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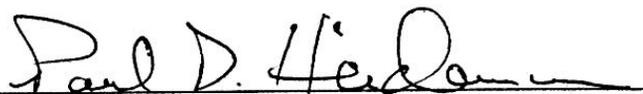
A Test for Heritable Variation in Reproductive Response to Bisphenol A in a Population of
White-footed Mice (*Peromyscus leucopus*)

A thesis submitted in partial fulfillment of the requirement
for the degree of Bachelor of Science in Biology from
The College of William and Mary

by

Lauren N. Canestrini

Accepted for Honors



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Williamsburg, VA
May 5, 2015

A test for heritable variation in reproductive response to Bisphenol A in a population of white-footed mice (*Peromyscus leucopus*).

Lauren Canestrini

Abstract

Bisphenol A, or BPA, is a plasticizer and a known endocrine-disrupting chemical in mammals that can interfere with functions controlled by hormones, including reproduction. This study tested for genetic variation in the reproductive response to the environmental toxin BPA. We used a genetically variable wild-source population of *Peromyscus leucopus*, from which two genetically different selection lines have been developed. These artificial selection lines of *P. leucopus* have either a strong reproductive response to short photoperiod or a weak reproductive response to short photoperiod. To measure the response of the reproductive systems to doses of BPA (50 and 250 mg/kg body mass), we measured food intake, body mass, gonad mass, and luteinizing hormone levels. In a previous pilot study, the selection lines differed in reproductive response, food intake, and body mass when given BPA. There was a suggestion for genetic variation in this response: the non-responsive line had higher food intake and body mass, less GnRH staining, and increased tyrosine hydroxylase staining relative to the photoperiod responsive line. This new experiment tested two different doses of BPA administered in food for 3 weeks. The effect of BPA on reproductive organ size, body weight, food intake, and a reproductive hormone was did not differ significantly at any dose or between lines. My data provide no evidence that the lines neither responded to BPA nor differed in reproductive responses to this environmental toxin, suggesting there may be no functional impairment resulting from this endocrine disruptor at these doses.

Introduction

Heritable Variation

Natural populations express genetic diversity that affects brain function, neuronal characteristics, and physiological processes, particularly reproduction. This genetic diversity can influence different aspects of reproductive function, from brain and hormonal regulation to sexual behavior (Geschwind 2000). Genetic variation results in intra-population phenotypic variation and these variable phenotypes may impact fitness of individuals. More diversity within a population can increase the average fitness of its individuals (Reed and Frankham 2003). Thus, natural populations with genetic diversity display phenotypic variation which in turn causes varying fitness among individuals and modifies the average fitness of populations.

Typical laboratory raised species such as lab rats or mice do not exhibit the normal variation that exists in nature. These laboratory-raised species can inaccurately represent human populations and wild populations of mammals (Smale et al. 2005). Unlike laboratory species, wild populations experience pressure from natural selection to eliminate detrimental alleles. As an alternative to the study of laboratory species, using a sample from a population of wild-caught animals allows the analysis of a naturally occurring range of behaviors and physiological responses. Natural heritable physiological variation is known in the response to seasonal changes in photoperiod in some species of wild rodents (Ebling and Cronin 2000, Prendergast et al. 2002). Studying wild-source mice allows the analysis of variation in endocrine mechanisms that give rise to naturally occurring individual variation in seasonal life history traits on which natural selection acts (Smale et al. 2005). This variation in endocrine mechanisms has been used as a

model to study heritable variation in reproductive regulation and other physiological processes (Ebling and Cronin 2000, Heideman 2004).

Photoperiod and Seasonality

Many animals use photoperiod, or the duration of hours of light in a day, as an indication of changing seasons to increase ultimate survival and reproduction. A shortening photoperiod is associated with the onset of winter and related reduction in temperatures, food availability, and cover from predators. Many species inhabiting temperate zones conserve energy in winter by limiting the amount of energy devoted to non-essential functions, such as reproduction (Heideman and Bronson 1990, Nelson et al. 1998, Martin et al. 2007, Kaseloo et al. 2012). The limited amount of energy and high demand on the energy available exerts selective pressure on organisms to adapt to the seasons. These selective pressures favor the use of short photoperiod to anticipate the approaching harsh conditions of winter (Moffatt et al. 1993, Jacobs 1996). During winter, reducing reproductive function saves resources to enhance immune function or predator avoidance, while delaying reproduction to resource-rich spring and summer months increases pup survival rate. Thus, responses to photoperiodic changes that signal a change in environmental conditions may increase an organism's fitness. (Mousseau and Roff 1987, Prendergast et al. 2001, Emerson et al. 2008). The use of photoperiod to trigger winter reproductive suppression in rodents is common, but there are some exceptions.

There are specific environmental conditions in which responsiveness to photoperiod can actually be detrimental to an organism's fitness. While photoperiod responsiveness is useful when winter is always harsh, it can be detrimental whenever winters are mild and suitable for

reproduction. In a mild winter, reproducing during winter instead of waiting for spring or summer months can increase individual fitness through production of more pups, resulting in higher fitness than in a reproductively suppressed photoperiod responsive individual. Different years have winters that differ in harshness, causing the strategy with the highest fitness to change from year to year.

Photoperiod is detected by the photoneuroendocrine pathway (Ebling and Cronin 2000, Prendergast et al. 2002). Light being received on the retina activates photoreceptors that transduce the signal to the pineal gland (Goldman 2001, Prendergast et al. 2002). The pineal gland releases melatonin only when there is no light and the pathway is inactive (Bartness et al 1993, Goldman 2001, Prendergast et al. 2002). The duration of melatonin secretion affects an organism's physiology (Silverman 1988, Bartness et al. 1993, Ebling and Cronin 2000), regulating many physiological processes, such as circadian rhythms and seasonal reproduction (Reiter 1991, Morgan et al. 1994, Li and Witt-Enderby 2000, Masson-Pevet et al. 2000, Witt-Enderby et al. 2003). Melatonin acts by binding to cells in the hypothalamus and pars tuberalis that regulate the master regulator of reproduction, gonadotropin-releasing hormone (GnRH). Thus, melatonin regulates reproduction through the action of GnRH. By limiting the secretion of melatonin to the dark period, an organism has a reliable way to transduce photoperiod and changes in season into physiological signals that regulate reproduction.

Though the duration of melatonin secretion varies by season, not all individuals within a population respond by regulating reproduction by season. Many species display intra-population seasonal variation in fertility due to environmental factors such as photoperiod (Dawson et al. 2001, Goldman 2001). Some species and some individuals within species will suppress reproduction in winter while others will not, representing a life strategy trade-off (Prendergast et

al. 2001, Zera and Harshman 2001, Heideman and Pittman 2009). This variation in reproductive seasonality is caused by variation at the post-pineal level, not in variation in melatonin secretion by the pineal gland (Blank et al. 1991, Prendergast et al. 2001). Since variability occurs at the post-pineal level, it is possible for the same duration of melatonin secretion to affect different pathways in distinct ways (Goldman 2001). Differences in signaling pathways can allow individuals to simultaneously stimulate and suppress two different traits (Prendergast et al. 2001). Differential stimulation and suppression of traits allows variation in response to seasonality within a population.

Endocrine Disrupting Chemicals

Endocrine disrupting chemicals (EDCs) are compounds that can interfere with the endocrine system. EDCs can disrupt the development of the endocrine system, causing permanent changes (Colborn et al. 1993). A general consensus developed from 1992 to 1999 highlighted the health hazards from endocrine disruptors to wildlife and humans (Bern et al. 1992, Bantle et al. 1995, Benson et al. 1997, Alleva et al. 1998, Brock et al. 1999). Health issues associated with endocrine disruptors include a wide range of reproductive problems, including reduced fertility, early puberty and reproductive tract abnormalities (Harrison et al. 1995).

Bisphenol A

Bisphenol A, or BPA, is a commonly found EDC. BPA increases the plasticity of products such as hard, clear, polycarbonate plastics, and epoxy resins. BPA-based plastics are used in a variety of consumer goods, including water bottles and DVDs. At high temperatures or

extreme pH, the compound can hydrolyze and leach from the containers. BPA is commonly found in household goods and its presence is well documented in humans. A 2011 study that investigated the number of chemicals to which pregnant women are exposed in the U.S. found BPA in 96% of women (Woodruff et al. 2007). Thus, BPA reaches nearly everyone, including developing fetuses. BPA has a short half-life in soil of only 1-10 days, but its ubiquity makes it an important pollutant. The hormone-like properties of BPA have raised concerns about its suitability in consumer goods, including leading the FDA and EU to ban the use of BPA in baby bottles.

The cumulative evidence from human biomonitoring studies indicate sufficient continued exposure of people to parent, unconjugated BPA to explain its effects on physiological systems in children and adults (Vandenberg 2011). BPA does not bioaccumulate (Tillet 2009) and BPA can be cleared rapidly from the body. In adults, parent BPA is eliminated relatively rapidly from the body via a detoxification process in the liver. The liver and gastrointestinal tract metabolize BPA to a conjugated form of BPA which does not display known estrogenic activity (Matthews 2001, Volkel 2002). However, this detoxification process is not fully developed in infants and children, resulting in a decreased ability to process BPA, making developing children more sensitive to the effects of BPA.

The HPG Axis

The hypothalamus-pituitary-gonadal axis (HPG axis) regulates processes such as reproduction through integrated and coordinated action of endocrine glands in the hypothalamus, pituitary gland, and gonads (Ebling 2005) (Fig. 1). In the hypothalamus, gonadotropin releasing hormone (GnRH) neurons release pulses of GnRH to the pituitary gland. These GnRH pulses

promote the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary gland. From the pituitary, LH and FSH travel through the bloodstream to the gonads, where FSH stimulates gametogenesis and LH triggers the gonads to release estrogen, progesterone, or testosterone. Testosterone inhibits the release of the gonadotropin hormones, partially by inhibiting the release of GnRH from the hypothalamus (Bronson 1981, Kalra and Kalra 1983, Smith and Neill 1987, Meredith et al. 1991, Freeman 1994).

The HPG axis can be altered and modified by environmental or metabolic cues. Metabolic cues such as glucose and leptin availability often modulate mammalian reproductive systems (Rowland and Moenter 2011, Smith et al. 2006), while a major environmental regulator of reproduction is photoperiod. Variations in the photoneuroendocrine pathway described above cause variable reproductive phenotypes in response to seasonal changes (Prendergast and Nelson 2001). In contrast, very little research has been conducted on natural variation in the response to other environmental regulators of the HPG axis.

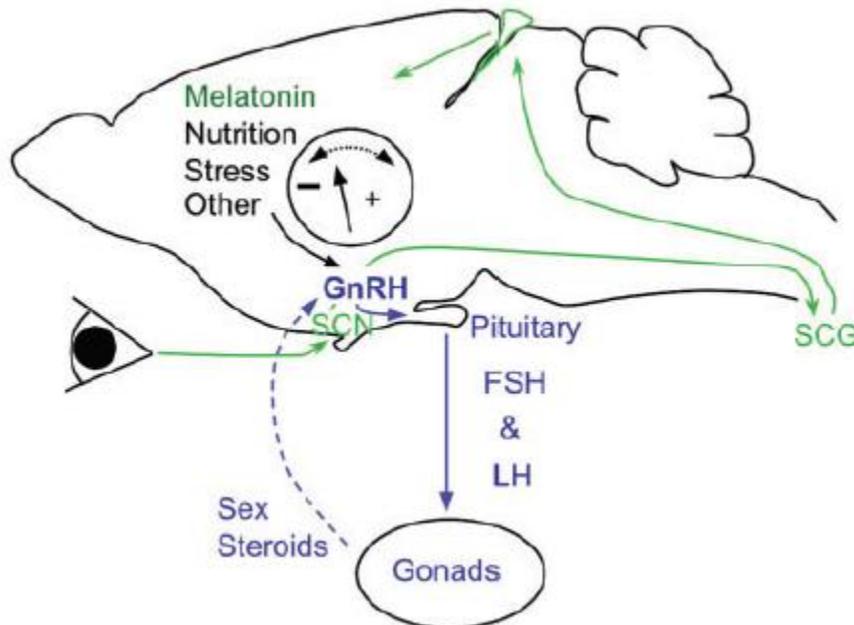


FIGURE 1. The HPG axis (in blue) of the reproductive system is regulated by multiple inputs, including season (photoperiodic pathway, in green), nutrition and stress (in black). Figure from (Heideman et al. 2009)

The HPG axis appears to be disrupted by BPA acting as an estrogen agonist. Evidence for the estrogenicity of BPA first came from experiments on rats in the 1930s by Dodds and Lawson (Dodds and Lawson, 1936). Subsequent work showed that BPA mimics the structure and function of estradiol, a sex hormone and steroid, allowing BPA to bind to and activate the same estrogen receptors (ER) as estradiol; ER-alpha and ER-beta. In 1997, adverse effects were reported in laboratory animals given low-doses of BPA (Erickson 2008). Numerous other studies have found that laboratory animals exposed to low BPA levels have elevated rates of diabetes, mammary and prostate cancers, lower sperm count, or other metabolic and reproductive problems (Gore 2007, O'Connor et al. 2003, Okada et al. 2008, vom Saal and Myers 2008). Early developmental stages appear to be the period of greatest sensitivity of its effects, but there is a shortage of research regarding the effects of BPA on adult animals.

Animal Model

The animals used in this study are a wild-source population of white-footed mice, *Peromyscus leucopus* that contains natural heritable variation. The colony is derived from a wild-caught population of white-footed mice originally captured in 1995 in Williamsburg, VA (Heideman et al. 1999). Within this colony, two selection lines were developed that differ in their reproductive response to photoperiod. The two selection lines were developed by selection for or against mature gonads in short day photoperiod (Heideman et al. 1999). One line labeled nonresponsive (NR) was selected for mature gonads in short day photoperiod. The other line

labeled responsive (R) was selected for immature gonads in short day photoperiod. In short day photoperiods mimicking winter, responsive mice respond by suppressing reproduction, resulting in immature gonads (Fig. 2). In the same short day, winter-like photoperiods, non-responsive mice respond weakly or not at all, resulting in mature gonads (Fig. 2). These two lines of *P. leucopus* represent two naturally occurring extreme phenotypes: strong reproductive responsiveness to photoperiod and slight reproductive response to photoperiod.

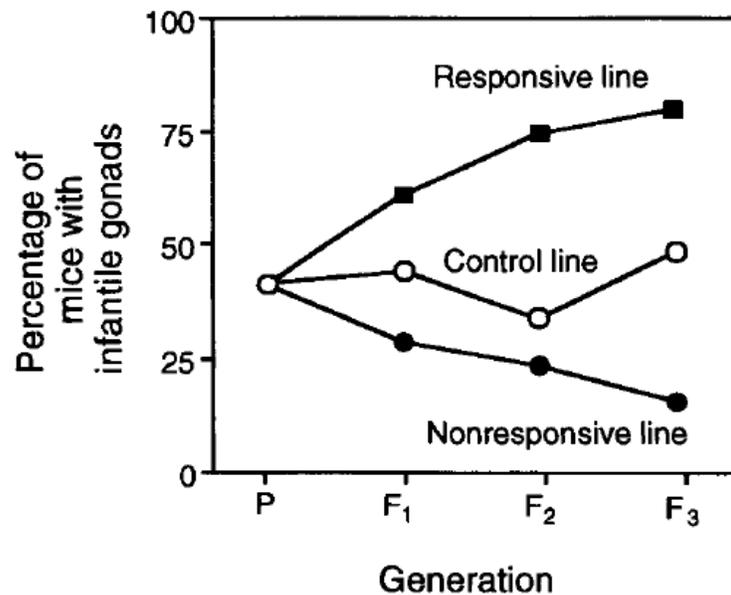


FIGURE 2. Percentage of individuals strongly responsive to photoperiod in each generation in each line (open circles, control line; solid square, responsive line, solid circles, nonresponsive line). Figure from (Heideman et al. 1999).

Amongst other differences, the two selection lines display differences in the HPG axis, including a significant differences in the number of immunoreactive (IR) gonadotropin releasing hormone (GnRH) neurons (Avigdor et al. 2005). While the responsive line had significantly fewer IR-GnRH neurons than the non-responsive line, there was no significant difference between lines in short day versus long day (Avigdor et al. 2005). This evidence of photoperiod-

independent differences suggests that the variation in photoperiod responsiveness may arise partially from the number of IR-GnRH neurons. Avigdor et al. (2005) also concluded that variation in counts of IR-GnRH neurons arises from genetic variation. Given the existing genetic variation in reproductive response in both long and short photoperiods, this suggests that the lines may differ not just in their photoneuroendocrine pathway, but also in the HPG axis itself. Given that the lines vary in both the photoneuroendocrine pathway and the HPG axis, it is important to examine possible genetic variation in response to other biologically and medically important stimuli, such as an endocrine disruptor.

Hypotheses and Predictions

In natural populations of *Peromyscus leucopus*, natural selection in a changing environment apparently results in genetic variation in endocrine signaling pathways. Organisms that have heritable reproductive variation in response to either long or short photoperiod may have heritable variation in reproductive response to other environmental stimuli. Previous studies indicate that the lines vary genetically in the endocrine pathway that regulates reproduction.

In this thesis, I test the following hypothesis:

1. There is variation in the reproductive response to an endocrine disruptor that is related to heritable variation observed in our artificial selection lines of wild-source white-footed mice, *Peromyscus leucopus*. The response to an endocrine disruptor would vary because the lines of mice vary in endocrine traits, such as the number of gonadotropin-releasing hormone neurons that would alter how an EDC affects the HPG axis processing of an environmental stimulus.

2. The null hypothesis is that there is no variation in reproductive response to an endocrine disruptor between the two selection lines.

Based on this hypothesis, I predicted:

1. In short-day photoperiod, mice from the responsive line would have a stronger reproductive response to the endocrine disruptor than the mice from the non-responsive line because mice in the responsive line have already display differences related to lowered fertility.
2. Under the null hypothesis, there would be no difference in reproductive response to an endocrine disruptor

Materials and Methods

Development of Selection Lines

Forty-eight wild founders of the laboratory colony at the Population and Endocrinology Laboratory were captured in the winter of 1995 in Williamsburg, Virginia. Wild-caught animals were paired in a long-day photoperiod (LD; 16 hours of light; 8 hours of dark) to produce a parental generation as stock for selection experiments. To establish short-day reproductively inhibited and photoperiod-nonresponsive lines, animals from the parental generation were transferred to short-day photoperiod (SD; 8 hours of light; 16 hours of dark) at birth and raised in SD conditions. Mice were examined at 70 ± 3 days of age and assigned a reproductive index based on testis size or the size of the ovaries, uterine diameter, and presence or absence of visible corpora lutea (Heideman et al. 1999). Females with ovaries ≤ 2 mm in length, lacking visible corpora lutea, and uterine diameter of ≤ 0.5 mm were classified as reproductively inhibited (R) by SD. Females with large ovaries (usually >3.5 mm in length), large visible follicles or corpora

lutea, and uterine diameter >1 mm were classified as nonresponsive (NR) to SD. Males with a testis index (length × width of testis) < 24 mm² were classified as R; those with a testis index > 32 mm² were classified as NR. R males and females were paired in LD to produce offspring for the R line. NR males and females were paired to produce offspring for the NR line. Selection was continued on the offspring of each line to further develop the R and NR selection lines. The experiments in this study were conducted on mice from generations 15-21.

Experimental Design

For this experiment, 42 male mice between 56 and 84 days of age were selected from the responsive and non-responsive lines in short day photoperiod (8 hr light, 16 hr dark). Brothers were included, and familial relationship was used as a variable to determine its effect on the results. Multiple runs were completed from August 2014 to February 2015, with each run consisting of 6 mice balanced for line, weight, age, and testes size.

Mice were individually housed in standard BPA-free polypropylene cages with mouse chow and water available *ad lib*. Before the experiment began, mice were housed at the Population Laboratory on the campus of William and Mary. After obtaining subjects for the experiment, mice were housed within their BPA-free cages in Millington Hall in light-tight ventilated plywood boxes. The six treatment groups were as follows: non-responsive selection line given 0 mg/kg BPA, responsive selection line given 0 mg/kg BPA, non-responsive selection line given 50 mg/kg BPA, responsive selection line given 50 mg/kg BPA, non-responsive selection line given 250 mg/kg BPA, and responsive selection line given 250 mg/kg BPA.

Measures were taken to avoid uncontrolled BPA exposure. Mice were housed from birth in standard polypropylene cages, which are BPA free. Food was placed in metal food hoppers

and water was available in glass bottles. When creating BPA stock solution, glassware was used. Fruit Loops which were to be dosed with BPA were contained in a BPA-free container. Throughout the process, plastic was avoided unless it was known to be BPA free.

Treat Preparation

To prepare dosed treats, the sugar layer on one side of the Fruit Loops was scraped or drilled off. Stock solutions of BPA were prepared before every run based on the average weight of the mice in the run. The appropriate amount of stock solution was then pipetted into the exposed fruit loop. Fruit loops were allowed to dry a minimum of 5 hours before being placed in the food hopper. Treats were sometimes prepared well in advance, but never more than 21 days in advance.

Treat Administration

BPA-dosed and BPA-free treats were placed in the food hopper daily approximately 1 ± 2 hours before the dark period began; when that timing was not practical, treats were placed under dim red lighting within four hours after the dark period had begun. Mice were given a random mix of colors. Animals were unobserved until the next dosage time. We recorded whether or not animals ate the entirety of their treat. My records indicate that as the dose increased, animals were less likely to eat the full dose. If over the course of 3 weeks the animal had not eaten a significant amount of the treats or had begun to hoard them, an extra day of treatment was added (N= 3 mice out of the total N= 42 experimental mice; all three were in the highest dose treatment).

Controls

Control treats were initially unmodified Fruit Loops. Midway through the experiment, the control preparation was altered. At the midpoint of the experiment, control treats were shaved to match the BPA-treated Fruit Loops and dosed with pure ethanol using the same procedure used for BPA-dosed treats. Consumption appeared unaltered, as mice continued to eat the ethanol-dosed Fruit Loops as frequently as they had consumed the plain Fruit Loops.

Blood collection, testes mass, body mass, food intake, and reproductive organs

On day 0 mice were lightly anesthetized and initial estimated testes volume (ETV) was measured using a caliper (performed by Paul Heideman). Mice were weighed on day 0 and day 21 (Figure 1). The food in the food hopper was weighed on day 0 and day 21. Food intake data from mice that ate more than 6g per day was excluded from analyses as previous results suggest higher amounts is ground by mice but not eaten. Twenty-four hours after the final dose (day 22), mice were heavily anesthetized with isoflurane and blood was collected using a retro-orbital collection technique. Immediately afterwards, animals were euthanized using an overdose of isoflurane and were perfused transcardially using approximately 50 mL 0.1 M phosphate-buffered saline (PBS) at pH 7.4 for 4 minutes. Following perfusion with PBS, mice were perfused with approximately 100 mL Zamboni's Fixative for 20 minutes. After perfusions, reproductive organs were extracted and weighed. Procedures were approved by the Institutional Animal Care and Use Committee of the College of William and Mary (IACUC-2014-03-25-9477-pdheid).

BPA ELISA

To test whether BPA was present in mice after dosing, a BPA mouse-specific enzyme-linked immunosorbent assay (ELISA) was obtained from MyBioSource, Inc. The double-sandwich ELISA kit had a detection range of 3.12--200 ng/mL BPA.

LH Assay

Levels of luteinizing hormone in plasma were obtained through a sensitive two-site sandwich immunoassay. The assay was performed by the UVA Center for Research in Reproduction Ligand Assay and Analysis Core (Charlottesville, VA).

Data Analysis

Analyses were done using a linear model analysis, with selection line and dose (0, 50, or 250 mg/kg body weight) included in each model, performed with R statistics software on a Dell laptop computer. Dosage groups were balanced for age, weight, initial testes volume, and line. All analyses included line, dose, and the interaction between line and dose.

Results

Experiment 1

With a two-way between-subjects linear model, the effects of line, dose, and interaction between line and dose were examined on food intake, weight change, seminal vesicle (SV) mass, leutinizing hormone levels, and testes mass.

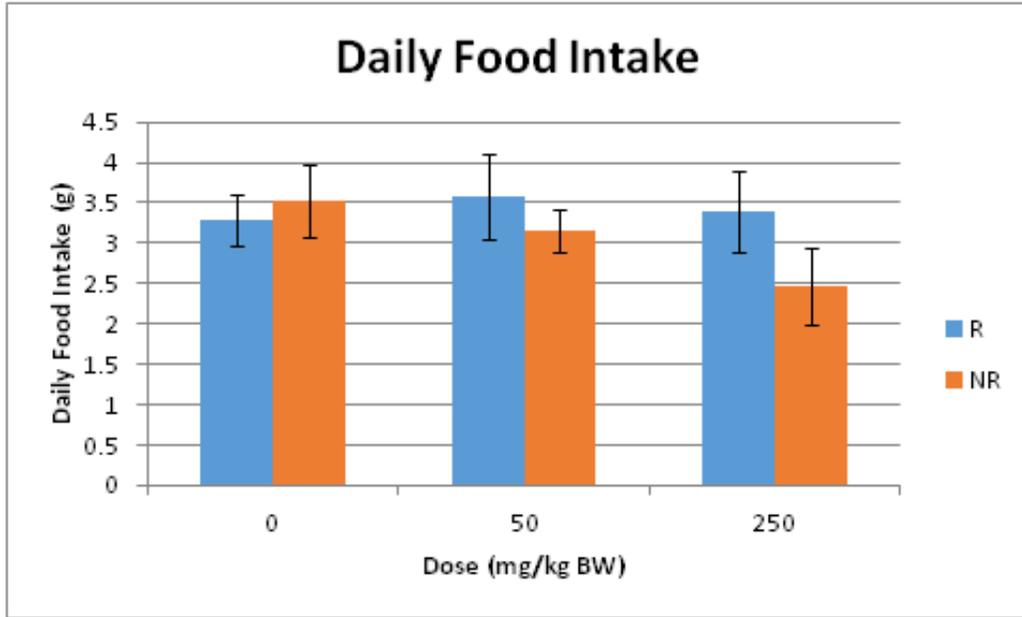


FIGURE 3. Mean (\pm standard error of the mean) weight of daily food intake from male mice in each of the six treatment group; $p > 0.1$.

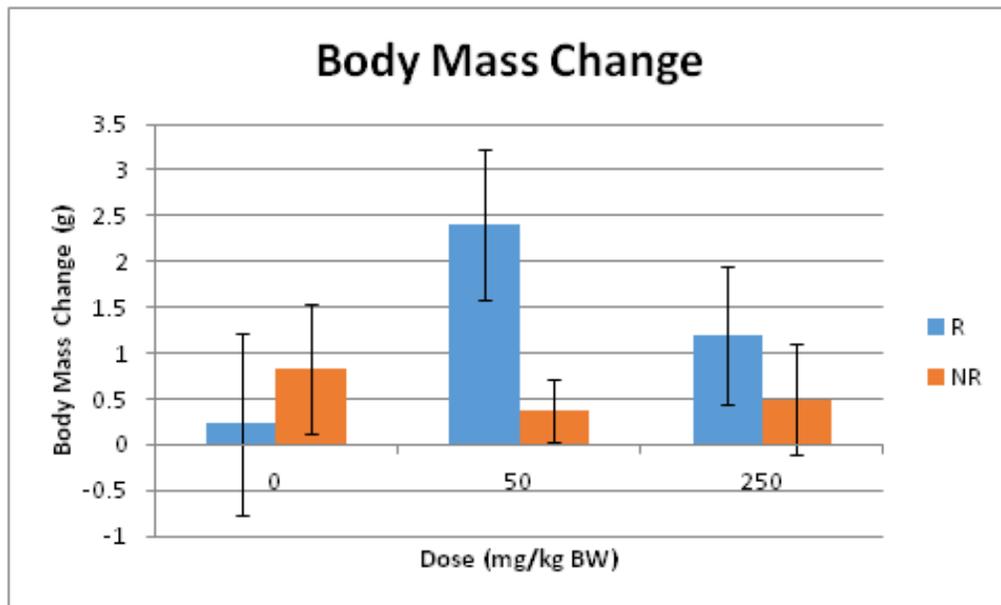


FIGURE 4. Mean (\pm standard error of the mean) weight of body weight change between day 1 and day 21 from male mice in each of the six treatment group; $p > 0.2$.

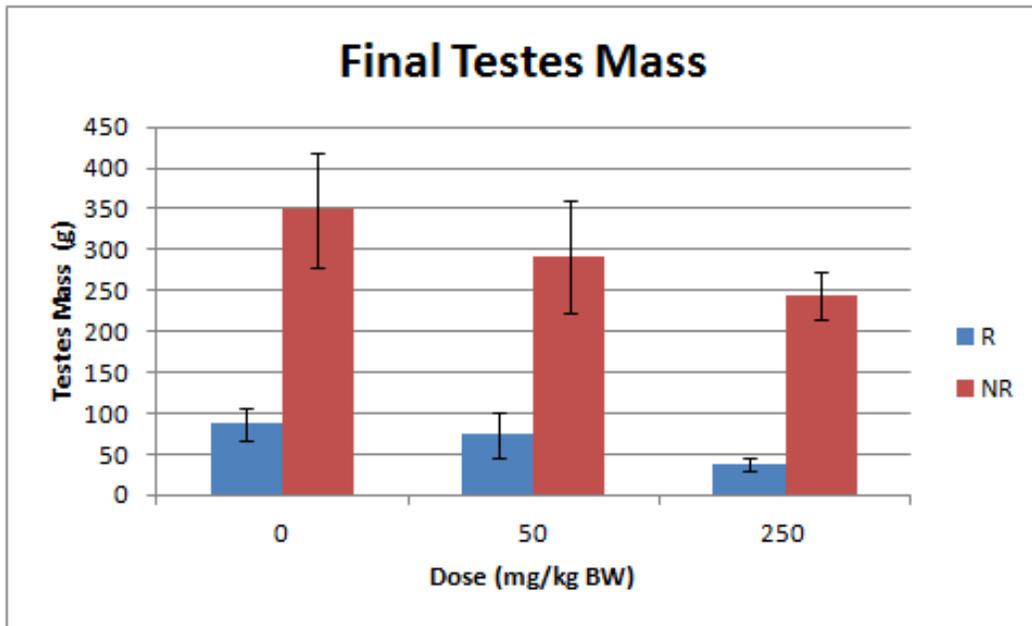


FIGURE 5. Mean (\pm standard error of the mean) weight of final testes mass from male mice in each of the six treatment group; $p = 0.07$.

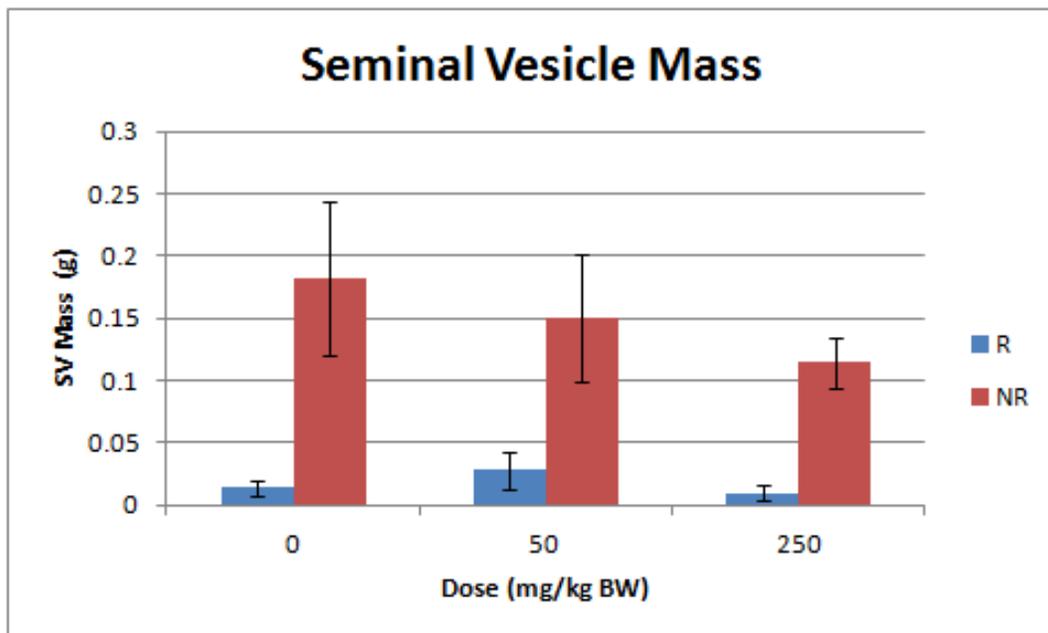


FIGURE 6. Mean (\pm standard error of the mean) weight of seminal vesicle mass from male mice in each of the six treatment group; $p > 0.2$.

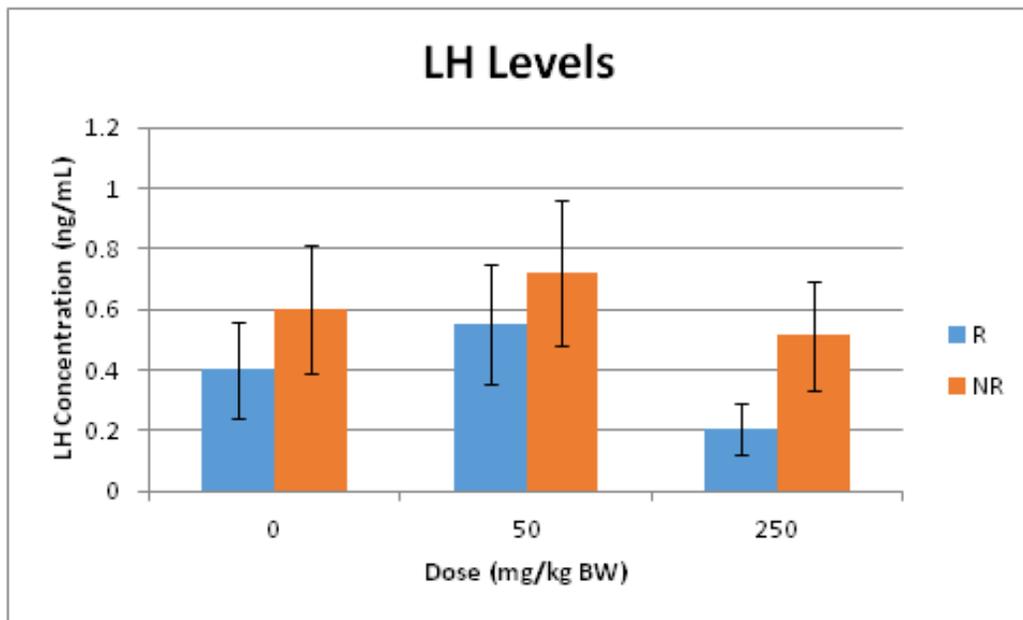


FIGURE 7. Mean (\pm standard error of the mean) concentration of LH in serum from male mice in each of the six treatment group; $p > 0.1$.

Final Testes Mass

Testes mass varied with line ($F=49.09$; $p < 0.001$), but not dose ($F=3.42$; $p=0.07$), and there was no significant interaction between dose and line ($F=0.33$; $p=0.57$).

Seminal Vesicle

Seminal vesicle mass varied with line ($F=27.2$; $p < 0.001$), but not dose ($F=1.45$; $p=0.24$) and there was no significant interaction between dose and line ($F=0.76$; $p=0.39$).

Daily Food Intake

Daily food intake did not vary with line ($F=1.35$; $p=0.25$), dose ($F=1.81$; $p=0.19$) and there was no significant interaction between dose and line ($F=1.82$; $p=0.19$).

Body Weight Change

Change in body weight over the 3-week period did not vary with line ($F=1.5$; $p=0.23$), dose ($F=0.002$; $p=0.97$) and there was no significant interaction between dose and line ($F=0.12$; $p=0.73$).

LH levels

Concentration of LH in serum did not vary with line ($F=2.63$; $p=0.11$), dose ($F=1.51$; $p=0.23$), and there was no significant interaction between dose and line ($F=0.15$; $p=0.7$).

BPA ELISA

The initial ELISA run did not detect any measurable BPA in any serum samples. All samples tested were below the detection limit of 3.12ng/mL.

Discussion

Functional Disruption

In the introduction I hypothesized that there might be variation in the reproductive response to an endocrine disruptor that is related to heritable variation observed in our artificial selection lines of wild-source white-footed mice, *Peromyscus leucopus*. The response would vary because the lines of mice vary in endocrine traits, such as the number of gonadotropin-

releasing hormone neurons. This would in turn alter response to an environmental inhibitory stimulus.

Based on this hypothesis, I predicted that in short-day photoperiod, mice from the responsive line would have a stronger reproductive response to the endocrine disruptor than the mice from the non-responsive line. The responsive line may be more sensitive to an inhibitory environmental stimulus due to already existing differences related to lowered fertility.

The selection lines differed in two reproductive traits in short photoperiod, testes mass (Fig. 4) and seminal vesicle mass (Fig. 5), as in previous studies (Heideman et al. 1999, Broussard et al. 2009, Heideman and Bronson 1991). The selection lines did not differ in food intake (Fig. 3) or adjustments to body mass (Fig. 4), as in many, but not all, previous studies (Heideman and Pittman 2009; White et al. 2014). In a previous study, females in the nonresponsive selection line had higher concentrations of luteinizing hormone than females in the responsive line (Heideman et al. 2010). In this experiment, males in the nonresponsive line appeared to have higher mean concentration (Fig. 7), but the differences were not statistically significant.

There were no detectable effects of BPA on adult animals, indicating a higher dose is required to elicit a reproductive effect. The lowest dose, 50 mg/kg, was administered because it is known to be the lowest-observed-adverse-effect-level (LOAEL). In the pilot study performed by Dr. Julian Pittman, both the current lowest dose and highest dose (250 mg/kg) had reproductive effects. For this experiment, we did not use an even higher dose because we were concerned that higher doses might be unnecessary and unrealistic and would approach lethal doses. The oral median lethal dose (LD50) for BPA in adult mice is 2,400 mg/kg. Rather than use higher doses in adults, I suggest that future experiments should test a different endocrine

disruptor or test effects during development. Using a different endocrine disruptor with a higher LD50 or more difference between the LD50 and LOAEL could allow the use of higher dosages more likely to elicit a response.

The effect of dose on reproductive organ size was not significant in any dosage group or line. There was no apparent functional disruption in reproductive organs, weight, or food intake after exposure to the endocrine disruptor. While the effects of BPA were not statistically significant, there was a general trend of decreasing reproductive organ size as dose increased (Figs. 5 and 6).

In addition, there may have been physiological or behavioral changes that were not observed or noted. For example, sexual behavior was not observed, despite the possibility that brief feminization of the brain may have resulted in reduced sexual behaviors. Additionally, liver weight was not taken, despite the possibility that different processing due to differing neurochemistry may have resulted in differences in liver weights.

Preliminary data collected by Dr. Julian Pittman had what appeared to be biologically significant differences between lines, but mice were dosed using oral gavage (Pittman unpublished data). Though the gavage procedure is widely used in toxicology studies, oral gavage is a more stressful procedure, especially for wild *P. leucopus*. Daily handling and gavage may have increased stress levels to combine with the effects of BPA such that reproduction was suppressed. The Fruit Loop delivery system dramatically reduced the potential confounder of stress from handling and gavage. Thus, the pilot experiment may have tested the combination of stress and BPA, while this experiment tested only the effects of BPA in voluntarily eaten food treats. If so, this could explain the lack of effect of BPA alone in this experiment.

Delivery system

Though the delivery system did not always deliver the full dose, most mice readily consumed their full dose on most nights. BPA was pipetted into six Fruit Loops, which the animals were allowed to eat at their own pace. Every animal ate at least the majority of the dose, but some individuals left chunks behind in their bedding. This occurred regularly at the 250 mg/kg dose, sometimes at the 50 mg/kg dose, and never at the 0 mg/kg dose. In addition to not eating the full fruit loop, when individuals didn't eat the full loop, mice may have been avoiding the section of the loop where the BPA was most concentrated. In the experiment, for mice that had not eaten the equivalent of 4 or more Fruit Loops during the study, that individual was given one extra day of BPA treatment. Overall, while some animals at the highest dosing group received less than the full dose, most individuals appeared to receive the majority of their dose.

While the Fruit Loop delivery system did not have reliable timing of the dose, it may represent a more realistic method of natural BPA exposure. When using oral gavage or injections, the full dose is given at one time, while with the Fruit Loop system, food was given close to the dark period for mice to eat over time. Since *P. leucopus* is mostly nocturnal, it was assumed that food was consumed during the 16 hour dark period, but the cages were not checked until the next feeding time. Thus, loops were consumed within a roughly 24 hour period. Excess treats were not removed from the cage, but were rarely eaten after the next day. Overall, it was difficult to ensure the entire treat was eaten and to determine when the treat was eaten. This made determining the appropriate time to euthanize and collect samples difficult and variable among subjects. Despite these issues, real world exposure to BPA in humans and other animals

is more similar to the Fruit Loop system because the majority of exposure comes from food or environmental pollutants, not an injection or gavage dosing (Sieli et al. 2011).

ELISA

BPA may have been undetectable in plasma collected approximately 24 hours post-dose because it may have already cleared and been excreted through urine or feces. Clearance rates of BPA in CD-1 mice after oral exposures are similar to that of humans and rhesus monkeys. Maximum serum concentration occurs about 1 hour after dosing, declining to low levels by 24 hours post-dose (Taylor et al. 2010; Figs. 8 and 9). Similarly, when Fischer 344 rats were dosed by oral gavage, BPA levels in serum peaked at 1 hour post-dose and decayed below the limit of quantification after 18 hours. However, BPA metabolites were detected up to 72 hours after oral gavage (Pottenger et al. 2000). Thus, sampling earlier might be necessary to detect BPA in blood.

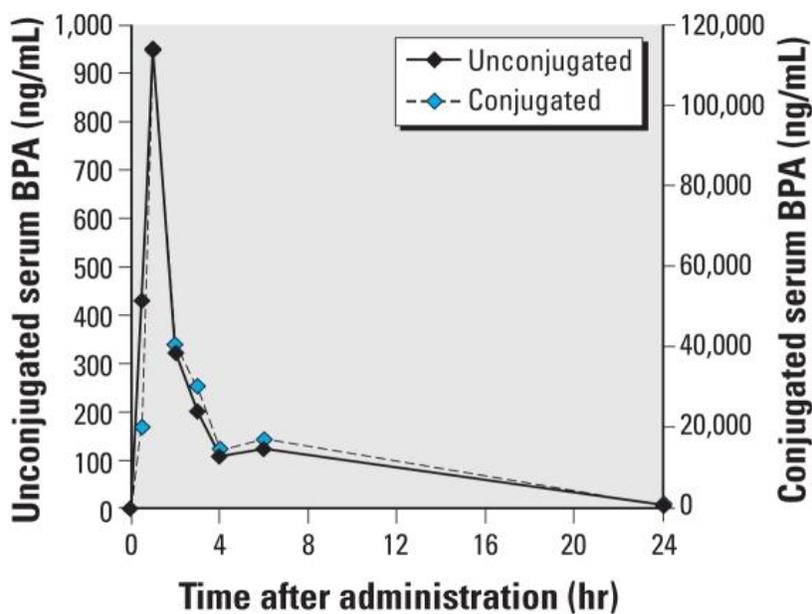


FIGURE 8. Unconjugated and conjugated serum BPA concentrations in adult female CD-1 mice (n = 4 per time point) during the 24 hr after a single oral dose of BPA (100,000 µg/kg). Figure from (Taylor et al. 2011).

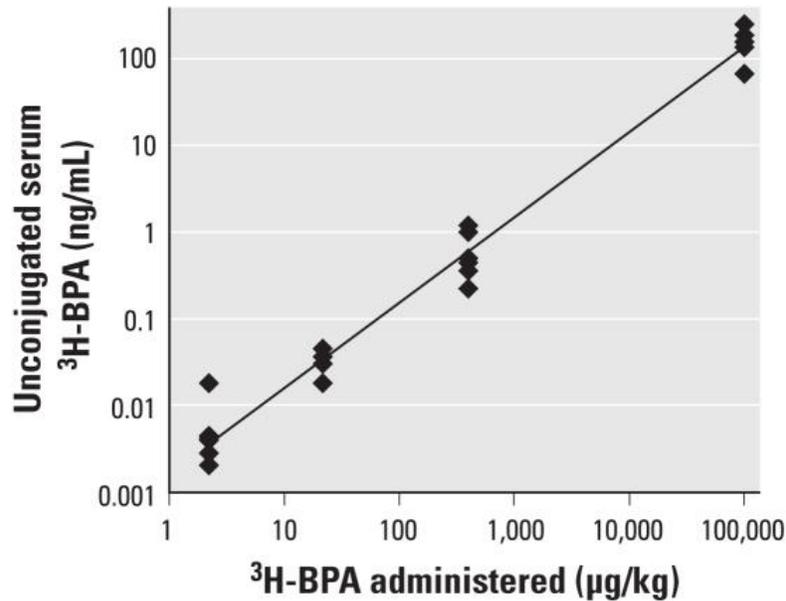


FIGURE 9. Concentration of unconjugated serum ³H-BPA in adult female CD-1 mice in relation to the administered oral dose of BPA over a 50,000-fold dose range (nominal dose: 2, 20, 400, and 100,000 µg/kg). Blood was collected 24 hr after administration. Figure from (Taylor et al. 2011).

The high peak and rapid clearance of BPA following oral gavage might lead to effects of BPA that would not be matched by the same dose of BPA administered in food treats. Instead of reaching a high, sudden peak, the concentration of BPA from a food treat would create a lower and more gradual rise (Fig. 10). Whether or not this more gradual rise would result in a physiological response would depend on the organism of interest's biological threshold for physiological response. An organism with a higher threshold may only have a brief physiological response from the gavage (Fig. 10, line A). In this case, the food treat method would not result in a high enough concentration to elicit a physiological response. However, in an organism with a lower threshold, both dosing methods would elicit a response. In this case, the food treat method

would elicit a delayed, but more sustained response when compared to the gavage dosing (Fig. 10, line B). Thus, the method of consumption can have significant effects on the onset, duration, and magnitude of physiological effects.

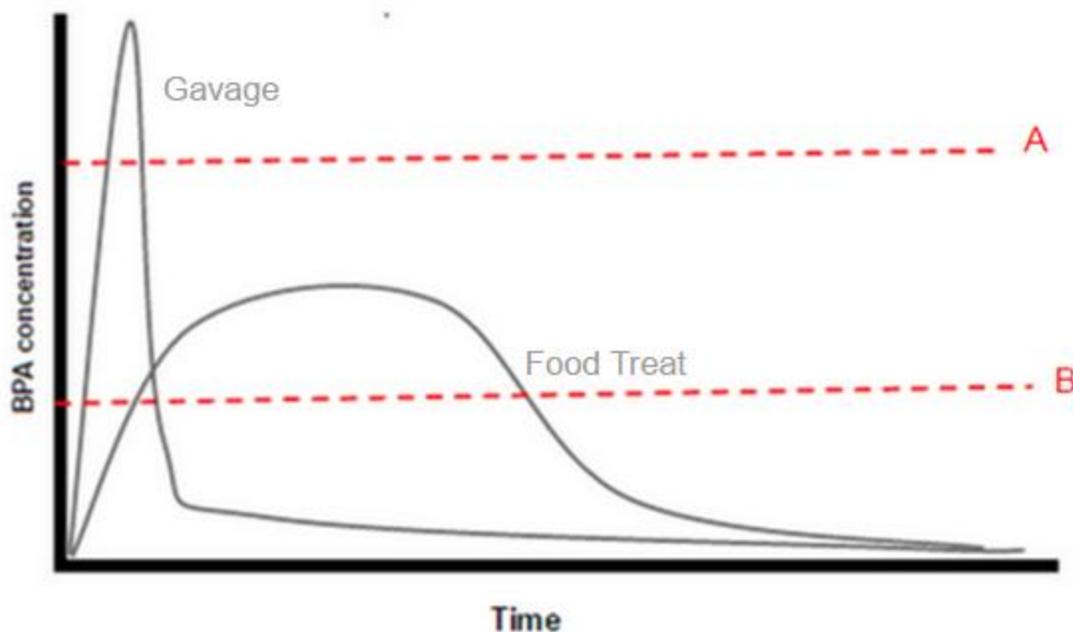


FIGURE 10. Potential concentration of BPA in serum or plasma as a function of time in two dosing methods: gavage and daily food treats. Dashed line A represents a higher biological threshold for physiological response, while dashed line B represents a lower biological threshold for physiological response.

Future Directions

In the future, experiments should investigate the effects of an endocrine disruptor on younger, developing mice. Currently a study has been started in our laboratory to test the endocrine disrupting effects of testosterone propionate on *P. leucopus* pups. Pups will be injected, which should provide a reliable delivery method. Additionally, most information

indicates that developing brains are the most susceptible and responsive to hormonal or environmental changes (Xu et al. 2011, Patisaul and Polston 2008). This would indicate that regulations banning endocrine disruptors are more effective in items designated for young infants or children than for adult consumption.

Additional investigations should be done on pregnant or nursing mothers intake on pups. A problem is that *P. leucopus* is not an ideal model for treatment of pregnant dams because it is difficult to tell when the females are ovulating or pregnant. In addition, while adult mammals have similar clearance rates of BPA, there may be important differences between rodent and primate metabolism of BPA in very young animals. This would impact the usefulness of using rodent models for understanding developmental effects of early BPA exposures in humans (Doerge, 2011; Taylor 2010).

Future experiments could examine the differences between response to toxins in long and short photoperiods. Differences in response to environmental toxins may vary with photoperiodic changes because mice may allocate resources differently in stressful “winter” conditions.

This experiment provided food *ad lib*, but this is not a reliable model of the actual environmental conditions facing wild animals. Under food or other energy restriction, animals may demonstrate differential allocation of resources, including response to endocrine disruptors (Schneider et al. 2013).

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