ENDOCRINE DISRUPTORS AND THE REGULATION OF VOLUNTARY PHYSICAL ACTIVITY IN MICE

A Dissertation

by

EMILY ELIZABETH SCHMITT

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Chair of Committee,	J. Timothy Lightfoot
Committee Members,	James D. Fluckey
	Michael P. Massett
	Weston W. Porter
	Roger Sansom
Head of Department,	Richard B. Kreider

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ABSTRACT

Physical inactivity is the underlying cause of a large variety of chronic health conditions, is the second leading actual cause of death in the United States, and is a major contributor to obesity and diabetes. In spite of the strong evidence favoring physical activity as a successful and cost-effective therapy, voluntary activity levels in the United States are extremely low. Given that the most potent biological regulators of daily activity known thus far are the sex hormones (specifically testosterone) and that there are multiple environmental endocrine disruptors (ED) that directly affect sex hormone functioning, it is an intriguing possibility that environmental exposure to ED may directly affect physical activity levels. Mouse dams were treated with benzyl butyl phthalate (BBP) or a control substance (sesame oil) on days 9-16 of pregnancy. The resulting pups were weaned at three weeks of age and were randomly assigned to a four week, 10 week or 20 week sacrifice group. Daily distance, duration, and speed were measured in pups. All mice were measured weekly to determine body composition. Additionally, blood samples were extracted via a heart stick to be used for later analysis of testosterone in male pups and estrogen in female pups.

Vaginal openings in female BBP mice were significantly delayed compared to control mice. Anogenital distances in male BBP mice were significantly smaller compared to control mice at 10 and 20 weeks. There was no significant differences observed in the physical activity distance ran, duration, or speed between male and female control and treatment BBP mice. Overall, there was a significant difference in

weight, lean mass, and fat mass in BBP and control male and female mice. Upon post hoc analysis, males in later life showed a significant difference in fat mass compared to controls. Lastly, there was a significant difference in serum testosterone concentration values in male mice between control and BBP at 10 weeks and at 20 weeks but no significant differences in estrogen concentration in female mice. This study was the first to analyze the effect of phthalates on physical activity in mice.

DEDICATION

This dissertation is dedicated to the two most wonderful parents I could ask for: Steve and Melanie Schmitt. Dad, I have learned so much you. Your hard work in corporate America, persistence in exercising every day, and selflessness when it comes to taking care of your family are just a few of the reasons your love is so much appreciated. Mom, I'll never forget the tree I was sitting under my first semester of Elon, terrified, explaining I had no idea what I wanted to do with my life. You assured me I could handle the science classes in the Exercise/Sport Science major and look where I have ended up! Your faith in my abilities, constant support and love, even 1,000 miles away for all of these years, makes you the most wonderful mother on the planet. Thank you both. I hope to continue to make you all proud.

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1. INTRODUCTION AND LITERATURE REVIEW

Physical inactivity is the underlying cause of a large variety of chronic health conditions, is the second leading actual cause of death in the United States (in conjunction with poor diet), and is a major contributor to obesity and diabetes (86; 91). Voluntary physical activity has been positively correlated with decreases in cardiovascular disease, obesity, type II diabetes and some forms of cancer (78). In spite of the strong evidence favoring physical activity as a successful and cost-effective therapy, voluntary activity levels in the United States are extremely low. Studies using direct activity measurements have suggested that as few as 3.5% of adults over 20 years old complete at least 30 minutes of moderate activity daily (137) even though national guidelines recommend the completion of 150 minutes of moderate intensity aerobic activity per week for healthy living (20). Thus, identifying mechanisms that could enhance voluntary physical activity levels or lead to a better understanding of what factors regulate physical activity is extremely important. Not only will identifying these mechanisms help to increase physical activity in those who do not exercise, but it will also potentially reduce health care costs due to inactivity related diseases and conditions.

The regulation of physical activity, previously assumed to be mostly voluntary, has been shown to be primarily influenced by genetic/biological mechanisms (25-92% influence; reviewed in (78)) and unique environmental exposures (8-52% influence; (64; 132)). What is not known is whether an interaction between biological mechanisms and environmental exposures play a significant regulatory role in physical activity. Given that the most potent biological regulators of daily activity known thus far are the sex

hormones (14; 77) and that there are multiple environmental endocrine disruptors (ED) that directly affect sex hormone functioning (88; 119; 147), it is an intriguing possibility that environmental exposure to ED may directly affect physical activity levels (137). This literature review and subsequent dissertation will focus on environmental toxicants, or ED, the effect on sex hormones, and subsequent effects on the regulation of physical activity. It is well documented (8; 18; 24; 25; 38; 61; 73; 82; 89; 127) that disruptions in hormones have caused a variety of hormonal problems since the 1970s, yet no one has investigated as to whether these disruptions in our hormones are having an impact on voluntary physical activity levels of the human population.

1.1 Endocrine Biology

The mammalian endocrine system is a collection of glands that produce and secrete hormones that travel through the bloodstream. The major endocrine glands include the pancreas, parathyroid gland, thyroid gland, gastrointestinal tract, gonads (ovaries and testes), adrenal cortex, hypothalamus, and pituitary gland (90). In addition, the skin, liver, and kidneys work together to produce vitamin D (11; 103) and the stomach and small intestine secrete hormones to aid with eating and digestion (44). Also, the literature suggests that adipose tissue - "fat" - is an endocrine organ (3; 7; 67; 117) because it is regulated by hormonal signals (129), specifically the secretion of leptin (a hormone) (69), and is also characterized as a major site for sex steroid metabolism and synthesis (125). The complexity of the endocrine system requires the

glands and organs that secrete hormones to work in harmony with each other and this synchronization is susceptible to disruption.

Hormones are secreted by glands of the endocrine system and are responsible for regulating a range of physiological and behavioral activities, such as, metabolism, respiration, development, reproduction, and mood. Specifically, the sex hormones function at the genomic level meaning they work through intracellular receptors by binding to the appropriate hormone-receptor complex to activate or repress transcription of target genes (51). The classic receptors for sex steroids are produced in the cytoplasm and form complexes with other proteins to ensure protein folding leads to the formation of chaperone proteins (51). For example, the action of receptors promoting this complex formation occurs by either direct interaction with mechanisms of transcriptional factors or through an intermediate factor like a coactivator (138). These complexes modify and interact with the transcriptional initiation complex that encompasses many transcriptional factors (10). The length of time from sex steroid entry into the cell to the production of new proteins usually takes a few hours and can be disrupted by inhibitors like actinomyocin D or cycloheximide (83).

Testosterone is the major circulating hormone in males produced in the Leydig cells and the adrenal cortex through the conversion of cholesterol into androgens which begins at the mitochondrial level and results in the production of pregnenolone (98). The conversion starts the steroidogenic casade that produces viable testosterone via the biosynthesis of active androgen and degradation of inactive pregnenolone (98). 3β-hydroxysteriod dehydrogenase is the catalyst for this process (conversion of

hydroxysteriods to ketosteriods) (98), and the final step in this biosynthetic pathway of testosterone is the reduction of the 17-keto-group by 17 β-hydroxysteriod dehydrogenase (17 β HSD) (98). Under normal conditions the total capacity of the pregnenolone-converting enzyme system in humans is insufficient to convert all available progesterone into testosterone (98). Therefore, many of the progesterone derivatives leak out of the Leydig cells and cause a rate limiting step to this process (98). In males, testosterone is converted to its useable from of dihydrotestosterone through an aromatization process using the *cyp19* enzyme complex.

The sex steroid, estrogen, is produced in the ovary and is the major circulating hormone in females that regulates estrogen receptor-α and estrogen receptor-β (16). The proteins in this superfamily of nuclear receptors function as ligand-inducible transcription factors (45). The biosynthesis of estrogen begins when G-protein receptors bind to follicle stimulating hormone causing cyclic-AMP (cAMP) levels to rise. This rise in cAMP enhances the binding of steroidogenic factor-1 and cAMP response element binding protein located in the aromatase gene to activate estrogen secretion from the follicle (16; 126). Finally, progesterone is the major secretion product in the female ovary but can also be secreted by the adrenal gland as well (37). Progesterone helps mediate the synthesis of other estrogens through the synthesis of glucocorticoids and mineralocorticoids (37).

Another estrogen producing pathway is when testosterone is converted to estrogen by aromatase, and this pathway contributes to the circulating estrogen levels in males and females (81) in both animals (111) and humans (130). The conversion of

testosterone to estrogen can happen in the liver, ovary, testis, placenta, brain, and/or adipose tissue (93). The conversion was first discovered in the 1930s by Steinach and colleagues (130) who treated five men with testosterone doses of 50mg three times weekly for a total of 20 injections. Each morning before treatment, the urine of each subject was analyzed to determine excretion of an estrogen substance (130). As the treatment continued weekly, excretion of this estrogen substance continued to increase (130). Once injections stopped there was a sharp decline in estrogen and after a few weeks the normal levels of estrogen were detected (130). This study was the first evidence that testosterone was converted to estrogen.

Sex hormones can also act on the non-genomic level, thus not following the "classical" genomic model described above. Non-genomic actions of sex steroids usually involve intracellular second messengers and promote a rapid effect on signaling mechanisms occurring in seconds to minutes instead of hours that is characteristic of the genomic model of sex hormone activation (83). Estrogen can also act on endothelial cells and activate nitric oxide synthase to assist in vasodilation (83). Lastly, testosterone has been shown to have similar effects as estrogen does on endothelial cells, as well as regulating vascular function (83; 146). Testosterone has also been shown to induce mitogen-activated protein kinase phosphorylation in the kidneys (10). The relative lack of comprehensive knowledge regarding the non-genomic effects of sex steroids present the possibility that there are additional unknown effects of these sex steroids.

1.2 Endocrine Disruptors (ED)

An environmental toxicant is defined as a man-made synthetic substance that alters an organism's environment and presents a risk of death, disease, or birth defect in living organisms through absorption, ingestion, and inhalation (43). Similarly, endocrine disruptors (ED) are chemicals that interfere with the body's endocrine system and produce harmful developmental, reproductive, neurological, and immune effects in humans and wildlife (101). As such, ED are a form of environmental toxicant and as such, the terms "ED" and "environmental toxicants" will be used interchangeably in his manuscript. Examples of ED are found in, but not limited to metals, personal care products, pesticides, pharmaceutical drugs, and industrial chemicals. Exposure to ED are more dangerous if the exposure occurs during "critical periods" of life when organisms are still developing and are more sensitive to hormonal disruption (43). The placenta is not impenetrable to ED like it once was thought and it cannot fully protect the developing fetus from the toxicants (100). In fact, the fetus can be more sensitive and more susceptible to environmental hazards than the adult (100). Examples of the "critical periods" when ED may do harm are intrauterine, perinatal, or puberty periods; however, exposure to certain ED in adulthood can alter physiology as well (43).

It is known that obesity occurs from eating an excess of calories, not exercising properly or enough, and genetic predisposition, but there is still some uncertainty regarding the etiology of obesity. Heindel (55) describes in a toxicological highlight that it is very clear obesity is extremely difficult to treat in most cases and that we must be diligent as scientists in the preventative medicine aspect for this epidemic. It has been

postulated that *in utero* and early developmental ED exposures may lead to obesity later in life (55) suggesting other potential research models by which to study and understand obesity, physical activity levels, and related diseases need to be developed.

Some common examples of ED include: diethylstilbestrol (synthetic estrogen DES), dioxin and dioxin-like compounds, polychlorinated bipehenyls (PCBs), bisphenol A (BPA), pesticides like dichlorodiphenyltrichloroethane (DDT), and phthalates (described in detail below) (102). Numerous official environmental and scientific organizations have attempted to develop suitable methods for determining the prevalence of these ED in the environment; however the complexity and low concentrations of certain substances have made it difficult to accurately quantify these environmental compounds (85). Nonetheless, The Endocrine Disruptor Exchange has identified (as of October 2013) almost 1,000 named ED (136).

1.3 Endocrine Disruptors' Effect on Physiology and Sex Hormones

Endocrine disruptors effect the response of hormones by essentially mimicking hormones and binding agonistically or antagonistically to the hormone receptor. ED may turn off, turn on, or modify hormonal sigals, which could affect the normal functions of tissues and organs (102). For example, ED can mimic estrogens, androgens, and thyroid hormones by competitively binding to the appropriate receptor and potentially producing over- or under-stimulation of these receptors (102). The blocking of the hormone/receptor complex can lead to reduction in male fertility and number of

males born, abnormalities in reproductive organs for both males and females, and increases in mammary, ovarian, and prostate cancers (102).

1.4 Endocrine Disruptors' Effect on Cancer Development

Research has shown that pesticides and PCB residues (38) and phthalates (82) have contributed to the incidence of breast cancer. The correlation between breast cancer and persistent chemicals present in our atmosphere has lead researchers to study this phenomenon (15). Falck et al. (38) evaluated levels of chemical residues in mammary adipose tissue with malignant cancer and compared those tissues to nonmalignant breast tissue. The researchers found that elevated levels of PCBs were found in fat samples from women with cancer compared to the women without cancer (38). In addition to the biphenyls, phthalates have also been shown to cause breast cancer (82). Researchers in northern Mexico examined the association between urinary concentrations of phthalates and breast cancer disease in women. They found that phthalate metabolites were detected in at least 82% of the women who had diagnosed breast cancer (82). These two studies (38; 82) were based on correlations so Hsieh et al. (61) investigated the cause of phthalates effects on breast cancer and their role in the development of hormone-dependent cancer in an animal model. They found that breast cancer formed in estrogen receptor-negative breast cancer cell lines (61). This finding was significant because it opened the door to a possible nongenomic pathway involved with the promotion of breast tumors.

It is also well documented (8; 24; 89) that DES, a synthetic estrogen that was used by physicians from 1948-1971 to prevent spontaneous abortions in women, caused the daughters of mothers who took DES to have reproductive organ dysfunction, reduced fertility as adults, and even promoted cancer growth of the vagina, ovary, or uterus. In an interesting case report by Blatt et al. (8), they showed that not only the offspring of the mothers were having complications, but the granddaughters of women who had used DES had a higher incidence of cancer. This type of multigenerational effect suggests that past family usage of hormonal drugs can induce transgenerational changes in hormonal profiles of the offspring (8).

Another documented problem from these ED (phthalates, bisphenols, pesticides) involves the testicular dysgenesis syndrome in male animal (31; 49; 59; 96; 110) and human models (28; 109; 134). This syndrome encompasses the rapid increase of reproductive disorders of poor semen quality, testicular cancer, undescended testis and hypospadias that are attributed to environmental changes over the past century (127). Testicular cancer is highest in younger men aged 20-40 years old, and it is hypothesized that this is due to estrogen exposure *in utero* (25). In addition, a meta-analysis of 61 sperm count studies from 1940-1990 have proven male sperm counts have significantly decreased over the past 50 years (18).

1.5 Phthalates

Based on our pilot studies with several ED (see results), this dissertation focused on the study of phthalates. Phthalates, or phthalate esters as they are sometimes referred

to in the literature, encompass a large group of compounds that share a similar chemical structure and are considered ED (65). Examples of common phthalates include: diethylhexyl phthalate (DEHP), dibutyl phthalate (DBP), butyl benzyl phthalate (BBP), and di-isononyl phthalate (DINP, DiBP, DIDP), dipentyl phthalate (DPP) and all have been shown to have adverse effects on human health (70; 110). For example, high DEHP exposure in rodents caused cancerous liver tumors with lifetime exposure (70). Phthalate compounds have been in our environment since the 1930s and are often found in personal care products, such as, perfumes, lubricants, aerosols, and nail polish products (148). Food is also a major source of phthalate exposure, specifically DEHP, for humans with diets high in meat and dairy resulting in two-fold increases in exposure (124). The concern over phthalate exposure in humans continued when DINP was found in the plastic used to make toys for infants and children (65). Koo and Lee (71) determined that four individual phthalates (DEHP, DEP, DBP, BBP) were detectible in cosmetics. DEP was present in the highest concentrations in perfumes and deodorants and DBP was highly detectable in nail polishes (71). Similarly, Hubinger and Havery (62) tested the same four common phthalates in 48 consumer cosmetic products and found that most contained at least one phthalate ester with DEP being the most frequent (62).

Another source of phthalates are dust particles that leak from building products, furniture, toys, household products, clothing, and even inside of cars (122). Rudel et al. (120) identified several hormonally active agents in air and dust samples collected from a small number of residential and commercial environments to characterize the extent of

exposure to phthalates. Significant amounts of phthalates were present in air (0.005-2.8 $\mu g/m^3$) and dust (0.3-524 μg/g) (120). A similar study by Otake et al. (108) measured phthalate esters from indoor air samples from 27 houses in Tokyo, Japan and found the median concentrations of diethyl phthalate, DBP, BBP, dicyclohexyl phthalate, and DEHP were 0.10, 0.39, 0.01, 0.07 and 0.11 $\mu g/m^3$, respectively. Although these values are less than that what the European Commission on health specifies as "hazardous" (detailed below) this study was significant because it showed another way in which humans are exposed to environmental hazards. Therefore, since humans are exposed to ED in the air, in beauty products, as well as in food, then exposure to harmful environmental chemicals could potentially pose a serious and cumulative health problem to humans.

An expert panel convened in 2000, The American Council on Science and Health (ACSH), to assess the studies and data on phthalates to determine if there was a biological concern (72). The ACSH, chaired by the former Surgeon General C. Everett Koop, concluded the evidence suggested "negligible concern" over human risks from phthalate exposures (72). The panel did suggest, however, a number of studies that could be carried out on exposure to phthalates and toxicity levels that would be helpful in making more confident statements in relation to the risks of phthalate exposure to humans (65).

Since the ACSCH report, the Centers for Disease Control along with the National Health and Nutrition Examination Survey have collected samples from 2500 participants to conduct ongoing assessments of the levels of environmental chemicals affecting the

U.S. population (19). Results so far indicate that BBP levels in the population above the age of 6 years old correspond to mean exposures less than 1ug/kg/day and the 95th percentile exposures are approximately 3ug/kg/day (65). A maximal safe exposure to BBP has not been set; however, the intraclass correlation coefficients of 0.04 has been proposed as a cutoff for sufficient reproducibility in a biomarker to justify if the substance is harmful enough to be used for epidemiologic analysis (118). Yet, relying on a single sample per subject may lead to unreliable results so it is suggested that repeated measures be taken over time to correctly assess phthalate exposure (2). Gledhill et al. (46) concluded in 1980 that there was insufficient published data to make any determination on the safety evaluation of BBP in the U.S. and that is still the case today. However, the current, tolerable daily intake from the European Commission (in mg per kg body weight per day) for humans for the phthalates DEHP, BBP, DBP, DINP, and DIDP is 0.05, 0.5, 0.01, 0.15 and 0.15 mg/kg/day respectively (121). Lastly, the Environmental Protection Agency's (EPA) reference doses for the general population for acceptable daily intake (ADI) values for DEHP (considered the most hazardous) for short-term, intermediate, and long-term exposure are: 0.1 mg/kg/day, 0.024 mg/kg/day, and 0.058 mg/kg/day, respectively (33). Products that contain DEHP may be considered "hazardous" to female populations (13 to 49 years of age) if the ADI for DEHP exceeds 0.011mg/kg/day (33).

In addition, the National Health and Nutrition Examination Survey conducted from 2001-2010 combined data on 11 phthalate metabolites for 11,071 participants and calculated percent changes and least square geometric means using a regression model

(150). The data showed that urinary metabolite concentrations of DEP, DnBP, BBP, and DEHP decreased about 20-50% but urinary metabolite concentrations of DiBP and DiNP increased by more than 100% (150). Biomonitoring studies are important to give researchers and the general population a sense of trends. Data should be continually gathered on human exposure to environments ED because the end goal is to understand how these phthalates affect human health; however we should continue to study how phthalates affect the animal model, where data gaps will be less and conditions can be better controlled.

1.6 Metabolism of BBP

Researchers have investigated the *in vivo* metabolism of BBP to understand the active agents involved in the abnormalities of physiological development due to BBP administration. Eigenberg et al. evaluated BBP after oral and IV dosages of 2, 20, 200, or 2000 mg/kg in male rats (29). It was determined that 24 hours after injection (2-200 mg/kg), 61-74% of the dose was excreted through the urine and 13-19% in the feces. At the 2000 mg/kg dose, 16% was excreted in the urine and 57% in the feces (29). The excreted metabolites in the urine were made up of monophthalate derivatives: monobutyl phthalate and monobenzyl phthalate. Also, the half-life of BBP and its derivatives at a dose of 20 mg/kg were 10 minutes and 5.9 hours, respectively (29). This study was of importance because it signified that BBP was rapidly metabolized and the majority of BBP was excreted through urine and feces (29).

Nativelle et al. (97) sought to discover how BBP was metabolized in female rats. Oral doses of BBP at levels of 150, 475, 780, and 1500 mg/kg/day were administered for

three consecutive days to female rats. In order to investigate conjugated metabolites of BBP, alkaline and enzymatic hydrolysis were used. Alcaline hydrolysis breaks down ester bonds of conjugates and enzymatic hydrolysis frees glucuronidies and sulfo-conjugates. Urinary extracts were analyzed using gas chromatography and the analysis found that oral administration of BBP to female rats yielded six metabolites. The six metabolites identified were hippuric acid (51-56%), monobutyl phthalate (29-34%), monobenzyl phthalate (7-12%), monobutyl phthalate co-oxidized (1-2%), phthalic acid (2-3%), and benzoic acid (% not given). Treatment with the lowest dose of 150 mg/kg resulted in a steady-state urinary excretion of metabolites within 72 hours suggesting a lack of a dose-response. Multiple dosing with the highest dose of 1500 mg/kg showed identical levels of urinary excretion of all metabolites the first 48 hours but by the last day there was a 1.9, 2.4 and 1.4-fold increase in monobutyl phthalate, monobenzyl phthalate, and hippuric acid-respectively, suggesting a slight time dependency at a higher level. This study was significant because it added to the body of knowledge of how BBP was metabolized after administration and what metabolites could adversely affect mammalian physiological development (97).

1.7 Phthalate Studies in Animals

The majority of studies on phthalates have been conducted in animal models at doses far higher than those present in the atmosphere to induce metabolic and physiological changes. It should be noted that any value over 0.5 mg/kg/day for BBP is considered higher than the EPA standards (19); therefore the animal studies dose well

over the tolerable limit. The gavage technique is the most widely used and accepted way to dose the animals (31; 49; 59; 96; 110) because the most common way humans are susceptible to the effects of ED is through ingestion (breathing, food intake, etc). Therefore, gavage closely mimics the way humans are exposed to ED.

Using gavage, Nagao et al. (96) studied the effects of BBP in rats on multigenerational reproduction. The researchers exposed the F0 (parent) male rats to 0, 20, 100, 500 mg/kg/day from 6 to 23 weeks of age and noticed that the males did not gain as much body weight in the 500 mg/kg/day group compared to the 100 and 20 mg/kg/day and control groups. Another major finding in the parent generation was that daily subchronic exposure to BBP increased the liver and kidney weights of both the male and female rats in the 500 mg/kg/day group (96). Also, the parent generation showed a decreased serum concentration of testosterone in the males. In the F1 generation (the first offspring to the parents), the body weight of male and female offspring at birth were decreased in the 100 and 500 mg/kg/day group. Anogenital distance (AD) at birth was decreased in the male group and was increased in the female pups in the 500 mg/kg/day group. AD is a standard clinical measurement that determines the distance from the anus to the genitalia. It is regulated by dihydrotestosterone and can be disrupted by phthalate exposure and as such can be clinically significant (22). Nagao's et al. (96) study was significant because 20 mg/kg/day of BBP had no adverse effects on the parent or next generation animals exposed to BBP but exposure rates of 100 mg/kg/day and 500 mg/kg/day did demonstrate adverse effects on the animal (96).

Another study assessing AD in male rats determined that there was a significant increase in the incidence of undescended testes and a decrease in AD in male fetuses dosed at 250mg/kg and higher (31). Pregnant rats were given MBeP by gavage on days 15-17 of pregnancy at a dose of either 167, 250, or 375 mg/kg. Data from the fetuses were taken on day 21 of pregnancy (31). This study was important because it demonstrated harmful effects to the male reproductive tract from an administration of a BBP metabolite in male rats.

Howdeshell et al. (59) was the first to study multiple phthalate (BBP, DBP, DEHP, DiBP, dipentyl phthalate F (DPP)) exposure on testosterone production in the Sprague-Dawley rat. His rational to study multiple phthalates was that many of these endocrine disruptors do not act alone but in combination. Pregnant female rats were dosed by gavage on gestation days 8-18 with a mixture of five different phthalates. Following the last dosage on gestation day 18, the rat dams were sacrificed along with the fetuses (59). It was found that testosterone production was reduced in a dose-additive manner in the fetuses (59). It is also important to note that some of the individual phthalates (DPP and DiBP) and mixtures of BBP, DBP, DEHP, and DiBP also significantly induced fetal mortality (59). This study was the first to confirm that phthalates work together to produce a cumulative or dose additive inhibitory effect on fetal rat testosterone production.

Another study on phthalates and the harmful effects seen in the male rat is by Parks et al. (110). Parks and colleagues studied the plasticizer DEHP to determine if any significant changes occurred in testicular testosterone production and reproductive

development with DEHP exposure during late gestation and neonatal life (110). The researchers found that DEHP disrupts male rat sexual differentiation because during a critical stage of male development it reduces testosterone to female levels (110). Gray et al. (49) also studied exposure of these phthalates prenatally and found certain phthalates reduced pregnancy weight gain and pup weight at birth. Male (but not female) pups from the DEHP and BBP groups displayed a reduced AD and reduced testis weights. The male pups in the DEHP, BBP, and DINP groups also displayed female-like characteristics of areolas and nipples (49) suggesting prenatal exposure to phthalates disrupt sex steroids in males and cause them to develop female traits.

It is important to note that females are also adversely affected by exposure to phthalates *in utero*. Ramirez and Sawyer found that short-term treatment with low physiological doses of estradiol benzoate (0.05 ug/100g body wt/day) delayed vaginal opening by more than a week in the immature female rat (113). The opening of the vagina signifies the start of puberty, ovulation, and the initiation of the estrous cycle (113). This study was significant to the field because it showed that exposure to estrogen *in utero* can delay puberty leading to a number of adverse health effects. To relate this to humans, puberty and reproduction are different between different ethnic groups. The difference in the groups is due to genetic factors like family, ethnicity, and gender. But as we gain a better understanding of how the environment plays a role in the physical development of humans, we are learning that puberty and hormones are greatly affected which can lead to health disparities later in life. For example, researchers used data from the National Longitudinal Study of Adolescent Health to

study a sample of school-based young people with Cuban, Puerto Rican, Chinese, African-American, and European-American descent to analyze puberty rates. The researchers found that African-American and Hispanic girls experienced menarche before 11 years of age, while European-American girls experienced puberty between 8-14 years of age, and Asians went through puberty at 14 year or later (1). The study also found that early maturing girls were twice as likely as average maturing girls to be overweight in all ethnicities (1).

1.8 Phthalate Studies in Humans

Since it is unethical to inject phthalates into humans, epidemiological studies have been done in humans to assess the potency and detrimental effects on human biology of ED. Literature has demonstrated that human exposure to ED is ubiquitous and there may be detrimental effects on human health (28; 60; 109; 133; 134). Specifically, most of the human literature reports associations between prenatal and postnatal phthalate exposure in males (28; 133; 134) with the effects of a shorter AD correlating to low fetal testosterone levels that can adversely affect male reproductive development. In addition, alterations in the female genital tract (i.e. start of menses and/or development of cancer) are not obvious until puberty or later into adulthood (133). Thus, it is imperative that studies continue to gather information in an epidemiological manner to better understand the effects of phthalates on human health.

Several scientists have developed studies to examine the effects of phthalates on human biology. For example, Duty et al. (28) demonstrated that some phthalate

monoesters in the environment contributed to lower sperm concentration, lower motility, and increased percentage of sperm with abnormal morphology in humans (28). By analyzing 168 male subjects who were part of couples struggling to conceive, the researchers concluded that at least two of the phthalate exposure (mono-butyl phthalate and non-benzyle phthalate) humans were consistently subjected to were testicular toxins and decreased sperm production in males (28). Another study consisting of 74 men who worked in a factory producing unfoamed polyvinyl chloride (PVC) flooring in China examined the harmful effects of phthalates through dust inhalation and contact on their skin (109). Phthalates are the major compound used to soften PVCs. A comparison group of 63 male workers was chosen from a nearby construction company. The groups were matched for age, smoking, marital, and alcohol status, while urine and blood samples were also collected from each participant along with questionnaires. It was determined that levels of urinary phthalate markers were 5-100 times higher in the exposed group who worked with the PVC compared to the unexposed group. Furthermore, the exposed workers had significantly lower free testosterone levels than the unexposed group suggesting that poor environmental working conditions in factories making PVC led to adverse health consequences in male populations (109). On February 13, 2012 the EPA issued a final ruling to set limits for air toxins from PVC exposure in the U.S. The annual emission reductions from the manufacturing of PVC are estimated to be 135 tons of vinyl chloride and 33 tons of hydrogen chloride (34). Several rules were set in place to limit emission and improve air quality for factory workers exposed to these harmful chemicals (34).

Swan and colleagues (134) collected data on 134 male babies in the United States from mothers who had live births and performed physical examinations that included standard anthropometric measurements, as well as AD at a mean age of 15.9 months. A urinary phthalate metabolite analysis was also carried out for mothers of 85 of these boys (134). The researchers then compared the median and 75th percentile of the AD associated phthalate metabolite concentrations among two groups of mothers (those whose boys fell in the short anogenital group and then all the others) and used those concentrations as predictors of age-adjusted AD in regression and categorical analysis (134). It was concluded through regression analysis that the adjusted AD decreased significantly with an increase in phthalate score (134). Therefore, the associations between male genital development (AD) and phthalate exposure indicate that phthalates may contribute to the incomplete male development (134) that has also been noted in rodent studies (31; 49; 96).

1.9 Measurements of Physical Activity

Measuring physical activity in rodents using a running wheel has been studied as a behavioral characteristic for over a century (131). Stewart in 1898 was the first to scientifically use wheel running by examining the effects of diet, alcohol, and barometric pressure on wheel running in rats (131). Early studies in pharmacology in the 1950s also utilized voluntary activity levels in mice to study side effects of drugs. Dews (27) was curious about activity in mice after administration of certain drugs like epinephrine, nicotine, and cocaine. The apparatus he used to assess the activity of the mice consisted

of a rectangular cage with a wooden floor with transparent plastic sides (27). A beam of light passed through the transparent sides and when a mouse broke the beam of light a photogenic cell activated the digital counter (27). Rearing activity (when a mouse stands up on the hind legs) has also been examined in caged rodents as a measurement of physical activity. Van Abeelen (140) used a similar tracking system as Dews (27) to quantify rearing responses in mice selected for frequency of rearing responses. He utilized three horizontal light beams activating the photocells recorded on counters and determined that rearing was a genetic trait in mice (140).

For this dissertation, we used a running wheel to determine voluntary levels of physical activity in mice. Throughout the 20th century researchers have studied physical activity in rodents and have drawn parallels to human activity (30). The suggested motivations of wheel running include (for both humans and rodents): weight regulation, social benefits, and positive affective state (i.e. endorphins) (30). Even though the motivations of wheel running are heterogeneous scientists still use the animal model to shed light on human physical activity because multiple factors between humans and rodents in responses to voluntary exercise are similar (30) such as the use of exercise to control weight gain. Also, voluntary wheel running activity in mice has been our standard lab protocol, as we have published studies to assess reliability and validity to these methods (80; 139).

1.10 Sex Hormones and Regulation of Physical Activity

The concept of sex hormones regulating physical activity was studied as early as the 1920s. Wang (142) first noticed, in 1923, that there was a cyclical relationship between content inside the vagina of female rats and activity levels. He noted female mice were most active when they were in "heat" (142). Another study that came directly after the publication of Wang's paper concluded that physical activity in females was driven by "copulation and successful stimulation of the cervis uteri" or what we presently refer to as sex hormones (128). Slonaker (128) studied activity patterns in female albino rats and confirmed that activity levels were affected during significant reproductive changes throughout the rats lifetime (128). Scientists have also studied the effects of male sex hormones on activity levels. In 1925, Hoskins (58) observed that male rats that were castrated did not run as much as intact males. Richter (115) quantified running distances in the male and female white rat. He studied animal behavior and the internal drives to investigate the reasons why rats run. He hypothesized that spontaneous activity occurred because of some physiological drive due to his observations of nest building, oestrus cycling, and food gathering (115).

Scientists have also studied the effects of implanting or replacing hormones in mice after removal of sex steroids. In 1925, Wang and colleagues (143) implanted ovaries from females into castrated male rats at different age points. They found that 17 out of the 24 castrated animals with implanted ovaries demonstrated an increase in activity (143). They also found that the younger the castrated male rat was implanted with the ovaries the better chances they had for a successful transplantation. The

successful transplantation was most likely due to the fact the animal had not gone through puberty yet (143). These studies indicate that even before the sex hormones had been identified, it was suspected that a factor inherent to sex was driving daily activity. More recently, Gorzek et al. (48) sought to investigate whether estrogen (or tamoxifen) could reverse the decline in physical activity in mice after surgical ovariectomy. Mice were surgically operated on to remove the ovaries and placed on a running wheel for four weeks to track voluntary activity (48). The ovariectomized mice then received implanted pellets of tomoxifen or a placebo and were allowed to run for 4 more weeks after implantation (48). The results suggested that ovariectomized mice ran about 80% less and carried approximately 20% more body mass compared to the controls (48). In addition, replacing the lost estrogen increased running distances after one week (48). These results are important because it shows that removing the ovarian hormones decreases physical activity, but replacing the hormones though implantation can increase physical activity levels again (48).

1.11 Estrogenic Control of Physical Activity

Whether testosterone or estrogen was the primary mediating hormone responsible for physical activity was unknown until the mid-70's at which time, Roy and Wade hypothesized that there was a primarily estrogen-driven activity regulating mechanism (119). Roy and Wade (119) performed three experiments to test whether testosterone played a role in regulating wheel running activity in rats. One experiment compared the effects of estradiol benzoate, testosterone propionate, and

dihydrotestosterone propionate on wheel running activity in castrated male rats. They found that estradiol benzoate significantly increased the running activity of the male rats and that testosterone also increased the running activity in male rats (119). Dihydrotestosterone – which cannot be aromatized to estrogen like testosterone can - did not increase running activity above baseline (119) suggesting that testosterone must be aromatized to estrogen to activate activity mechanisms.

Roy and Wade's second experiment showed that anti-estrogen (MER-25) decreased running wheel activity while their third and final experiment showed that running-wheel activity of castrated males was not affected by the estrogen antagonist even when food was not available (119). From these results, the authors concluded that testosterone must be aromatized to estrogen in order to increase wheel running in rats (119). In support of the theory that estrogen drives physical activity, Watai et al. (144) found a decrease in running wheel activity in an estrogen-deficient aromatase knockout mouse model. Further support for the 'estrogen-central' mode came from studies by Ogawa and Morgan considering estrogen alpha and beta receptor knock-out mice (105).

Given the strength of Roy and Wade's results, no authors challenged the hypothesis of an estrogen-mediated activity for several years. Interestingly enough, Hill et al. (57) found that male aromatase knockout mice – which would be hypothesized to have greatly reduced physical activity – actually had significant increases in wheel running activity when compared to controls. This aromatase knockout mouse model is void of aromatase (the enzyme needed to aromatize testosterone to estrogen), so this

study contradicted the hypothesis that estrogen was the primary driver of physical activity.

The question of whether or not estrogen was the primary controller of voluntary physical activity led Bowen et al. (12) to evaluate the effects of aromatase inhibitors on wheel running activity in male mice. They injected irreversible aromatase inhibitor exemestane into intact (control), supplemented (with testosterone and 17 β-estradiol), or orchidectomized (removal of the testes or ovaries) mice (12). The researchers found that blocking aromatase did not alter physical activity levels in the mice receiving exemestane injections (12). In addition, exemestane injections in conjunction with implantation of testosterone or 17 β-estradiol did not yield any significant results. These results call into question the 'estrogen-central' hypothesis was not needed to control voluntary wheel running in male mice. Bowen et al. (13) then expanded their studies to examine the effects of supraphysiological doses of sex steroids on voluntary wheel activity in mice by performing a surgical gonadectomy (after baseline wheel running) to reduce circulating steroid levels in both males and females. The researchers then proceeded to replace the hormones by developing two sets of implants to release sex steroids into the bloodstream based on diffusion (13). Silastic implants containing 17 β-estradiol or testosterone were surgically placed back in the mice after the gonadectomy (13). The mice were then re-exposed to the running wheel and data was analyzed to see if the running activity could be recovered. Bowen et al. (13) concluded that mice implanted with 17 β-estradiol had a lower recovery of running activity while mice implanted with testosterone had higher recovery of running activity. Bowen's studies

(12; 13) suggested that physical activity was regulated via an androgen dependent mechanism as opposed to a primary estrogenic system as first hypothesized by Roy and Wade (119).

To expand on the androgen dependent mechanism, the androgen receptor (AR) was fist cloned in 1988 by Chang and colleagues (21) and important to this field of study because the appropriate regulation of the AR is necessary for a wide range of physiological processes. Some of these androgen-related processes include: male sexual development and maturation, maintenance of male reproductive organs, and spermatogenesis (87). In addition, testosterone and dihydrotestosterone are thought to mediate biological effects by binding to the AR (56). The activation of the AR in the presence of testosterone, dihydrotestosterone, and 17 β-estradiol can also be induced by selective tissues such as the breast, prostate, and liver (149). In addition to the transcriptional mode of action by steroids, a few papers suggest that progesterone and estrogen can also have nongenomic effects (39; 114; 145). These nongenomic effects can increase free intracellular calcium and activation of protein kinase, and activation of these second messenger cascades may be what also activates the AR (56). The observations remain unclear and more studies need to be carried out to confirm the nongenomic effects of the AR (56).

The role of androgen receptors in regulating physical activity could be elucidated by the use of androgen receptor knockout model (ARKO). However, at this time there are a few studies that have observed voluntary physical activity levels in mice using the androgen receptor knockout (ARKO) model. For example, Ophoff et al. (107) placed 5-

week old male ARKO mice on running wheels and tracked their voluntary activity until sacrifice at 16 weeks of age. At all time points, distance, speed, and duration were significantly lower in the ARKO mice compared to the wild-type controls, suggesting a role for the androgen receptor in regulating physical activity. In a similar study, Fan et al. (40) generated an AR null mouse line and studied spontaneous activity, body fat, oxygen consumption, glucose tolerance, and other variables. In terms of the physical activity data collected, cage activity was monitored at 8, 20, and 40 weeks of age using an infrared system, and they observed that the 20 week old AR null mice ran significantly shorter distances and showed almost half the number of rearing behaviors as compared with the wild type mice (40). The researchers concluded that the AR system plays a vital role in male metabolism and effects physical activity and energy balance (40).

1.12 Purpose of Study

This dissertation focuses on the endocrine disruptor, benzyl butyl phthalate (BBP) and its effects on the regulation of voluntary physical activity in mice. BBP is a clear, oily liquid used as a plasticizer and is commonly found in vinyl floor tiles, vinyl foams, and polyurethane. It is accepted that BBP is released into the air, and once in the environment, BBP migrates to the soil, surface water, and most commonly food. Historically, dietary intake from contaminated food is the biggest form of human exposure to phthalates (122).

Given that a decrease in circulating sex hormones, and specifically a decrease in testosterone, leads to a decrease in voluntary physical activity (12; 13), the purpose of

this study is to determine if BBP exposure decreased physical activity in mice because of a disruption in sex hormone production from prenatal exposure to BBP.

2. METHODS

2.1 Animals

This protocol conformed to the standards of humane animal care and was approved by the Texas A&M University Institutional Animal Care and Use Committee (AUP 2012-0274). C57BL/6J inbred mice (Jackson Laboratory, Bar Harbor, ME) were used in this study because of their consistent use in the scientific literature and genetic homogeneity of the strain.

2.2 Overview

To determine if ED impacted physical activity in offspring, we conducted three sets of experiments. The first set (hereafter referred to as Experiment 1) was used primarily to develop basic methods of ED administration as well as to determine the efficacy of the three ED in altering physical activity. The second set of experiments (hereafter referred to as Experiment 2) focused on the ED that most altered physical activity in Experiment 1 and incorporated several additional techniques and methods due to the results from Experiment 1. Finally, the third set (hereafter referred to as Experiment 3) was the main study and focus of this dissertation in which alterations to Experiment 2 were made to enhance the study design (addition of animal groups and time point sacrifices) and administration of ED techniques.

2.3 Experiment 1 Methods

We randomly assigned four pregnant female C57Bl/6J inbred mice to one of four groups: ED by Bisphenol-A (BPA, 395 µg/kg (135)), ED by benzyl butyl phthalate (BBP, 500 mg/kg (23)), ED by dichlorodiphenyldichloroethylene (DDE, 200 mg/kg (66)) or saline/control. All pregnant mice received an intraperitoneal injection on gestation days 9-16 to disrupt organ system development. Pups (BPA, n=6; BBP, n=2; control, n=3, DDE dam did not survive injections) were weaned at 3 weeks of age and placed in individual cages with running wheels. Daily distance, duration, and speed were measured daily for six weeks. Mouse weight (g) was measured weekly.

2.3.1 Experiment 1 Measurement of Wheel Running Activity

At four weeks of age physical activity measurements were determined by measurement of daily distance, duration, and speed of wheel running using our standard lab protocol (80; 139). In brief, running wheels were mounted to the cage tops of standard rat cages and were equipped with a cycling computer (BC500, Sigma Sport, Batavia, IL) to record running distance and duration. Running wheels had a 450mm circumference and a 40mm wide, solid running surface. Running distance and duration data were collected on a daily basis in the morning and average daily running speed was calculated from the corresponding distance and duration measures. The sensor alignment and freeness of the wheel were checked daily and adjusted as needed.

2.3.2 Experiment 1 Statistical Analysis

Physical activity data (distance, duration, and speed) was analyzed by an ANOVA with an alpha level of 0.05 set *a priori* (Jmp v.10, Cary, NC). A Tukey's *post-hoc* test was used to assess significant main effects or interactions.

2.4 Experiment 2 Methods

With the results of Experiment 1, we randomly assigned three pregnant female C57Bl/6J inbred mice to one of three groups: group one and two were endocrine disrupted by BBP and group three was by saline/control (Figure 1). All pregnant mice received an intraperitoneal injection of BBP on gestation days 9-16 of 500mg/kg (23). Fetuses were estimated to have been exposed to approximately 1/100 to 1/1000 (92) of the mother's dose which places exposure rates near the EPA safe dose for humans of 0.2 mg/kg/day (35). Pups (BBP, n=23; control, n=20; Table 2) were weaned at three weeks of age and placed in individual cages with running wheels at week four.

2.4.1 Experiment 2 Measurement of Wheel Running Activity

Daily distance, duration, and speed were measured for six weeks similar to in Experiment 1 until mice were sacrificed on week 10. In addition, locked wheels were also placed in some of the cages to prevent running activity, but to maintain a similarly enriched environment (6). Mice from the locked wheel cages were analyzed by histology analysis to examine breast morphology in the female mice.

2.4.2 Experiment 2 Measurement of Body Composition

All mice were measured weekly using magnetic resonance imaging using an EchoMRI (EchoMRI, Houston, TX) to determine body composition. This whole body analysis was done with the mouse awake and made direct measurements of total body fat (in grams) and lean mass (in grams) using magnetic resonance imaging (MRI). In brief, mice were first weighed (grams) and then placed in the appropriate MRI sized tube (based on their body weight), and then inserted into the MRI machine. Analysis took approximately 30 seconds and calculations of lean and fat mass were displayed on the computer screen. The tube was removed and the mouse was taken out of the tube and placed back in their cage.

2.4.3 Experiment 2 Histology

Mammary glands from female mice in the treated/control and locked/running wheel groups were extracted to determine if mammary tissue morphology was altered by the ED and exercise treatments. Histology samples were fixed using formaldehyde-based fixatives at a 4% solution in PBS. The samples were sectioned by the histology lab (Texas A&M University) and used to determine cell proliferation (Ki67), as well as estrogen.

2.4.4 Experiment 2 Statistical Analysis

Statistics were computed in a similar manner as in Experiment 1.

2.5 Experiment 3 Methods

Mouse dams were treated with BBP or a control substance (sesame oil) via oral gavage on days 9-16 of pregnancy. After using what we thought was the most appropriate dosing technique for the mice and reviewing our data from Experiment 2, we decided to change the way in which the ED was dosed. Therefore, we used the gavage technique which is the most widely used and accepted way to dose the animals (31; 49; 59; 96; 110). Most of the ways humans are susceptible to the effects of ED is through ingestion (breathing, food intake, etc). Therefore, the gavage closely mimics the way humans ingest ED. The resulting pups were randomly assigned to four week, 10 week or 20 week sacrifice groups. Our goal was to have at least six males and six females at each time point for statistical power (See Figure 3 and Table 3).

2.5.1 Experiment 3 Sentinel Control Animals

In this study, we used two groups of sentinel control mice to ensure that our animals were as active as past animals in this strain (79) and to eliminate the possibility that our breeding procedures were introducing an unknown variable that was altering physical activity. As such, one of the sentinel animal groups (three male and three female mice; Jackson Laboratory, Bar Harbor, ME) arrived at 3.5 weeks of age. The mice were placed on running wheels at four weeks of age and were sacrificed at 10 weeks of age to mimic past studies. The second sentinel control group were pups born from our male/female breeder pairs in our animal care facility that were randomly assigned to this group who received no treatment at all (i.e. no BBP, no oil). There pups

were placed on running wheels at eight weeks of age, ran for two weeks, and were sacrificed at 10 weeks of age.

2.5.2 Experiment 3 Breeding and Treatment

Two female mice (total of 12 female breeders) were housed in a single cage with one male breeder (total of six male breeders). Female mice were evaluated every 12 hours for the presence of a vaginal plug (gestation day 0). Upon gestation day 0, the female mouse was placed in an individual cage for the remainder of pregnancy. Pregnant female mice were administered a gavage treatment of either control oil or BBP on gestation days 9-16 (59; 96) when organ system development occurs. The treatment groups received 500mg/kg of BBP in a vehicle of 100µl of sesame oil and control groups received just 100µl of sesame oil. The resulting pups were weaned at three weeks of age and then housed individually for the remainder of the experiment until sacrifice.

2.5.3 Experiment 3 Measurement of Wheel Running Activity

Wheel-running activity was monitored similarly to Experiment 2 (and included locked wheels).

2.5.4 Experiment 3 Measurement of Puberty Development

Measurements reflecting the effect of the endocrine disruptors on puberty development were performed to determine the efficacy of the ED treatment. In female pups the beginning of mouse estrous cycling identification was indicated by the day of

vaginal opening (113). This identification was performed everyday by scuffing the mouse, holding the mouse face up, and examining the vaginal region with a spatula until vaginal opening was visibly present. To monitor male puberty development we measured anogenital distance (AD - distance from the anus to the base of the genitalia (84)) weekly using a digital caliper (Mitutoyo Digimatic). Also, upon sacrifice, the seminal vesicle was taken from the male animal and weighed (in grams).

2.5.5 Experiment 3 Measurement of Body Composition

All mice were measured weekly using the protocol described in Experiment 2.

2.5.6 Experiment 3 Sex Steroid Assays

On the day of sacrifice, blood samples were extracted via a heart stick using a 20 gauge needle, and the blood was spun down with cold centrifugation at 4°C for 30 minutes and then subsequently removed and aliquoted for later analysis of testosterone in male pups and estrogen in female pups. Testosterone (ng*ml) was measured in triplicate via ELISA per manufacturer's instructions (Alpco Serum Testosterone, Salen, NH) for all male samples, and estrogen (ng*ml) was measured in triplicate via ELISA per manufacturer's instructions (MyBioSource, San Diego, CA) for all female samples. Upon studying pilot data from Experiment 2, a coefficient of variation was run on the triplicate numbers for each sample - and it was determined that the highest coefficient of variance from the samples of the same treatment group was 40%; therefore, we used a 40% variance as our cut point for eliminating samples from the analysis. When

testosterone concentration values were computed, any value outside three standard deviations above or below the mean were eliminated from the analysis.

2.5.7 Experiment 3 Statistical Analysis

Physical activity data (distance, duration, and speed) and body composition data were analyzed by an ANOVA to assess if there were significant main effects or interactions. A Tukey's *post-hoc* test was then used to assess potential contrasts between the treatment groups. Descriptive data such as the sex hormone monitoring measures and the sex hormone ELISA data were assessed using a one-way ANOVA to determine potential associations between these sex-hormone measures and physical activity. Pearson product-moment correlation were used with the alpha level set *a priori* at 0.05 (Jmp v.11, Cary, NC).

3. RESULTS

3.1 Experiment 1

Ten total pups (male and females) were analyzed in Experiment 1 (Table 1). From the three ED used, the level of DDE given to the mother – in spite of this being the recommended dosage from the literature - resulted in the death of the mother on the fourth day of injection (thus, killing the pups). BPA showed no significant effects on physical activity compared to the control mice; however there was an observable decrease in physical activity with the BBP pups (Figure 3). This data caused us to stop pursuing DDE or BPA, and led us to expand the sample size of those mice receiving BBP prenatally in Experiment 2. We also learned from Experiment 1 that in order to have a true control sample, we need to use sesame oil instead of saline because in subsequent experiments sesame oil was used as the vehicle in which BBP is administered.

3.2 Experiment 2

Forty-three pups were analyzed for Experiment 2 (Table 2). There were no significant differences observed in the physical activity distance ran in either male ($p=0.9394$) or female ($p=0.7235$) groups that received BBP treatment (Figure 4 & 5, respectively). There was no change in lean mass ($p=0.09$) or fat mass ($p=0.85$) in males between the BBP and control group (Figure 6). There was a significant change in lean mass ($p=0.01$) and no change in fat mass ($p=0.10$) in females between the BBP and

control group (Figure 7). Female control and BBP treated mice indicated BBP treated mice had a higher percentage of tumor cells as shown by Ki67 ($p=0.0043$) but no change in estrogen receptor status ($p=0.1802$; Figure 8). In addition, H&E staining of BBP treated mice have ductal filling and cruciform structures (Figure 9) and a trichrome stain indicated localized invasion in to the surrounding stoma (Figure 10).

After reviewing the results of Experiment 2, it was determined that a third experiment needed to be conducted due to several observations made in Experiment 2. Importantly, our control mice in Experiment 2 ran approximately half the distance that the same strain had previously run (79). In considering all lab conditions during the experiment, it was unclear why the control animals were less active. Also, as noted earlier, we were concerned that that the intraperitoneal injection was not as physiologically relevant as the gavage technique. Lastly, the histology results suggested that perhaps there was a chronic effect affecting the female mice and this suggestion lead to investigating a longer time frame, i.e. 20 weeks.

3.3 Experiment 3

Eighty-nine pups were analyzed for this experiment with an average litter size of six pups in the control mice and four pups in the BBP mice (Table 3). In addition, mouse breeding indicates the male/female ratio for control, control oil, and BBP average 1.5/3, 3.8/2.6, and 2.0/2.4, respectively (Table 4). From here forward, mice from the different treatment groups that were sacrificed at 4 weeks, 10 weeks or 20 weeks are

referred to as “Control 4 weeks” or “BBP 4 weeks” and “Control 10 weeks” or “BBP 10 weeks” and “Control 20 weeks” or “BBP 20 weeks,” respectively.

3.3.1 Experiment 3 Sex Steroid Assays

There was a significant difference in serum testosterone concentration values in male mice between control and BBP at 10 weeks ($p=0.0390$) and at 20 weeks ($p=0.0217$) (Figure 11). The average coefficient of variance between triplicates from each sample was 2.23%. As noted earlier, when testosterone concentration values were computed, any value outside three standard deviations above or below the mean were eliminated from the analysis. We eliminated one data point from the BBP 4 week group and two data points from BBP 20 weeks and Control 20 weeks. Testosterone values (ng/ml) for sentinel control from Jackson Laboratory, sentinel control bred in animal facility at Texas A&M University, BBP 4 weeks, control 4 weeks, BBP 10 weeks, control 10 weeks, BBP 20 weeks, and control 20 weeks were as follows: 1.33, 2.64, 1.06, 0.81, 1.15, 5.72, 1.00, and 7.72, respectively.

There was so significance difference in estrogen concentration values in female mice between control and BBP mice at any time ($p=0.0918$; Figure 12). The average estrogen concentration values for all groups combined was 2.35 ng/ml. The estrogen kit (MyBioSource) states that it was able to detect 0.312 - 20 ng/ml and our values fell within this range. Estrogen values (ng/ml) for sentinel control from Jackson Laboratory 10 weeks, BBP 4 weeks, control 4 weeks, BBP 20 weeks, BBP locked 20 weeks, and control 20 weeks were as follows: 2.85, 1.85, 2.59, 1.70, 2.48, and 2.85, respectively.

The same coefficient of variation was run similar to testosterone explained above with estrogen concentration coefficient of variation values averaging 1.68%. No data points were eliminated from the samples due to values outside three standard deviations above or below the mean.

3.3.2 Experiment 3 Puberty Development

As indications of sex hormone disruption due to the BBP treatment, vaginal openings in female BBP mice were significantly delayed compared to control mice ($p=0.0007$; Figure 13). Additionally, in the male mice, anogenital distances in BBP mice were significantly smaller compared to control mice at 10 ($p=0.0006$) and 20 weeks ($p=0.0379$) (Figure 14). Lastly, the male mice seminal vesicle weights were not different at either 10 weeks ($p=0.4431$) or 20 weeks ($p=0.7156$; Figure 15).

3.3.3 Experiment 3 Wheel Running Data

The graphs generated to interpret the wheel running data were grouped by "early life" or "late life" for both males and females, separately. Early life indicates weeks 9-11 and late life indicates weeks 17-19. In male mice, overall there was a significant decrease in distance ($p=0.0084$), duration ($p=0.0047$), but not speed ($p=0.4949$) between control and treatment mice (Figure 16). Upon *post hoc* analysis there was no significant decrease in distance in early life ($p=0.0793$) or late life ($p=0.3344$) and no significant decrease in duration in early life ($p=0.2029$) or late life ($p=0.4724$). However, on average, BBP male mice ran 20% less than controls when averaged throughout the

lifetime (Figure 17). Lastly, paired correlation data between testosterone concentration and distance ran (km) demonstrated a correlation coefficient of r=0.5710 with no significance (p=0.1393) for BBP male mice at 10 weeks (Figure 18) and a correlation coefficient of r=0.7902 with no significance (but a trend) (p=0.0614) for control mice at 10 weeks (Figure 19). In addition, paired correlation data between testosterone concentration and distance ran (km) demonstrated a correlation coefficient of r=0.0453 with no significance (p=0.9547) for BBP male mice at 20 weeks (Figure 20) and a correlation coefficient of r=0.9890 with no significance (p=0.0963) for control 20 week mice (Figure 21).

In female mice, overall there was a significant decrease in distance (p=0.0423), duration (p=0.0153), but not speed (p=0.7424) between control and treatment mice (Figure 22). Upon *post hoc* analysis there was no significant decrease in distance in early life (p=0.2766) or late life (p=0.1014) and no significant decrease in duration in early life (p=0.0845) or late life (p=0.0618). However, on average, BBP female mice ran 15% less than controls when averaged throughout the lifetime (Figure 23).

3.3.4 Experiment 3 Body Composition

The graphs generated to interpret the body composition data were grouped by "early life" or "late life" for both males and females, separately. Early life indicates weeks 9-11 and late life indicates weeks 17-19. Overall, in the male mice there was a significant difference in weight (p=0.0001), lean mass (p=0.007), and fat mass (p=0.0001) in BBP and control mice (Figure 24). However, upon *post hoc* analysis there

was no significant decrease in weight in early life (p=0.4650) or late life (p=0.9314), no significant decrease in lean mass in early life (p=0.5595) or late life (p=0.9872), no significant decrease in fat mass in early life (p=0.2877) but a significant decrease in fat mass in late life (p=0.0257).

Overall, in the female mice there was a significant difference in weight (p=0.0159), lean mass (p=0.0091), and fat mass (p=0.0048) in BBP and control mice that had access to running wheels and BBP mice on a locked wheel (Figure 25). However, upon *post hoc* analysis there was no significant decrease in weight in early life (p=0.9968) or late life (p=0.9999), no significant decrease in lean mass in early life (p=1.000) or late life (p=0.9995), and no significant decrease in fat mass in early life (p=0.9942) or late life (p=0.9553) in control and BBP females who had access to running wheels. In addition, upon *post hoc* analysis there was no significant decrease in weight in early life (p=0.9988) or late life (p=0.9939), no significant decrease in lean mass in early life (p=0.8660) or late life (p=0.4449), and no significant decrease in fat mass in early life (p=0.2629) or late life (p=0.1272) in control females who had access to running wheels and BBP females who did not have access to running wheels.

4. SUMMARY

These experiments were designed to study the effect of ED on physical activity in mice. To our knowledge, this is the first study to examine the effect of a pharmacological-level dose of BBP, a phthalate, on voluntary physical activity levels in mice. These results suggest that there were differential effects of the BBP on both activity and body composition depending on the sex of the mice. In both sexes, prenatal BBP administration resulted in later decreases in sex hormone levels as observed by both direct measures of testosterone as well as indirect measures such as anogenital distances and vaginal opening. In general, voluntary wheel running activity remained largely unaffected with prenatal BBP disruption in female mice. However, the female mice exposed to BBP *in utero* who did not have access to a running wheel at any point during their lifetime had significantly more body fat compared to mice exposed to BBP *in utero* and controls that did have access to running wheels (i.e. exposure to exercise prevented this body fat gain). In regards to the male animals, there was an overall decrease in physical activity with prenatal BBP exposure. This decrease in voluntary wheel running activity in males was evident in the significant decrease in overall distance ran and speed of activity between BBP and control males particularly at nine weeks of age. However, unlike the female mice, body fat was not affected in any of the male mouse groups.

4.1 Puberty Development

To confirm that our experimental model worked and we had disrupted the sex hormones prenatally, we assessed puberty development and growth in both male and female pups using vaginal opening in the females (17; 113; 116) and anogenital distances in the males (31; 60; 84; 134) , both accepted measures in the literature. In female pups, the literature suggests that normal opening of the vagina occurs around 26 days old in mice (17). In our study, the control mice who were not exposed to ED *in utero* had vaginal openings occurring from 28 - 31 days, with an average of 29 days. The mice exposed to BBP *in utero* had vaginal openings significantly delayed ($p=0.0007$; Figure 13) by approximately three days, on average, compared to control counterparts with openings occurring from 30 to 41 days old. These data suggested that the female mice exposed to BBP prenatally entered puberty later. These results are supported by other researchers who have found a significantly delayed vaginal opening when female mice were exposed to phthalates *in utero* (92; 94). For example, Moyer and Hixson (94) studied the phthalate ester, mono-2-ethyl-hexyl-phthalate (500 mg/kg), on C57/Bl6 mice via oral gavage and found that the phthalate delayed vaginal openings in the F1 offspring to 30.7 days which was significantly delayed from control mice. In addition, Moral et al. (92) found that Sprague-Dawley rats exposed to 500 mg/day of prenatal BBP disruption had an average vaginal opening at day 33.28 which was delayed as compared to the control animals. In contrast, other authors have published studies which show no significant change in time of vaginal opening in female rats by administration of BBP at 400 mg/day (4) or 500 mg/kg (96). The discrepancy in the

literature could be due to ED affecting animals differently due to age of administration, number of pregnancies, or route of administration. Our data suggest that 500 mg/kg of BBP *in utero* disrupted normal female development as evidenced by the significantly delayed vaginal openings.

In our male pups we determined weekly AD and then averaged the distances measured at five weeks, (when a C57Bl/6J mouse is considered sexually mature and able to reproduce (63)), until the sacrifice date for each mouse (either 10 weeks or 20 weeks of age). The literature supports phthalates disrupting major male reproductive systems, specifically testosterone production, in animals from prenatal disruption near the end of the second week of gestation in mice (9) and rats (42). Therefore, since AD is regulated by testosterone (22) we used it as a measurement to determine if our model of disrupting testosterone production was successful. Our study showed that male BBP mice had significantly smaller ADs compared to control mice at 10 weeks and 20 weeks ($p=0.0.0390$ and $p=0.0217$, respectively; Figure 14), suggesting that our experimental model worked. Further, our results are supported by other researchers who had observed a decrease in AD in animals after phthalate oral gavage *in utero* (31; 95; 96; 110). Nagao et al. (96) found male AD at birth were decreased with 500 mg/kg doses of prenatal BBP. In other studies, AD in male fetuses of rats were significantly decreased when given DBP, a phthalate, prenatally at either 250 or 375 mg/kg (31) and 500 or 750 mg/kg (95). In addition, mice exposed to BBP at either or 1000 or 1500 mg/kg (32) demonstrated a significant decrease in AD as well. Only one study was found noting no significance in AD in either control or mice treated with DEHP, a phthalate, at 0.05-5

mg/kg in dietary chow (112). The studying citing no significance in AD indicated there could be a dose dependent effect with no significant change in AD at lower doses but a significant decrease in higher doses. At the time of writing, only one article exists denoting what normal AD in adult mice are with which to compare our control measurements (84). This study (84) was done in CD-1 mice (age unspecified) and the mean AD was 15.3±1.68 mm (84) which is similar to our control mice AD measurements of 13.8±0.056 mm (Figure 14). Thus, not only did our study show a significant decrease in AD suggesting that the BBP interfered with testosterone, but our study was also the first to assess AD throughout the first 20 weeks of a mouse's lifespan after being prenatally BBP dosed.

The seminal vesicle is an important accessory sex organ that contributes to the majority of seminal fluid into the ejaculatory ducts during ejaculation (36), and the weight of the seminal vesicle is an indicator of reproductive system strength (74). The seminal vesicles induce 5-alpha reductase activity which means it primarily converts testosterone to the useable form of the sex hormone, dihydrotestosterone (47). Therefore, we also measured seminal vesicle weights in our male mice at 10 weeks and 20 weeks, depending on sacrifice date to determine if prenatal BBP affected these weights. We did not find a significant difference in seminal vesicle weights between the BBP and control mice at either the 10 week or 20 week sacrifice time point ($p=0.4431$ and $p=0.7156$, respectively; Figure 15) suggesting that seminal vesicle weight was not altered by prenatal BBP administration which conflicts with some of the literature (104). For example, researchers conducted a mating study in which male rats were exposed to

BBP in their diet, but not prenatally. Males in the group exposed to 500 mg/kg had decreased seminal vesicle weights compared to the controls (104). In another study assessing the effects of phthalates on rat sexual function, Lee and Koo (75) found seminal vesicle weights were significantly decreased by oral administration of DEPH at 100 and 500 mg/kg, DINP and MEHP at 50 and 250 mg/kg, and DIDP at 500 mg/kg. In another, it was also discovered that seminal vesicle weights were significantly lighter by approximately 20-25% compared to controls in male mice exposed prenatally to 0.05/5 mg/kg of DEHP in the mother's food (112). Interestingly, coadministration of testosterone or gonadotrophins did partly reverse the depression of seminal vesicle weight in rats given phthalate esters at puberty (not prenatally) (49). It is important to note that even though the seminal vesicle is important for sperm motility, once sperm leaves the seminal vesicle it may not be viable or healthy (47). Even though we did not observe a reduction in seminal vesicle weights, this does not mean that the mice necessarily had viable sperm or would have been able to reproduce.

4.2 Sex Hormones

Fetuses in the present experiment were estimated to have been exposed to approximately 1/100 to 1/1000 (92) of the mother's dose of BBP which places exposure rates near the EPA safe dose for humans of 0.2 mg/kg/day (35). We know that our experimental model worked from the significant decrease in AD (Figure 14) and a significant decrease in testosterone production in BBP treated mice via ELISA analysis (Figure 11). More specifically, we did not see a wide range of variance in any of our

male BBP treated mice in testosterone concentration. There was, however, a wider variance in testosterone in control mice suggesting that BBP exposure prenatally significantly decreases testosterone production and keeps it suppressed throughout the 20 week time point. The testosterone kit used calculates testosterone ranges from 1.7 – 14.4 ng/ml (52). Our standard curve was normal and all values were able to be detected. Other researchers have found a decrease in testosterone production following phthalate exposure (750 mg/kg by DEHP) (110) and (50-500 mg/kg by DBP) (76) in male rat fetuses.

We also attempted to measure estrogen in the female mice to detect if estrogen production was affected in female mice exposed to BBP *in utero.* While determination of estrogen levels in mice is often considered non-trivial due to the small plasma sample sizes and the lack of available ELISA kits (the kits we had previously used in studies done by our lab (13) are no longer available), we attempted these measurements with an available kit that provided little clear direction or standard validity. Given these limitations, we found no significance in estrogen concentration in any of the female groups (control, locked, running; Figure 12) suggesting that in spite of a delayed vaginal opening, estrogen levels were not significantly decreased by prenatal BBP exposure. Perhaps due to the difficulty of assaying estrogens in female mice, to our knowledge, there are no papers citing circulating estrogen levels in pups exposed to phthalates prenatally. One author admits to not measuring circulating steroid hormones, but suggests "poor gamete quality from exposed animals, together with the down regulation of *cyp19a1* and *pgr* (genes involved in the steroid signaling pathway) expression in adult

offspring of both sexes, may suggest low serum estrogen levels, which, in turn, may affect the hypothalamus-pituitary-gonadal negative feedback mechanism" (112). Thus, while the BBP exposure did decrease testosterone levels in the male mice, we did not observe decreased sex hormone levels in the female mice.

4.3 Running Wheel Activity

Past studies in our lab (12-14) have shown that testosterone is a primary regulator of physical activity in both male and female mice with estrogen also affecting physical activity, albeit at a lower level. Therefore, our overall goal was to determine if disruption of the sex hormone production prenatally with BBP would subsequently decrease physical activity levels in our mice. BBP male mice that showed a decreased testosterone and AD distance – both indicative of disrupted testosterone production, had an overall decrease in daily distance ran across the 20 weeks of the study. However, while the BBP mice generally exhibited lower daily distances run, these differences were not significantly different (Figure 16). It is possible that the lack of significant differences between groups in more weeks was due to the variability in testosterone level we observed in the control group, which has been noted in the literature previously (52). When correlating the testosterone values with the activity values in the control mice, we found a trend toward significant correlations (r^2=0.79 and p=0.06), suggesting that indeed, the variable range of testosterone possibly led to the variability in daily activity in the control animals which hindered our ability to find significant differences between groups in other weeks (Figure 19). However, in total, our data does suggest that

interfering with testosterone production with prenatal BBP administration will reduce testosterone and activity levels.

In the female mice, we observed no significant differences in any of the physical activity indices (distance, speed, duration) between control and prenatal BBP dosed animals (Figure 22). However, much like the male mice, the prenatally dosed BBP mice showed a general trend toward less activity compared to controls (Figure 23) that would be supported when considered in conjunction with the delay in vaginal openings. However, the relative lack of difference in circulating estrogen levels between the treatment and control animals would support a relative lack of difference in daily activity levels in the female mice.

4.4 Body Composition

Obesity is an increasing problem in developed counties, specifically the United States where it is estimated that more than one-third of adults and 17% of adolescents are obese (106). The increased rate in metabolic diseases in the last 40 years coincides with enormous amounts of chemicals being released into our environment (5). Recently, investigators have given attention to this obesity epidemic hypothesizing that chemicals in our environment or "obesogens" incorrectly regulate lipid metabolism and could be to blame for the drastic increase in obesity across the lifespan (50). In particular, phthalates can modify gene expression by targeting the activity of family of nuclear receptors that are activated by intracellular lipophilic hormones (41) and can affect lipid metabolism by activating peroxisome-proliferator activated receptors (PPARs) (26).

PPARs act as metabolic detectors for lipophilic hormones (68) and phthalates can alter the sensing receptors possibly leading to obesity.

The available literature is inconclusive on how phthalates affect body composition in male and female mice, yet it has been hypothesized that a target of endocrine disrupting chemicals is the adipocyte (99). It has been speculated that a dose-response exists and depending on how much ED exposure and the time frame (i.e. prenatally, puberty) can alter physiology (141). In our male mice we did not see a significant difference in weight or lean mass in either the control or BBP groups at any time point, but there was a significant difference in fat mass between control and BBP male mice later in life (Figure 24). All male mice had access to a running-wheel at the beginning of week eight until sacrifice at either 10 weeks or 20 weeks. Hao et al. (53) found that male offspring born to mothers dosed with low doses of MEHP (0.05, 0.25, or 0.5 kg/mg) did alter body weight and fat storage. However, the researchers also found in the same study that a 5-10 fold higher doses of MEHP (much like the present study) did not promote obesity in treated males (53). Another study found that male offspring (wild-type mPPARα, PPARα null, or hPPAR tet-ff) born to mothers and fathers exposed to 10-145 mg/kg in their diet did not have a significant difference in body weight compared to controls (54).

In terms of body composition in female mice, we found no difference in body weight, lean mass, or fat mass in any group (control, running BBP, locked BBP) (Figure 25). Although not significant, we did see an observable increase in fat mass between control running mice and BBP locked wheel mice later in life. We interpret these results

to mean that female mice exposed to BBP *in utero* and who do not have access to a running wheel at any point in life are susceptible to an increase in fat mass later in life. Other investigators have found a significant increase in fat tissue and body weight in female mice exposed prenatally and during lactation by food with 5 mg/kg of DEHP (123). For example, after the mice exposed were weaned, they were then placed on a standard chow diet, yet even 9 weeks after the DEHP exposure, body weights were significantly increased in both sexes; yet fat storage in the females were still drastically increased (123). Additionally, exposure to the chemical DES has been linked to obesity later in life (99). In particular, a dose of 1 mg/kg during days 1-5 of neonatal life (in mice) caused a significant decrease in body weight during treatment, but it was followed by normal weight around puberty, and finally a significant increase in body weight by week 8 (100).

While it appears that body composition and particularly fat deposition is affected with to prenatal exposure to BBP, what is not known are the mechanisms behind this change in both sexes. Possible mechanisms that have been suggested include - binding to nuclear receptors like estrogen and PPAR agonists, disruption of enzymes that convert testosterone to estradiol, and altered signals from other endocrine organs to the adipocytes (99). However, the role of any of these mechanisms in the current study are unknown and must await further investigation. Further, the mechanisms behind the potential fat-sparing effects of daily activity are similarly unknown.

4.5 Potential Effects on Breast Cancer Tumorgenesis

A significant finding from Experiment 2 of this dissertation was the effect of prenatal BBP disruption on breast cancer morphology. Histological findings from the experiment between female control and BBP treated mice indicated that BBP treated mice had a higher percentage of tumor cells as shown by Ki67 staining ($p=0.0043$) but no change in estrogen receptor status ($p=0.1802$; Figure 8). In addition, H&E staining of BBP treated mice have ductal filling and cruciform structures (Figure 9) and a trichrome stain (Figure 10) indicated localized invasion to the surrounding stroma. Since phthalates and other endocrine disrupting chemicals are found in cosmetics, perfumes, and beauty products, it has become an increasing concern that these common chemicals could affect breast health. Several human studies have found significant levels of chemical residues in mammary adipose tissue with malignant cancer and compared those tissues to nonmalignant breast tissue (38) with elevated levels of PCBs found in fat samples from women with cancer compared to the women without cancer (38). Also, researchers in northern Mexico examined the association between urinary concentrations of phthalates and breast cancer disease in women. They found that phthalate metabolites were detected in at least 82% of the women who had diagnosed breast cancer (82). While these associations are interesting, histological examination of the tissues from Experiment 3, not originally planned for this dissertation, will be conducted in the future and will further illuminate this question.

4.6 Limitations

There are few limitations to the study that primarily include analyzing the offspring (F1 generation) as a whole, with litter number and size not taken into consideration. Additionally, conclusions regarding multiple generation effects from BBP are not possible due to the limited breeding scheme we used. Also, because of the difficulty in analyzing estrogen ELISA data, we cannot make firm conclusions with regards to circulating estrogen levels in our female mice.

4.7 Conclusions

From this study, we can draw several conclusions. First, prenatal exposure to a physiological dose of phthalate in male mice caused a later reduction of testosterone and altered reproductive characteristics as an adult. Further, there was a reduction in average daily activity – characterized by a reduction in distance and speed – that was especially evident at nine weeks of age. Next, in females, other than one indirect sign of endocrine disruption, there was no effect of prenatal BBP exposure on activity, but an increase in fat deposition, especially if exercise was not part of the mouse's life. Further, we observed potentially worrying signs of early mammary tumorigenesis in these mice. Taken together, our observations suggest that physiological level, prenatal exposure to phthalates can significantly stunt reproductive development as well as decreasing activity, increasing body fat, and perhaps, triggering early mammary tumorigenesis.

While it is unclear if these results would translate to the human model, our data do provide a foundation for further research in these areas in humans.

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APPENDIX A

FIGURES

Figure 1: Experimental 2 Design

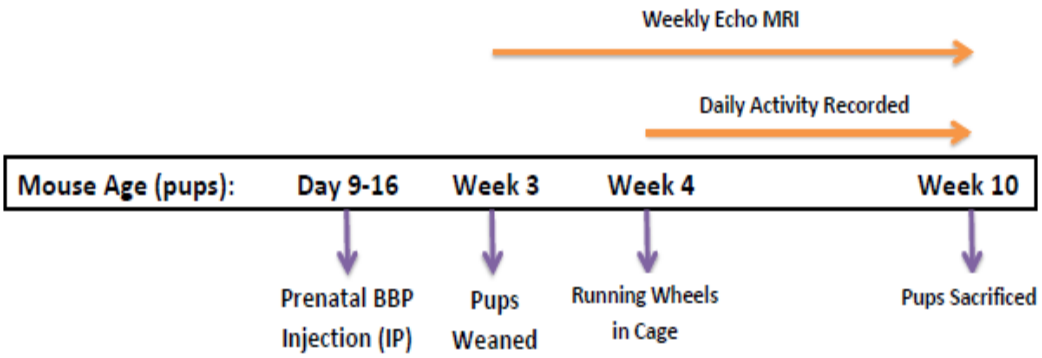


Figure 2: Experimental 3 Design

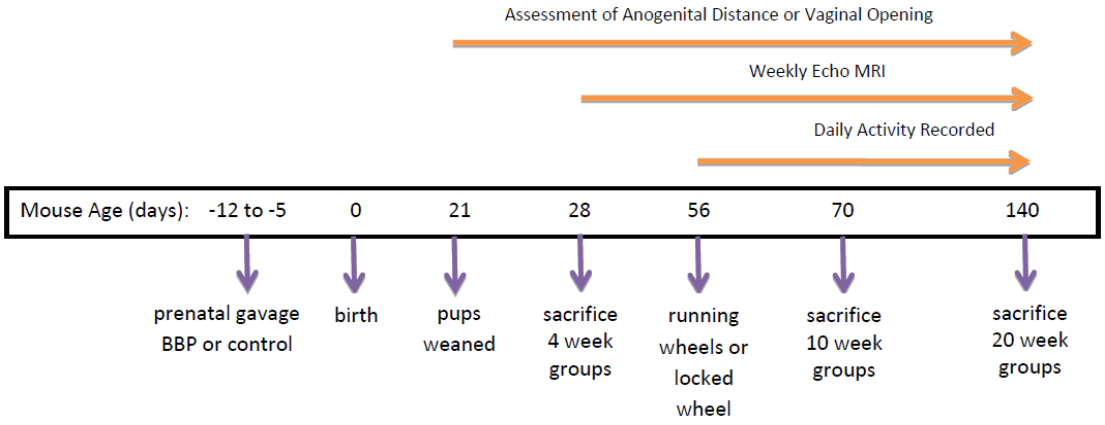
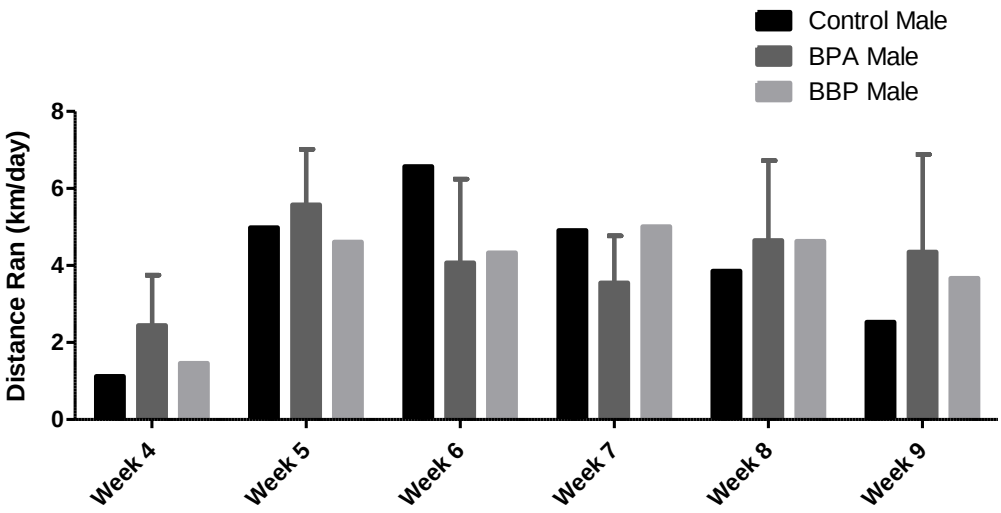


Figure 3: Male & Female Physical Activity Data Experiment 1



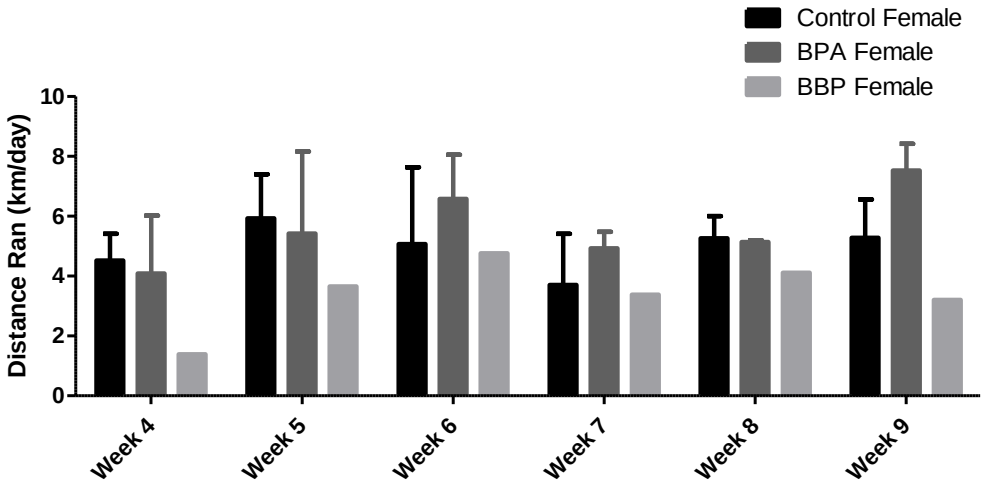


Figure 4: Male Physical Activity Data Experiment 2

There were no significant differences observed in the physical activity in male mice (p=0.9394).

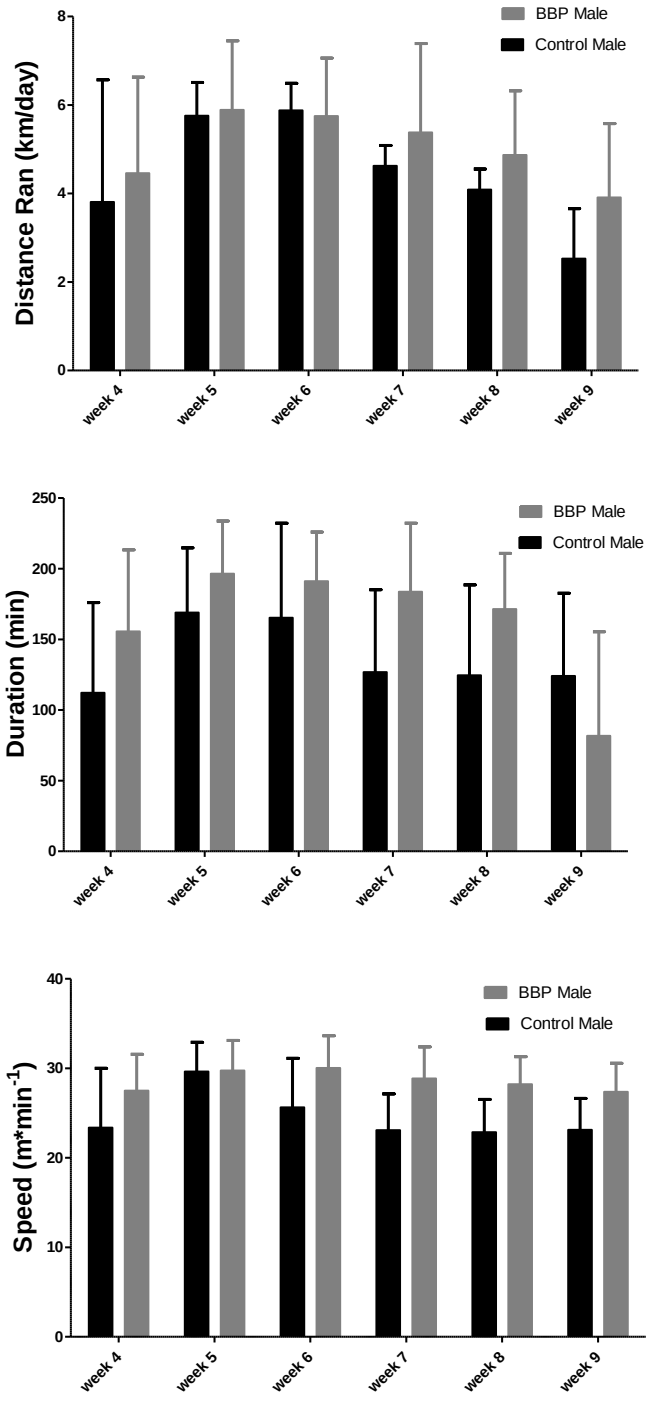


Figure 5: Female Physical Activity Data Experiment 2

There were no significant differences observed in the physical activity in female mice (p=0.7235).

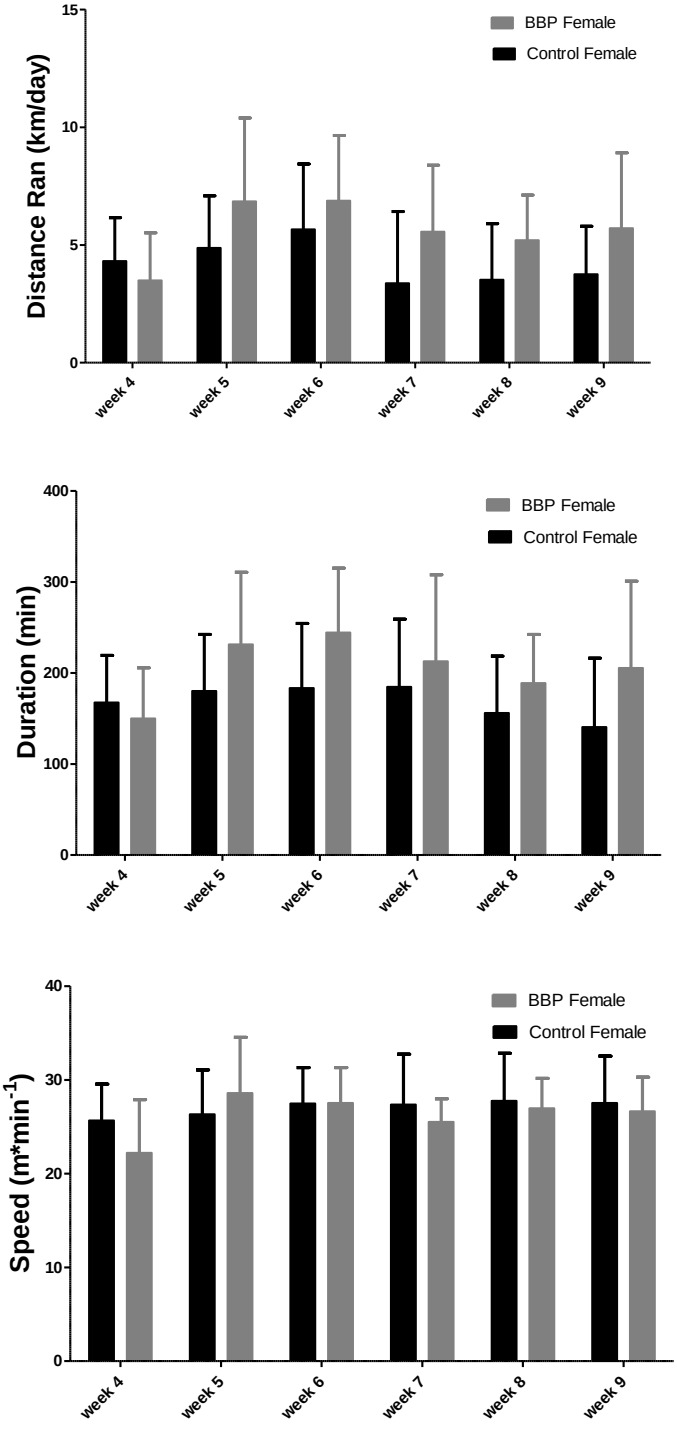


Figure 6: Male Body Composition Experiment 2

There was no change in lean mass (p=0.09) or fat mass (p=0.85) in males between the BBP and control group.

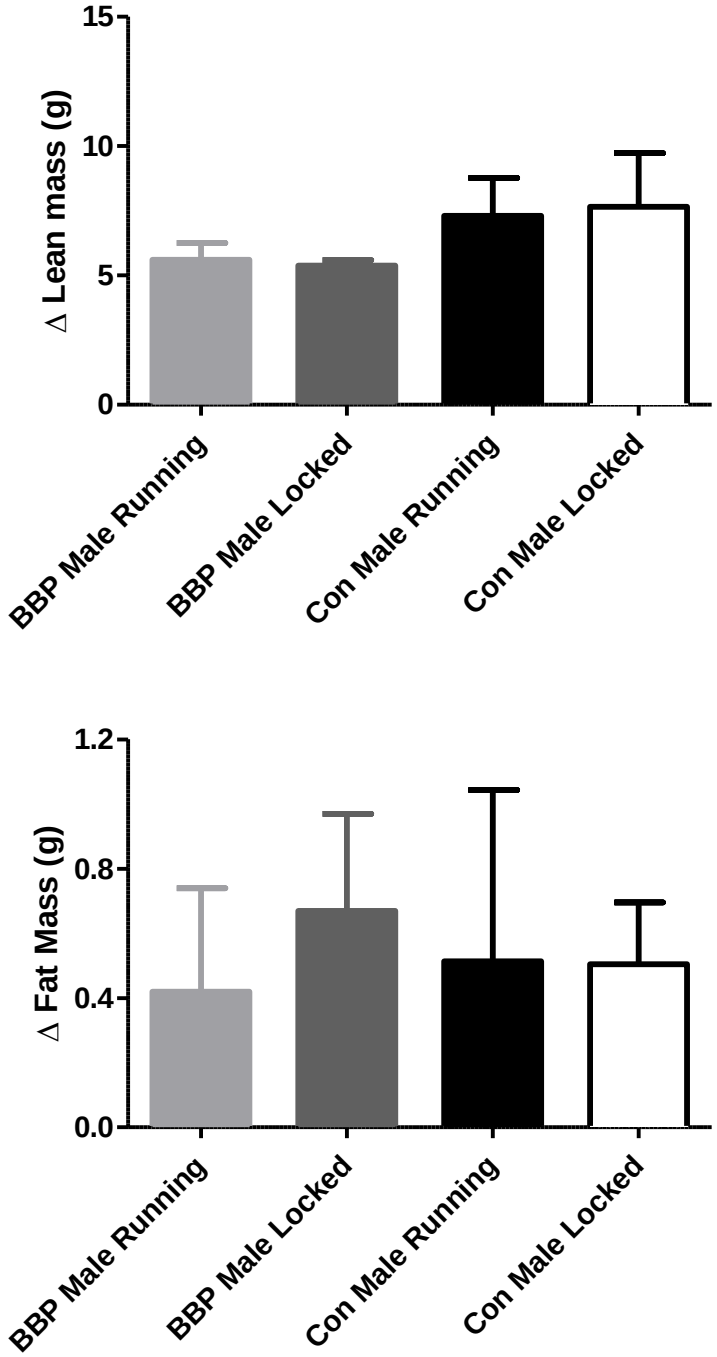


Figure 7: Female Body Composition Experiment 2

There was a significant change in lean mass (p=0.01) and no change in fat mass (p=0.10) in females between the BBP and control group.

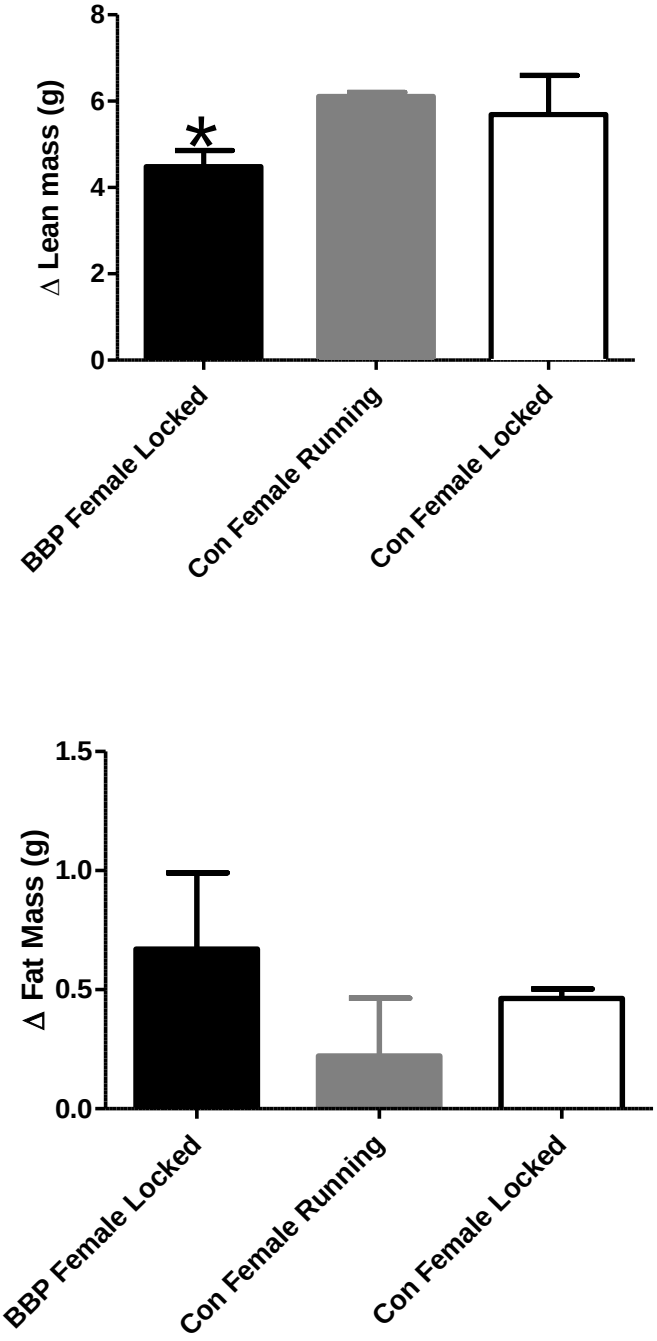
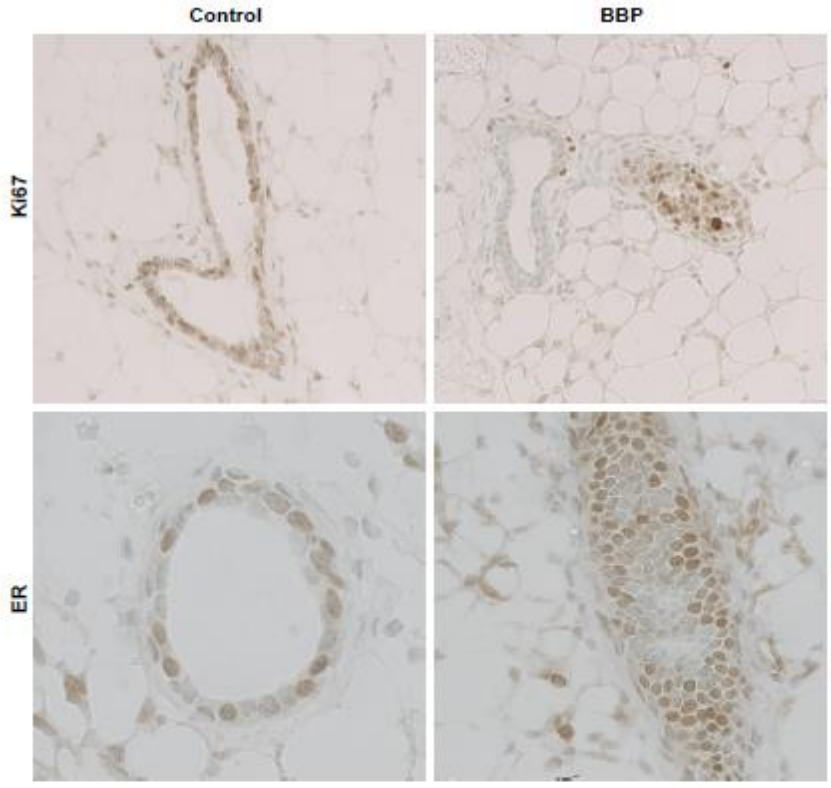


Figure 8: Female Histology Ki67 & Estrogen Receptor Experiment 2

BBP female mice had a higher percentage of tumor cells compared to controls as shown by Ki67 (p=0.0043) but no change in estrogen receptor status (p=0.1802).



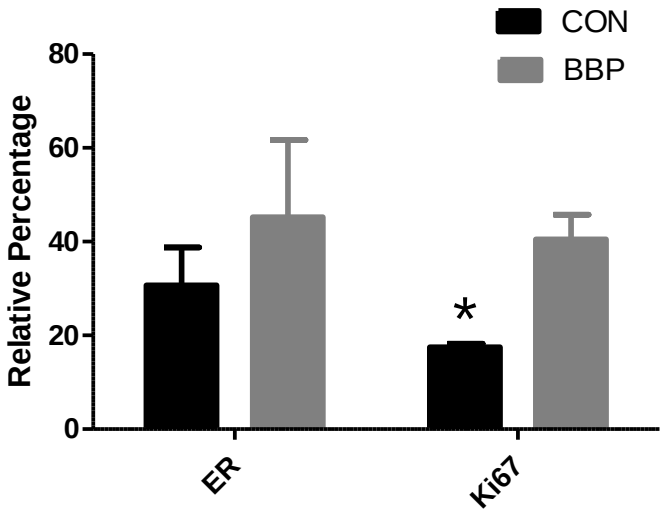


Figure 9: Female Histology H&E Staining Experiment 2

H&E staining of BBP treated female mice have ductal filling and cruciform structures.

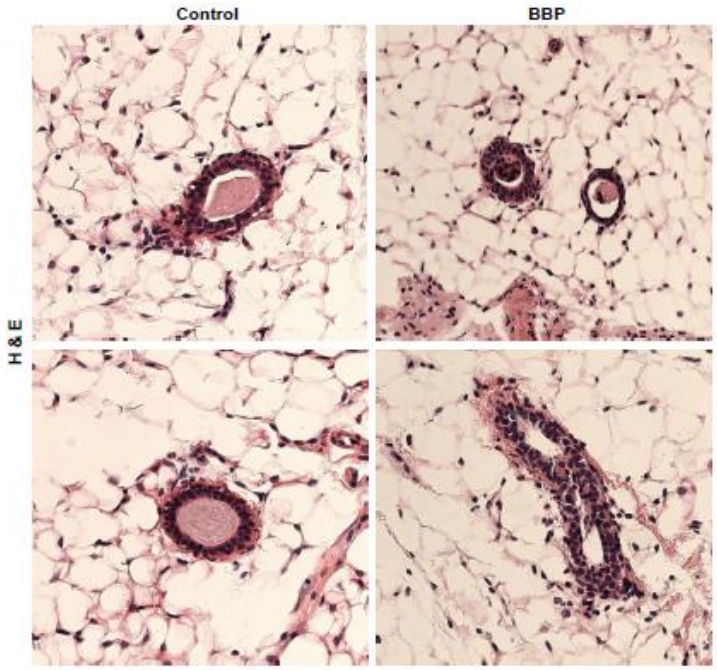


Figure 10: Female Histology Trichrome Stain

Trichrome stain indicates localized invasion in to the surrounding stroma.

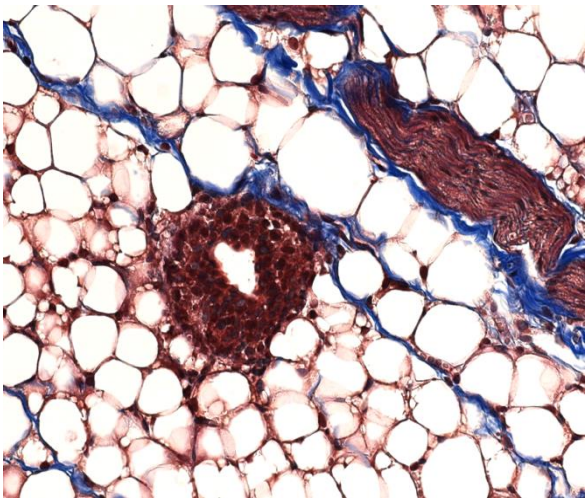


Figure 11: Male Testosterone Concentration Experiment 3

There was a significant difference in serum testosterone concentration values in males mice between control and BBP at 10 weeks (p=0.0390) and at 20 weeks (p=0.0217). The average coefficient of variance between triplicates from each sample was 2.23%.

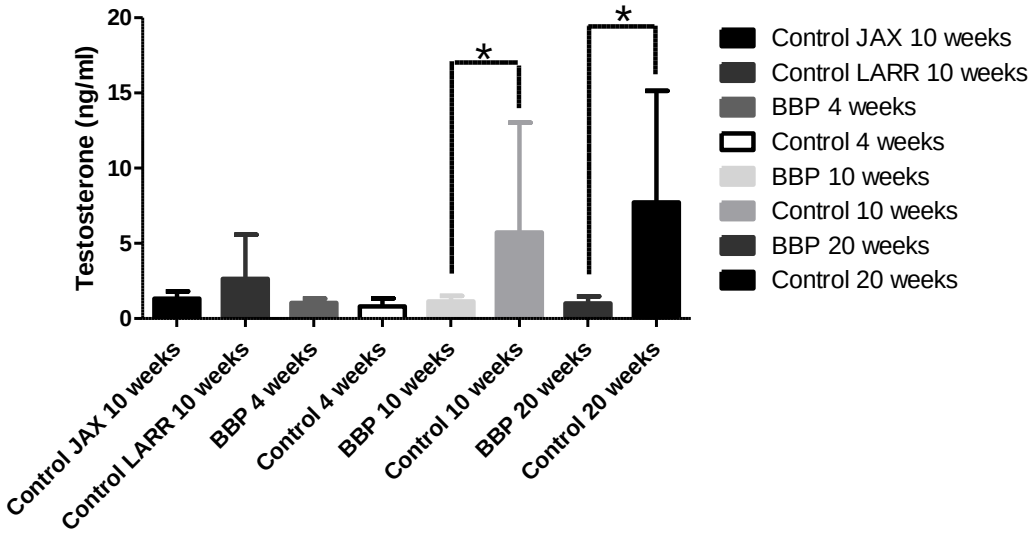


Figure 12: Female Estrogen Concentration Experiment 3

There was no significant in estrogen concentration values in female mice between control and BBP mice at any time point (p=0.0918).

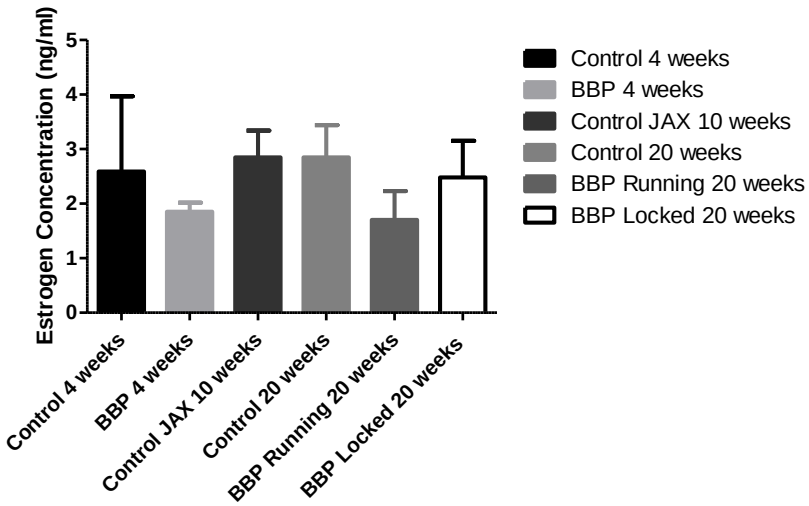


Figure 13: Female Vaginal Openings Experiment 3

Vaginal openings in female BBP mice were significantly delayed compared to control mice (p=0.0007).

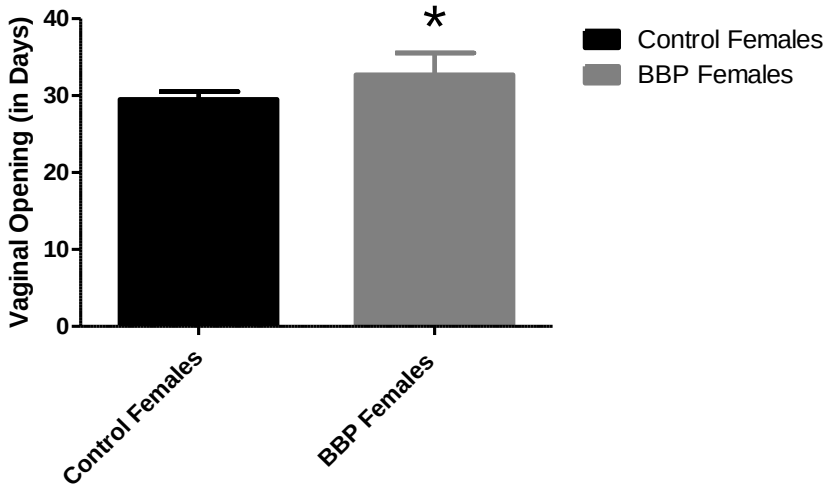


Figure 14: Male Anogenital Distances Experiment 3

Anogenital distances in the BBP male mice were significantly smaller compared to control mice at 10 weeks (p=0.0006) and at 20 weeks (p=0.0379).

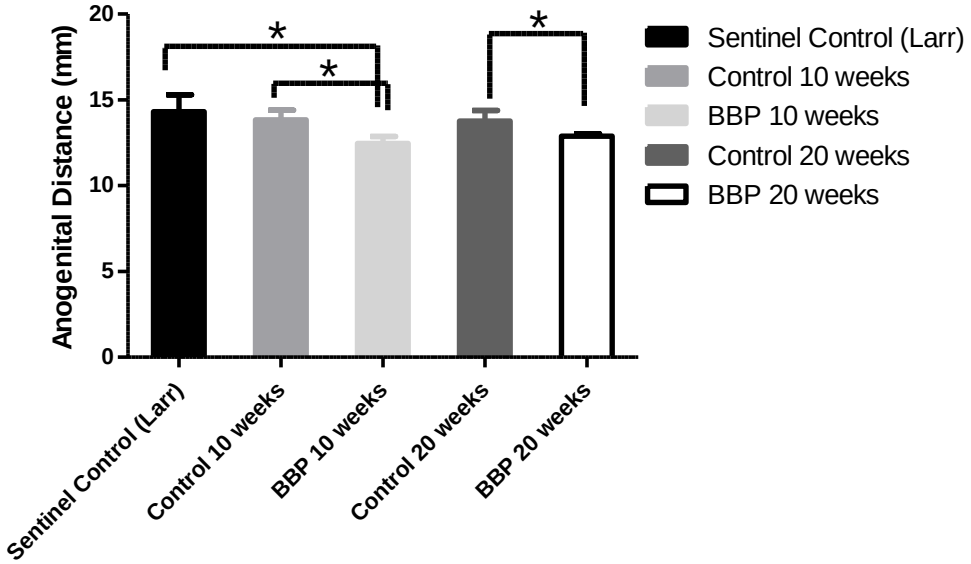


Figure 15: Male Seminal Vesicle Weights Experiment 3

Seminal vesicle weights in male mice were not significantly different at either 10 weeks (p=0.4431) or 20 weeks (p=0.7156).

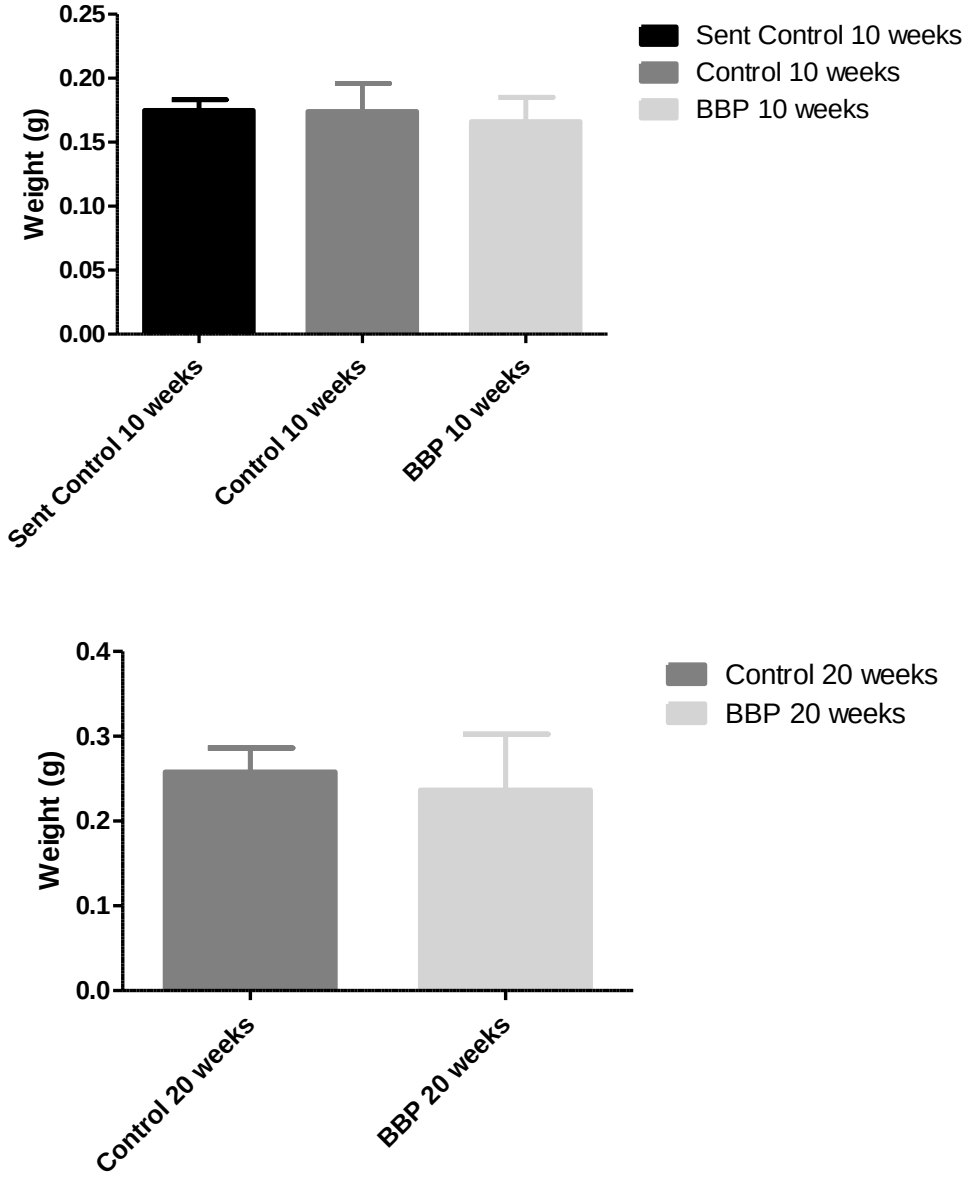


Figure 16: Male Physical Activity Data Experiment 3

In male mice, there was no significant decrease in distance in early life (p=0.0793) or late life (p=0.3344), no significant decrease in duration in early life (p=0.2029) or late life (p=0.4724), and no significant decrease in speed (0.4949).

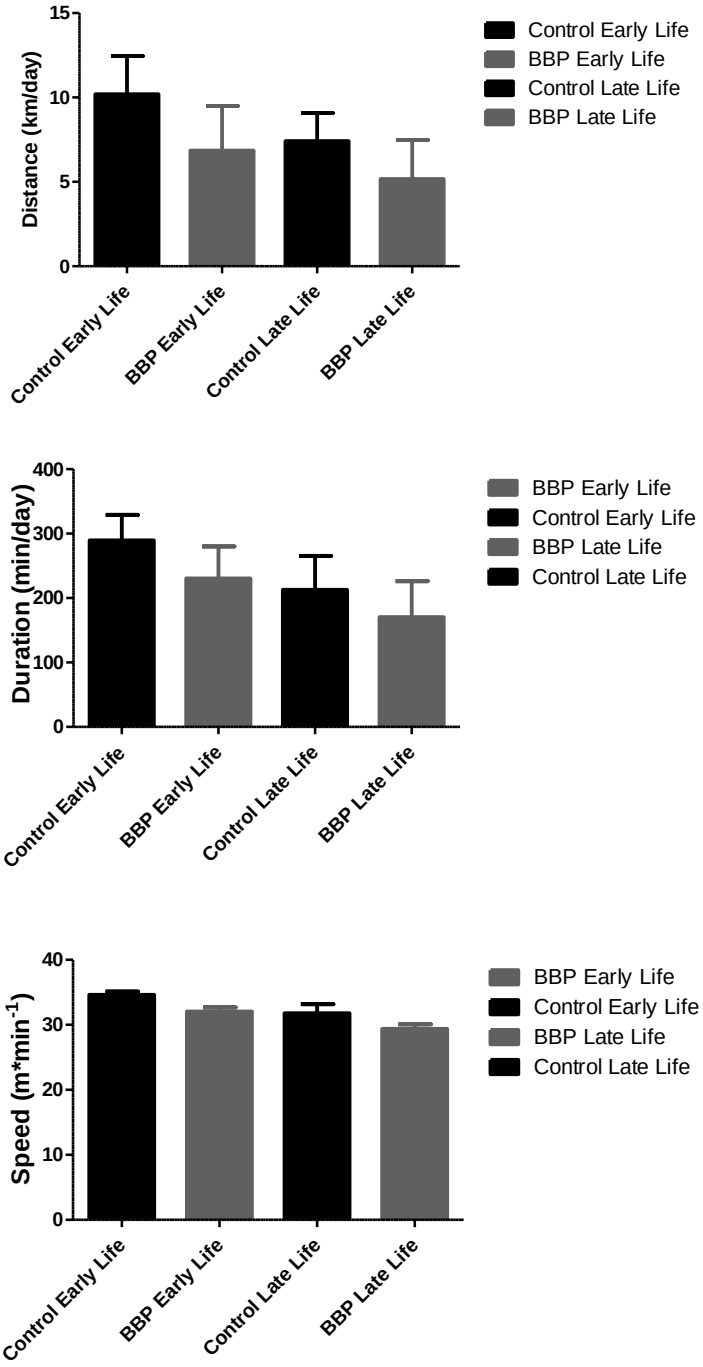


Figure 17: Male Distance Ran (represented in percentages) Experiment 3

On average, BBP male mice ran 20% less than controls when averaged throughout the lifetime.

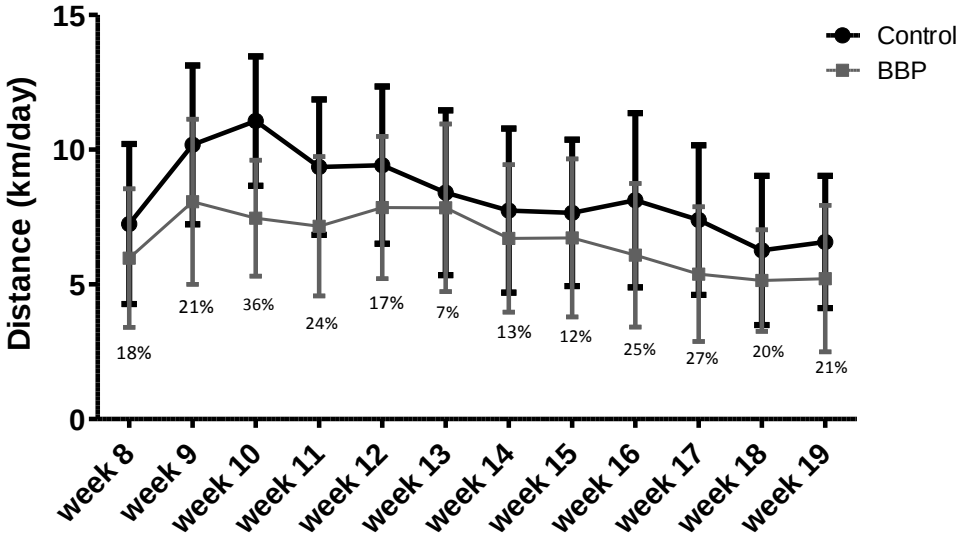


Figure 18: BBP Male Paired Correlation Week 10

Paired correlation data between testosterone concentration and distance ran (km) demonstrated a correlation coefficient of r=0.5710 with no significance (p=0.1393) for BBP male mice at 10 weeks.

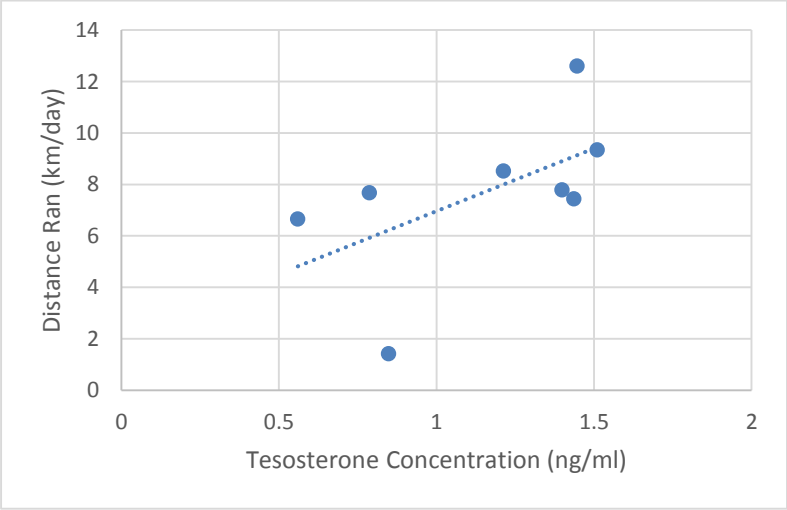


Figure 19: Control Male Paired Correlation Week 10

Paired correlation data between testosterone concentration and distance ran (km) demonstrated a correlation coefficient of r=0.7902 with no significance (p=0.0614) for control male mice at 10 weeks.

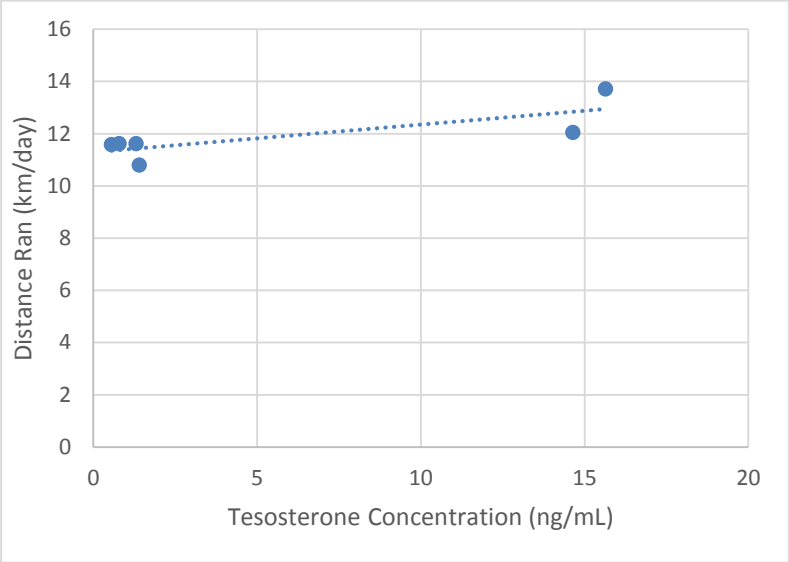


Figure 20: BBP Male Paired Correlation Week 20

Paired correlation data between testosterone concentration and distance ran (km) demonstrated a correlation coefficient of r=0.0453 with no significance (p=0.9547) for BBP male mice at 20 weeks.

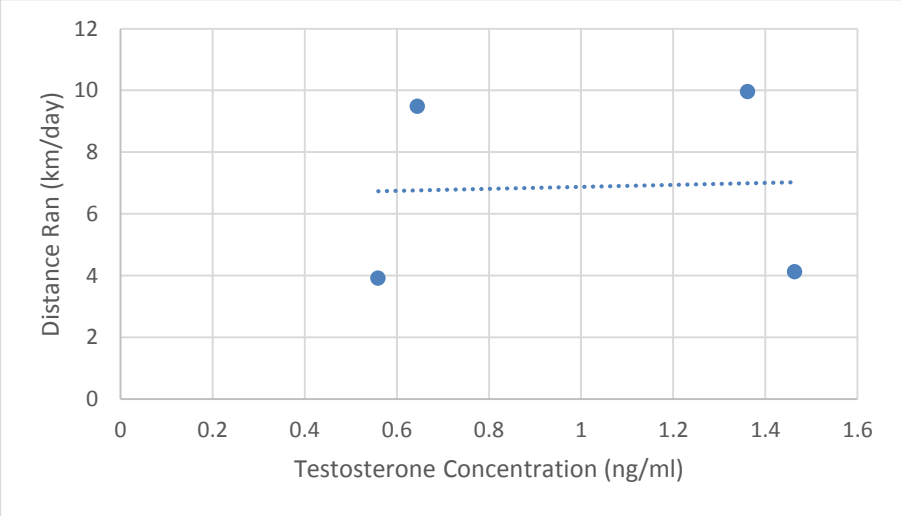


Figure 21: Control Male Paired Correlation Week 20

Paired correlation data between testosterone concentration and distance ran (km) demonstrated a correlation coefficient of r=0.9890 with no significance (p=0.0963) for control male mice at 20 weeks.

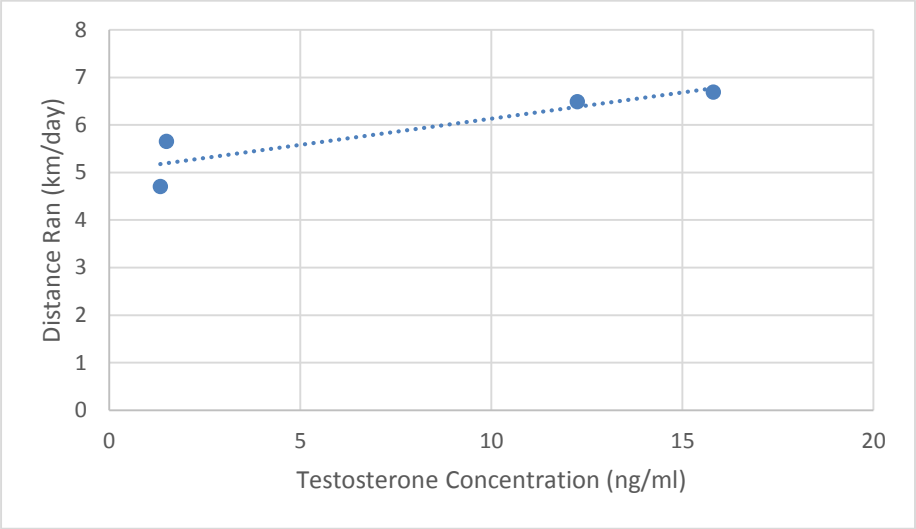
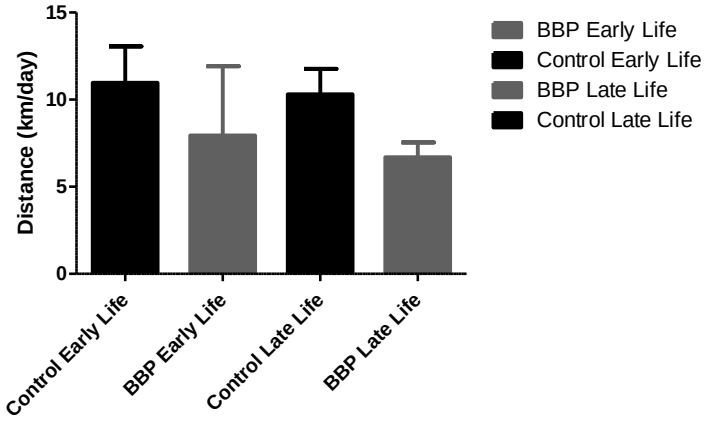
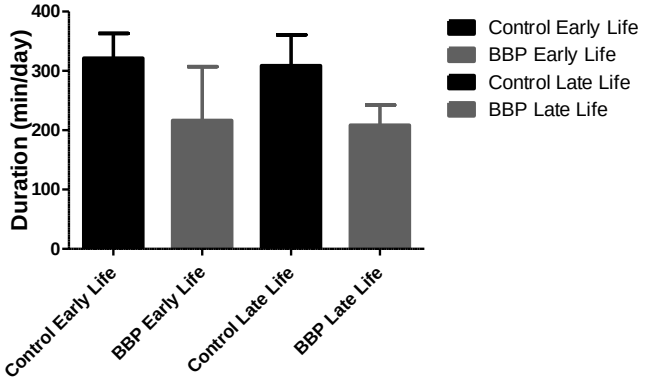


Figure 22: Female Physical Activity Data Experiment 3

There was no significant decrease in distance in early life (p=0.2766) or late life (p=0.1014), no significant decrease in duration in early life (p=0.0845) or late life (p=0.0618), and no significant decrease in speed (p=0.7424).





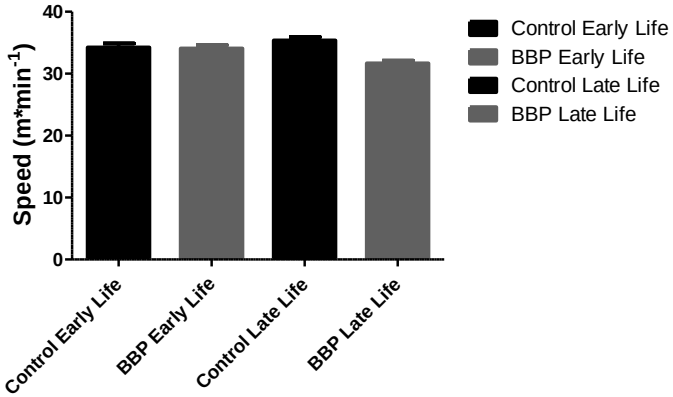


Figure 23: Female Distance Ran (represented in percentages) Experiment 3

On average, BBP female mice ran 15% less than controls when averaged throughout the lifetime.

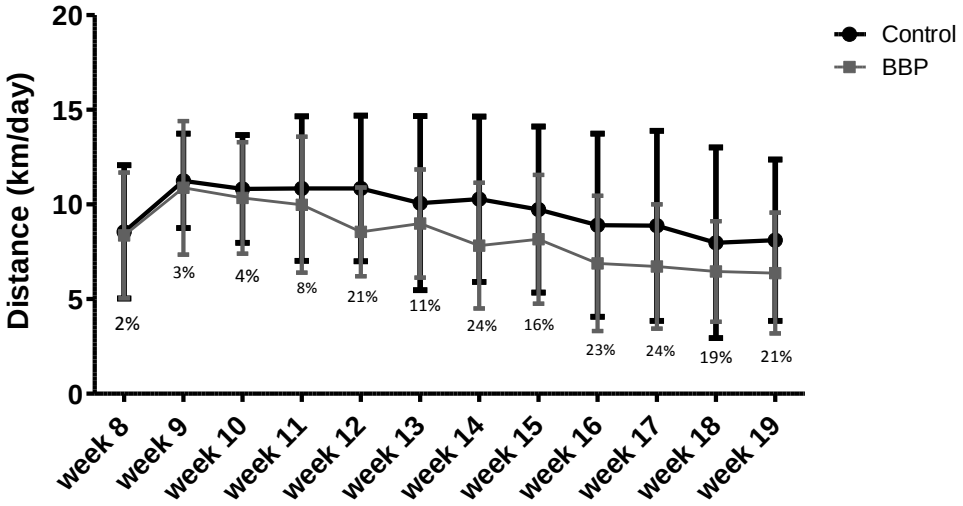


Figure 24: Male Body Composition Experiment 3

There was no significant decrease in weight in early life (p=0.4650) or late life (p=0.9314), no significant decrease in lean mass in early life (p=0.5595) or late life (p=0.9872), no significant decrease in fat mass in early life (p=0.2877) but a significant decrease in fat mass in late life (p=0.0257) for male mice.

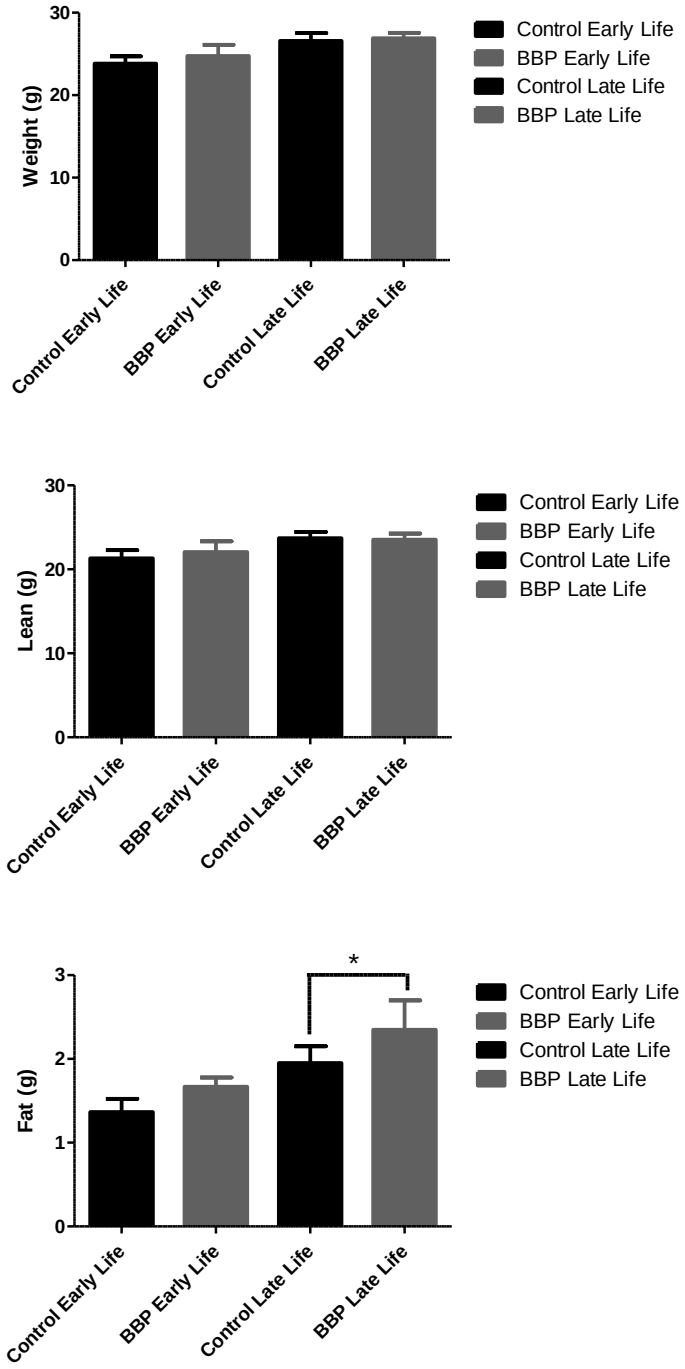
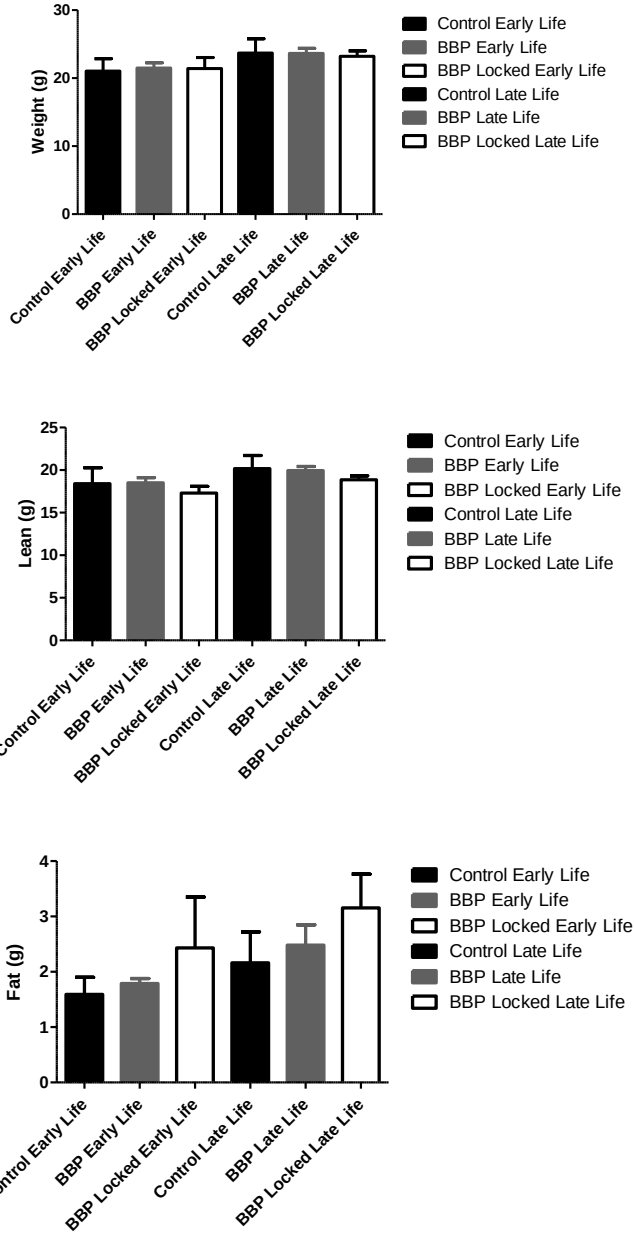


Figure 25: Female Body Composition Experiment 3

There was no significant decrease in weight in early life (p=0.9968) or late life (p=0.9999), no significant decrease in lean mass in early life (p=1.000) or late life (p=0.9995), and no significant decrease in fat mass in early life (p=0.9942) or late life (p=0.9553) in control and BBP females who had access to running wheels. Also, there was no significant decrease in weight in early life (p=0.9988) or late life (p=0.9939), no significant decrease in lean mass in early life (p=0.8660) or late life (p=0.4449), and no significant decrease in fat mass in early life (p=0.2629) or late life (p=0.1272) in control females who had access to running wheels and BBP females who did not have access.



APPENDIX B

TABLES

Table 1: Offspring Demographics for Experiment 1

	Control (Saline)	**BPA**	**BBP**
Male	1	3	1
Female	2	3	1
Total #	3	6	2

Table 2: Offspring Demographics for Experiment 2

Sex	**Treatment**	**Type of Wheel**	**n**
Male n=23	Control	Running	6
	Control	Locked	1
	BBP	Running	13
	BBP	Locked	3
Female n=20	Control	Running	10
	Control	Locked	3
	BBP	Running	4
	BBP	Locked	3

Table 3: Offspring Demographics for Experiment 3

Treatment	Type of Wheel	Age When Exposed to Wheel (weeks)	Age When Sacrificed (weeks)	Number of Males	Numbers of Females
Sentinel Animals ordered from Jackson Labs	Running	4	10	3	3
Sentinel Animals bred in Animal Facility (Texas A&M University)	Running	4	10	4	4
BBP	--	--	4	7	3
BBP	Running	8	10	8	6
BBP	Running	8	20	6	5
Control	--	--	4	6	2
Control	Running	8	10	6	6
Control	Running	8	20	6	4
BBP	Locked	8	10	--	5
BBP	Locked	8	20	--	5
			Total	**46**	**43**

Table 4: Descriptives of Breeding Experiment 3

Litter	Control	Control Oil	Control Oil	Control Oil	BBP	BBP	BBP	BBP	BBP	BBP	BBP
1	1 M 3 F	7 pups all died	4 pups all died	5 pups all died	Gavage	6 M 1 F	Gavage	Gavage	Gavage	1 M 4 F	1 M 1 F
2	2 M 2 F	3 M 1 F	3 M 2 F	5 M 4 F	Gavage	0 M 2 F*	3 M 2 F	Had liter, No inject	Gavage	Gavage	3 M 4 F
3	2 M 4 F		4 M 4 F	4 M 2 F	3 M 1 F		1 M 5 F	3 M 2 F	0 M 2 F	1 M 1 F	
4	2 M 3 F										
Average Ratio Male/ Female	1.5/3	3.8/2.6			2.0/2.4						