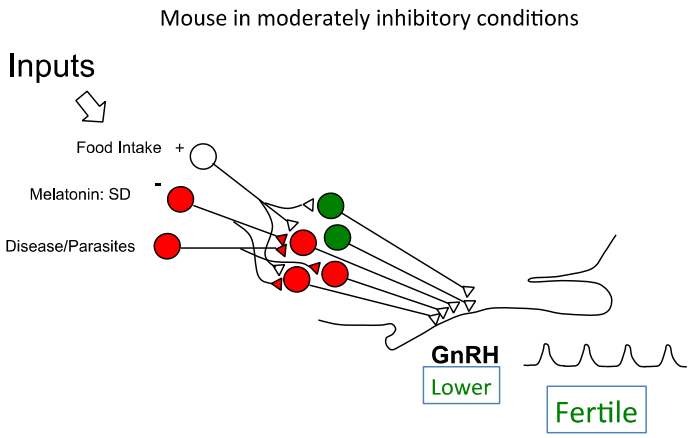


Figure 2c. Representation of an average number of gonadotropin releasing hormone (GnRH) neurons in a mouse under moderately inhibitory conditions. Decreased levels of melatonin due to a short-day (SD) photoperiod, combined with disease or parasites will result in a decreased secretion of GnRH. This reduction is not enough to bring GnRH below threshold, so the mouse is still fertile.

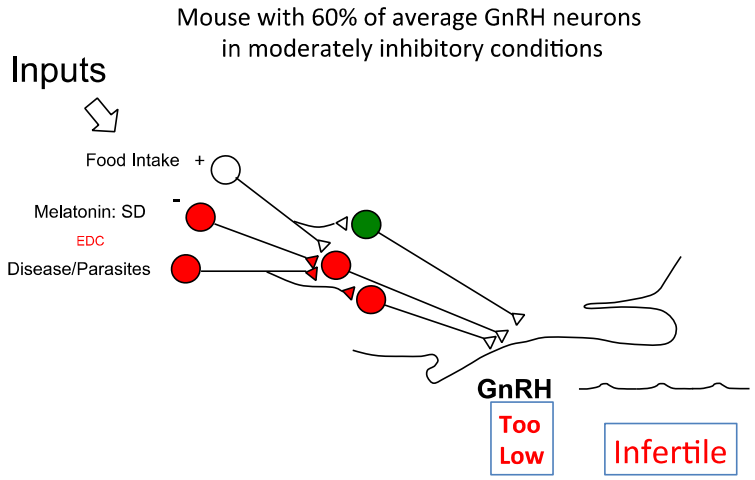


Figure 2d. Representation of a mouse with 60% of average number of gonadotropin releasing hormone (GnRH) neurons under moderately inhibitory conditions. Decreased levels of melatonin due to a short-day (SD) photoperiod, combined with disease or parasites will result in a decreased secretion of GnRH. This reduction is enough to bring GnRH below threshold, so the mouse becomes infertile. A hypothesis in this thesis is that endocrine disrupting chemicals (EDCs) are another input that could be inhibitory or excitatory.

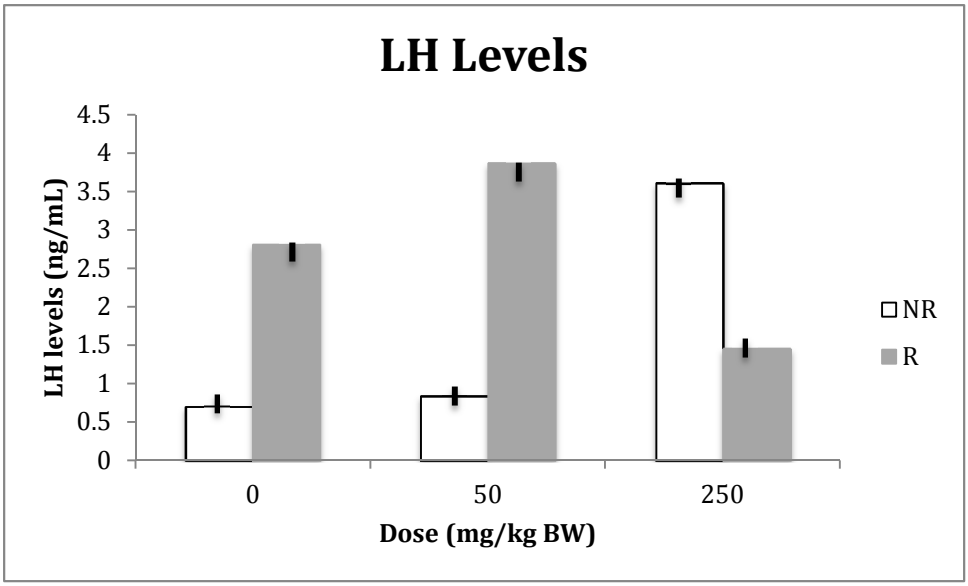


Figure 3. Levels of luteinizing hormone (LH) in adult male mice in a responsive line (R) and a nonresponsive line (NR) dosed with bisphenol-a (BPA). No significant difference was found (see results). N = 7/treatment group (Mean +/- SEM).

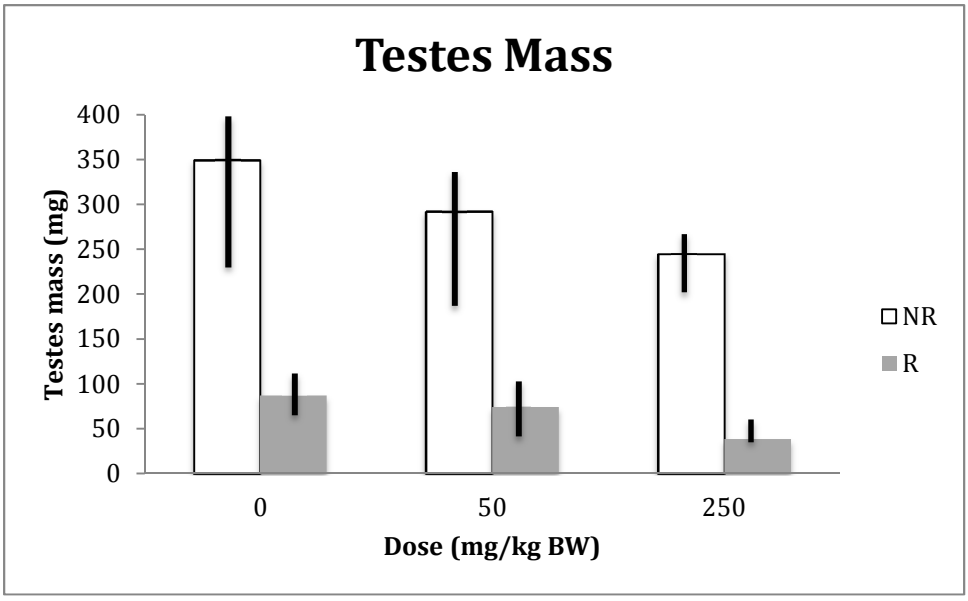


Figure 4. Testes mass in adult male mice in a responsive line (R) and a nonresponsive line (NR) dosed with bisphenol-a (BPA). There was a significant difference between the R and NR lines, but no difference between doses or line crossed with dose (see results). N = 7/treatment group (Mean +/- SEM).

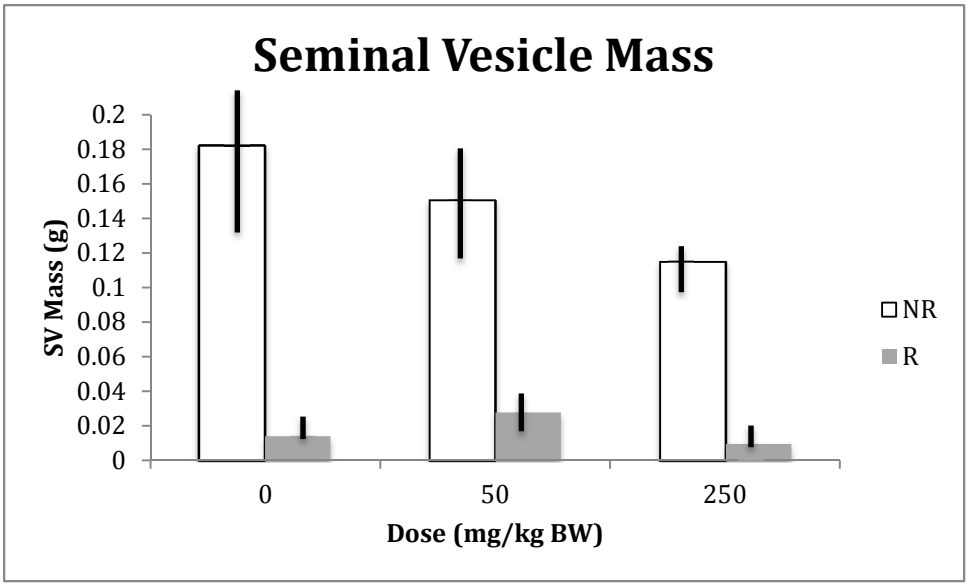


Figure 5. Seminal vesicle mass in adult male mice in a responsive line (R) and a nonresponsive line (NR) dosed with bisphenol-a (BPA). There was a significant difference between the R and NR lines, but no difference between doses or line crossed with dose (See Results). N = 7/treatment group (Mean +/- SEM).

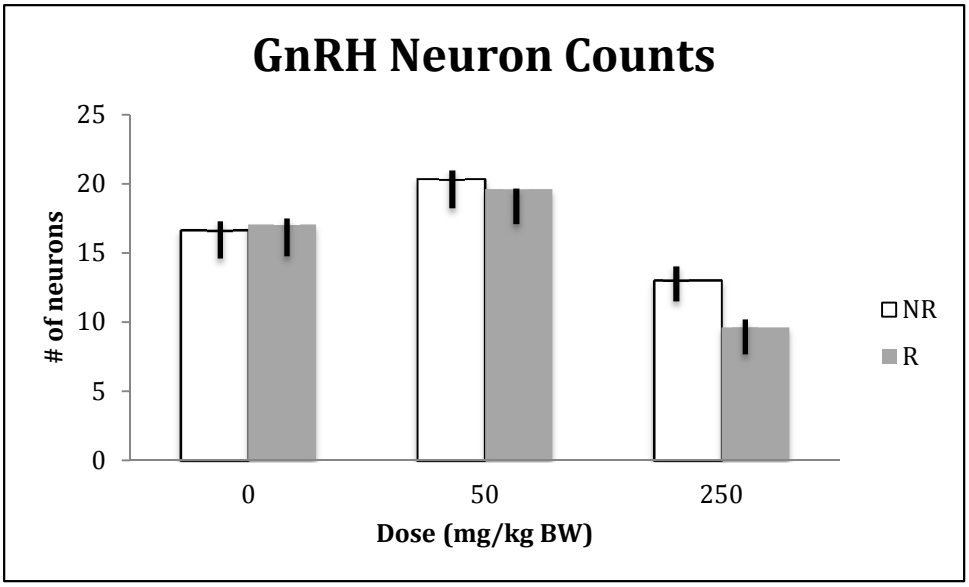


Figure 6: IR-GnRH neuron counts in adult male mice in a responsive (R) and non responsive (NR) line after BPA dosing. No signficant difference found between line, doses, or line crossed with dose (see results). N = 5-7/treatment group (Mean +/- SEM).

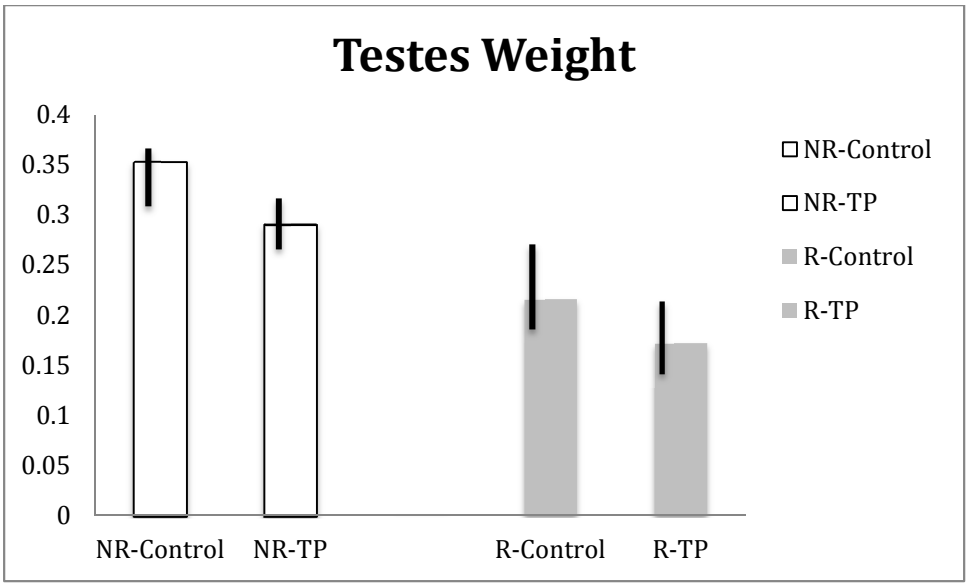


Figure 7: Testes Weights after testosterone propionate (TP) dose in long photoperiod. There was a significant difference between responsive (R) and nonresponsive (NR) lines, as well as between control and TP dose (see results). No significant difference of line crossed with treatment. N = 3-4 litters/treatment group (Mean +/- SEM).

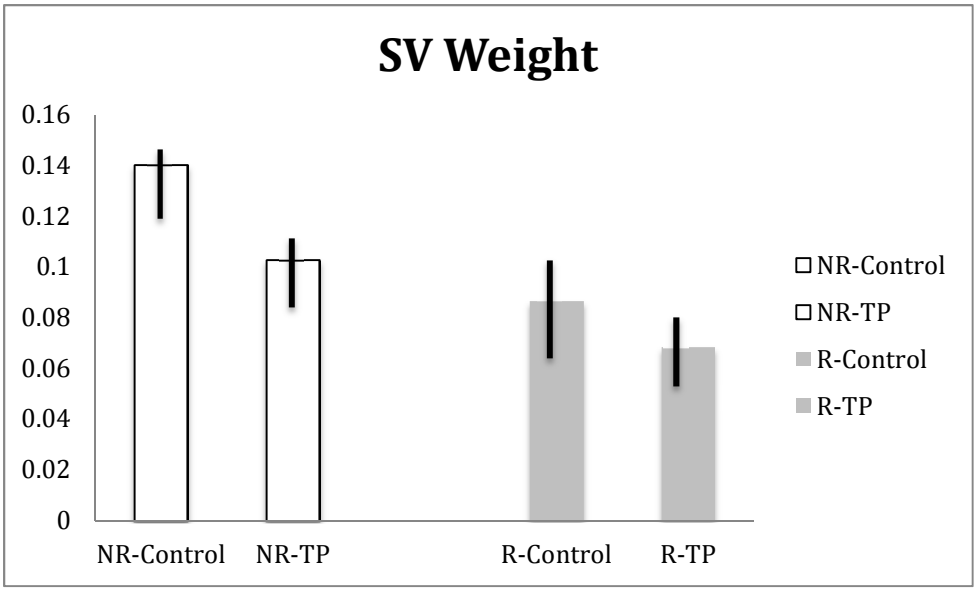


Figure 8: Effects of selection line (responsive: R; nonresponsive: NR) on seminal vesicle (SV) mass in a long photoperiod. There was a significant difference between lines and between doses, but no significant effect of line crossed with dose (see results). N = 3-4 litters/treatment group (mean +/- SEM).

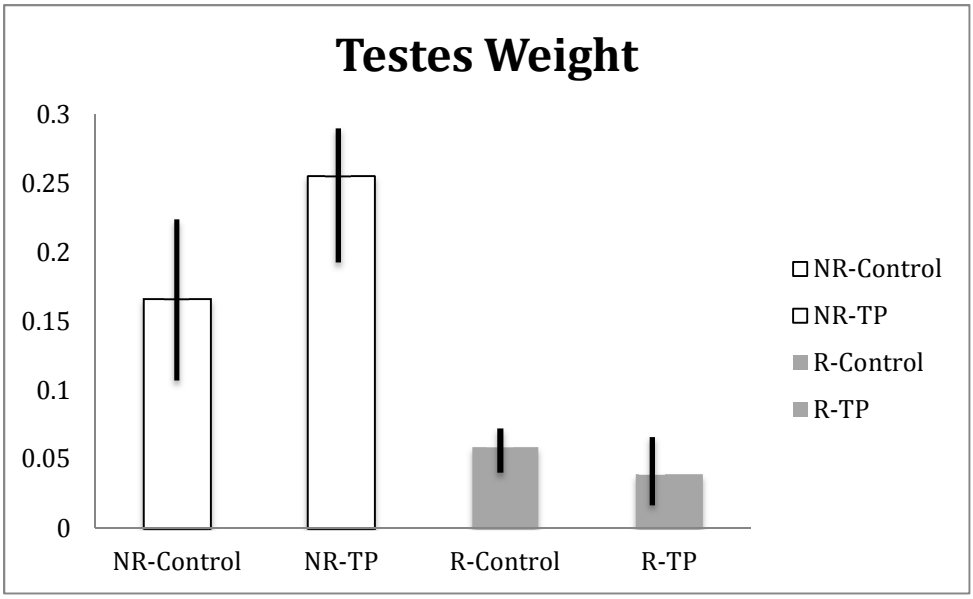


Figure 9: Effects of selection line (responsive: R; nonresponsive: NR) on testes mass in a short photoperiod (see results). There was a significant difference between the responsive and nonresponsive lines. There was no significant difference between the control dose and the testosterone propionate (TP) treatment and no significant difference for line crossed with treatment. N = 7-8 litters/treatment group (Mean +/- SEM).

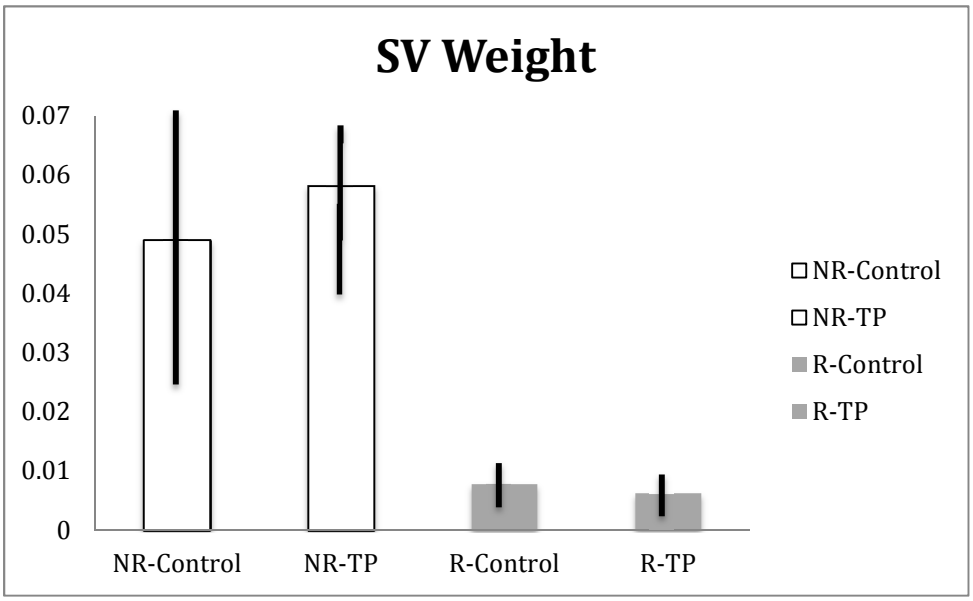


Figure 10: Effects of selection line (responsive: R; nonresponsive: NR) on seminal vesicle (SV) mass in a short photoperiod (see results). There was a significant difference between lines, but not between doses or in line crossed with dose. N = 7-8 litters/treatment group (Mean +/- SEM).

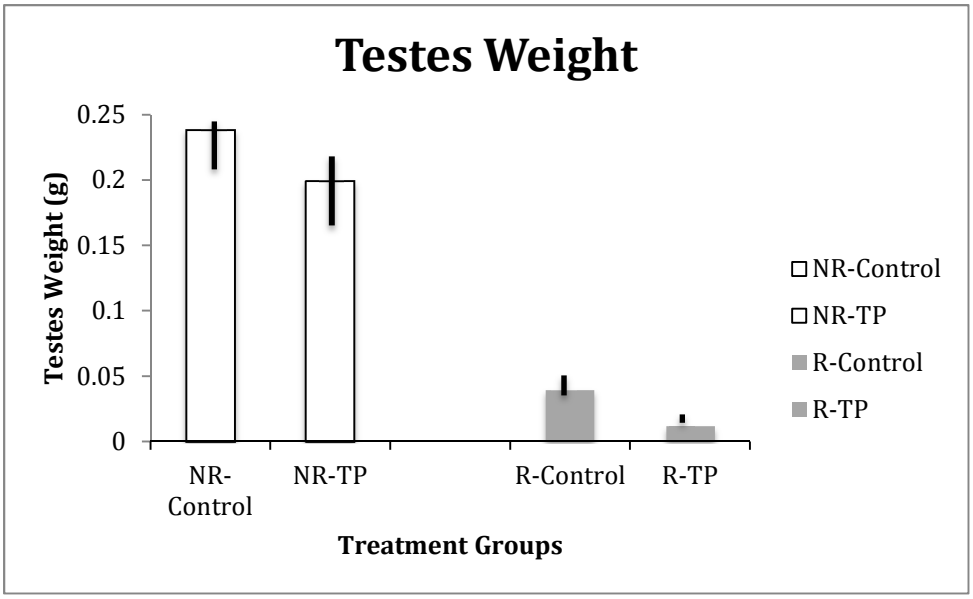


Fig 11: Testes Weights after higher testosterone propionate (TP) dose in short photoperiod (see results). There was a significant difference between lines, but not between doses or between line crossed with dose. N = 8 litters/treatment (Mean +/- SEM).

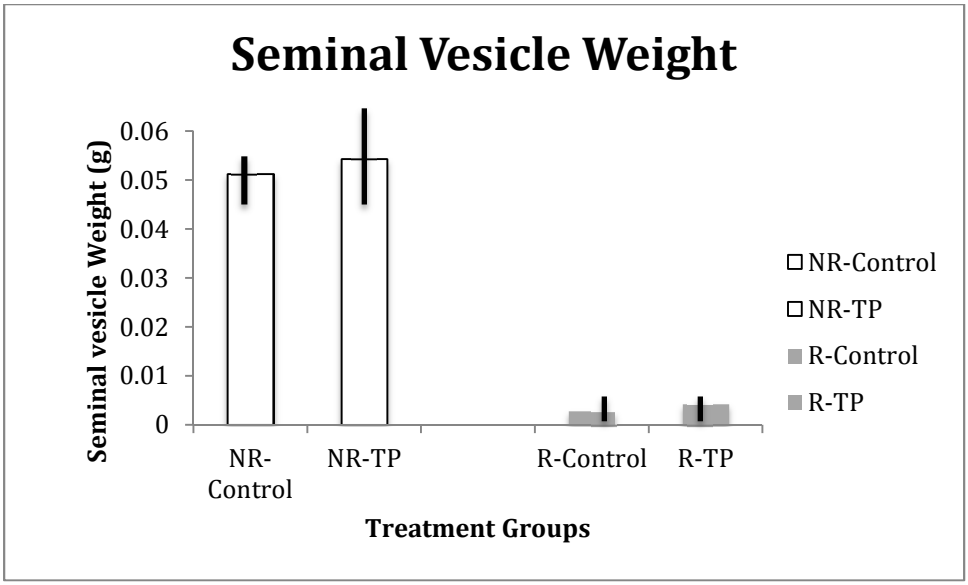


Fig 12: Seminal vesicle weights after higher testosterone propionate (TP) dose in short photoperiod (see results). There was a significant difference between lines, but not between doses or between line crossed with dose. N = 8 litters/treatment (Mean +/- SEM).

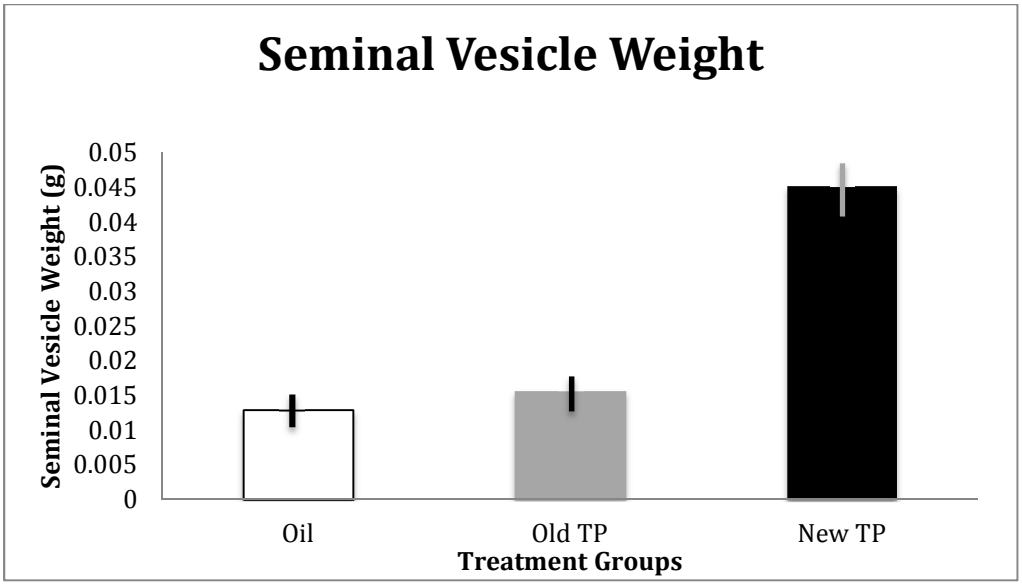


Fig 13: Seminal vesicle weights comparing testosterone propionate (TP) treatments from experiments 2a (old TP) and 2b (new TP). Significant difference between all three treatments. N=7-15 litters/treatment group (mean +/- SEM).

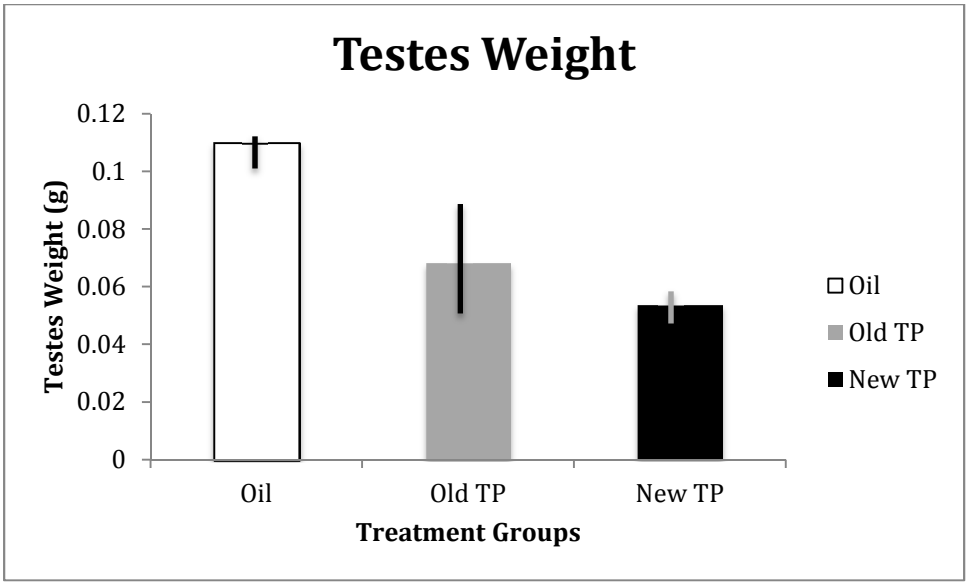


Fig 14: Testes weights comparing testosterone propionate (TP) treatments from experiments 2a (old TP) and 2b (new TP).. Significant difference between oil and new testosterone propionate (TP). No difference between old TP and new TP or between oil and old TP. N = 7-15/treatment group (mean+/-SEM) litters

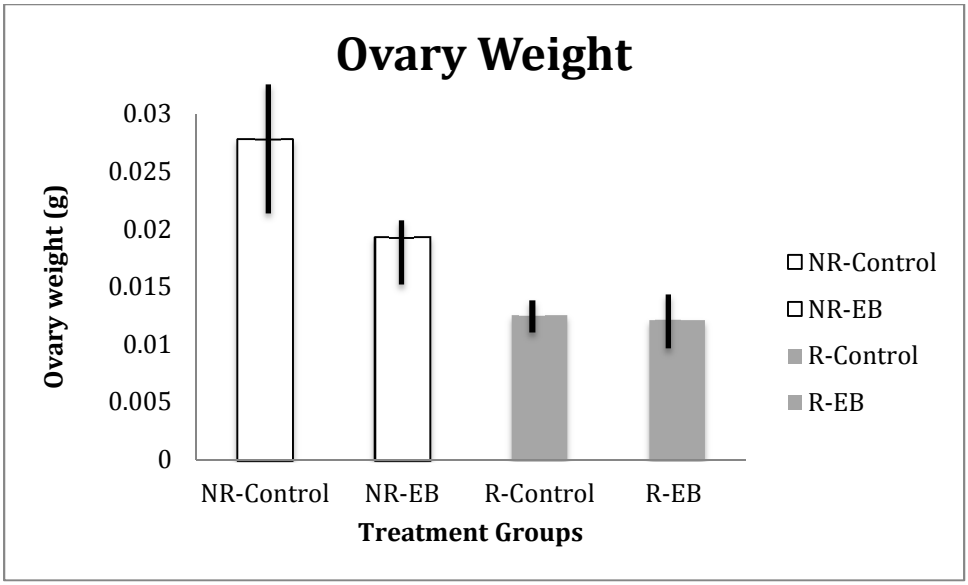


Fig 15: Ovary weights after estradiol benzoate (EB) in long photoperiod (see results). There was no significant difference between lines or doses. N = 5-8 litters/treatment (Mean +/- SEM).

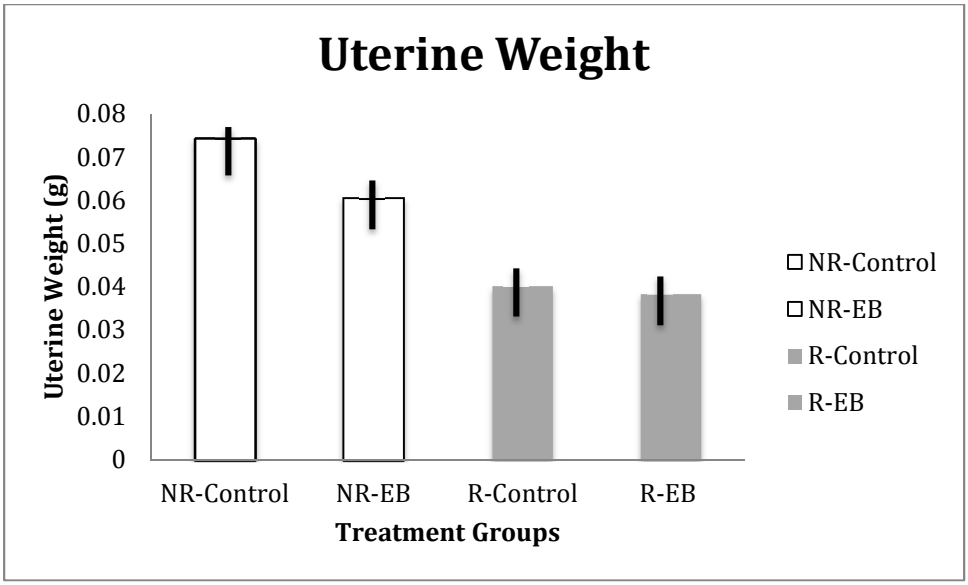


Fig 15: Uterine weights after estradiol benzoate (EB) in long photoperiod (see results). There was no significant difference between lines or doses. N = 5-8 litters/treatment (Mean +/- SEM).

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