EFFECTS OF BISPHENOL S, A POTENTIAL ENDOCRINE-DISRUPTING CHEMICAL, ON TYPE 1 DIABETES, BEHAVIOR AND REPRODUCTION

by

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(Under the Direction of Tai Guo)

ABSTRACT

Bisphenol S (BPS) is a common chemical found in plastics and epoxy-resins as an alternative for bisphenol A (BPA). Several studies have shown that BPA's ability to mimic endogenous hormone signaling can lead to immunomodulation, exacerbation of type 1 diabetes (T1D), reproductive toxicity and behavioral changes. However, little is known about the effects of BPS. The current research objective was to determine the reproductive, neurological, and immunologic effects surrounding bisphenol exposure by utilizing NODEF mice and *C. elegans*. Male mice fed a soy-based diet and exposed to BPS during adulthood exhibited hyperactivity, increased anxiety-like behavior, decreased short-term memory, increased insulin sensitivity, impaired glucose tolerance, resistance to fasting and increased proinflammatory markers. Adult female mice fed a phytoestrogen-free diet exhibited hyperactivity, increased anxiety-like behaviors and increased proinflammatory markers. Mice exposed *in utero* exhibited increased hyperactivity and anxiety-like behavior in male offspring and decreased working memory in female offspring. *C. elegans* exposed to BPS exhibited an ability to recover in 1-2 subsequent generations following one generation of exposure and an accumulative effect when exposed for up to three generations.

In summary BPS causes behavioral changes, immunomodulation, and decreased fertility and can have a multigenerational effect.

INDEX WORDS: bisphenol S; immunomodulation; type 1 diabetes; behavioral changes; neurotoxicity

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DEDICATION

This thesis is dedicated to all the mice who sacrificed their lives to make this research possible.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW:

BISPHENOL A AND BISPHENOL S; RELATIVE TOXICITY AND MECHANISMS[1]

Introduction

Endocrine-disrupting chemicals (EDCs) are a class of chemicals that can interfere with an organism's endocrine system, disrupting normal homeostasis. Due to their widespread effects, EDCs can cause toxicity in multiple organ systems including the reproductive system, immune system, and nervous system. In addition, several factors such as the structure of the EDC, exposure window, and amount of exposure contribute to the potency of these chemicals. This chapter will examine the impact of bisphenol A (BPA), and its analogue bisphenol S (BPS) on the reproductive, immune, and nervous systems and their mechanisms of action.

Bisphenol A

Bisphenols are a class of chemicals found in plastics and epoxy-resins, with bisphenol A (BPA) being the most common. BPA is also the most studied bisphenol for its potential endocrine-disrupting properties (Eladak et al. 2015). For instance, BPA binds to estrogen receptors (ERs), including ERα and ERβ, and several estrogen-related receptors (Xu et al. 2016). Of particular concern is fetal or infantile exposure due to the tightly regulated hormone levels during normal development (Gore et al. 2015). Although BPA has a half-life of approximately 6 hours, its

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widespread use has resulted in a detection frequency of 95.7% in human urine samples with a median level of 1.24 µg/L (Stahlhut et al. 2009; Lehmler et al. 2018).

Bisphenol S

There have been growing concerns surrounding the estrogenicity of BPA, which has led to the commercialization of "BPA-free" products. These products often use BPA analogues, such as bisphenol S (BPS). These analogues are likely to behave akin to BPA because they share the basic bisphenol structure of two benzene rings separated by a short carbon or other chemical chain (Eladak et al. 2015). BPS has two phenol functional groups on either side of a sulfonyl group (Figure 1.1), and it is found in epoxy glues, inner coatings of food packaging, thermal paper receipts, and as an additive in dyes and tanning agents (Naderi et al. 2014). BPA analogues are presently not regulated and used without restrictions, largely because of the few studies to date that have examined their potential toxicity. BPS detection rates in human urine were found to be at 89.4% with a median level of 0.37 µg/L (Lehmler et al. 2018). Because BPS is much less biodegradable than BPA, there are growing concerns about its environmental prevalence and persistence. Both BPA and BPS have been detected in multiple environmental samples including soil, sediments, water, sewage sludge, and sewage effluents (Chen D et al. 2016).

Reproductive Toxicity

Reproductive and developmental toxicity in Caenorhabditis elegans (C. elegans)

Due to the ease of use, several studies have been conducted utilizing the *C. elegans* model to evaluate BPA analogues, especially fertility. Similar to BPA, BPS can also alter fertility. Mersha et al. (2015) reported that *C. elegans* embryos treated with BPS at concentrations as low as 0.5 µM or BPA at 1.0 µM for 4hr exhibited a significant decrease in the number of eggs laid. These concentrations correspond to a 1-2 µg/kg BW mouse dose because worms exposed externally to 1

mM of BPA resulted in an internal uptake of 2 µg/g worm pellet of this chemical (Allard and Colaiacovo 2011). A multigenerational BPS study showed L4 larvae exposed to BPS for 24hr at concentrations as low as 0.01 µM had a significant decrease in brood size for all four generations examined (Xiao et al. 2019). As a comparison, a parallel multigenerational BPA study showed a decrease in brood size in all four generations at concentrations of 1.0 and 10.0 µM BPA (Zhou, Yang, et al. 2016a). Like BPS, generations 2-4 also experienced a decrease in brood size at concentrations of BPA as low as 0.01 µM (Zhou, Yang, et al. 2016a). The decrease in brood size may result from altered plasma estradiol levels, causing reduced egg laying (Xiao et al. 2019). Because these studies only examined brood size by counting offspring when they reached the L3 stage, further research is needed to confirm these reports by counting the hatched offspring in the L1 phase. This additional information would also aid in comparisons with existing literature because most studies count offspring in this phase. Another study using prolonged BPS exposure of L1 worms for 72hr found that only the concentration of 10 µM significantly decreased brood size (Zhou 2018). Discrepancies in the effective concentrations from the above studies could be due to different exposure windows or the fact that worms exposed for multiple generations exhibited greater toxicity. A comparison study of BPA, BPS, and a mixture of BPA + BPS following exposure from the L1 larval stage to adulthood found that all three treatments induced a decrease in offspring survival at 500 µM (Chen Y et al. 2016). However, BPS caused an increase in embryonic lethality at all concentrations (125, 250, and 500 µM) tested, whereas BPA only did so at 500 µM (Chen Y et al. 2016).

Growth of *C. elegans* following bisphenol exposure was another endpoint examined in seven of the *C. elegans* studies (Supplementary Table 1.1). BPS decreased body length at concentrations as low as 0.01 µM (Xiao et al. 2019). A BPS multigenerational study showed that

body length decreased in all four generations at 0.01-100 µM when compared to control (Xiao et al. 2019). Interestingly, compared to the first generation, the fourth generation showed a significant increase in body length, suggesting an ability to recover from the effects through generations (Xiao et al. 2019). As a comparison, worms exposed to BPA at concentrations as low as 0.01 µM also exhibited a significant decrease in body length (Zhou, Yang, Li, Cui, et al. 2016; Zhou, Yang, et al. 2016b). The single published BPA generational study reported a decrease in body length in all four generations at 1.0 µM and above (Zhou, Yang, et al. 2016a). Even though the exposure window for BPA was more prolonged than BPS in these studies, BPS produced effects at lower concentrations, suggesting that it might be a more potent developmental toxicant. Further research using similar windows of exposure is needed to confirm this. Collectively, these studies suggest that BPS is equally if not more toxic than BPA in *C. elegans* in terms of reproductive and developmental toxicity.

Reproductive and developmental toxicity in zebrafish

Zebrafish are a popular vertebrate model due to their short lifespan, short periods of parturition and puberty, and low maintenance (Supplementary Table 1.2). Qiu et al. (2016) reported that there was no impact on the hatching time of zebrafish embryos following high level BPS exposure (100 µg/L; ~0.4 µM), which was in contrast to decreased hatching time, but no effect on embryo survival, following BPA exposure at lower levels (1 and 10 µg/L). However, both BPA (10 and 100 µg/L) and BPS (100 µg/L) elicited an increase in gonadotropin-releasing hormone (GnRH) expressing neuron GnRH3 in the hypothalamus, although BPA (100 µg/L) also increased GnRH3 in the terminal nerve, a structure located anterior to cranial nerve I and having processes extending to olfactory epithelium and telencephalon (Qiu et al. 2016). Additionally, both BPA and BPS increased specifically unique genes' expression, suggesting that the bisphenols

exhibited some selectivity in their disruptive actions on embryonic development (Qiu et al. 2016). The effects of BPA on the expressions of these genes largely depended on the duration of exposure and the stage of development; however, the same conclusion could not yet be made for BPS because this study only examined the effects at 25hr post-fertilization. Both BPA and BPS upregulated the expression of kiss1/kiss1r system, which could account for the increases in GnRH neuron numbers and *gnrh* mRNA levels. Under normal conditions, kiss1 is the primary regulator of the hypothalamic-pituitary axis in zebrafish (Qiu et al. 2016). Through the use of inhibitors, the authors further concluded that both BPA and BPS used ERα, thyroid hormone receptor, and the enzyme aromatase pathways to exert their effects (Qiu et al. 2016).

Moreman et al. (2017) compared the relative toxicity among BPS, BPF, BPAF, and BPA in terms of hatching delay and mortality in zebrafish embryos and found that BPAF was the most toxic BPA analogue, followed by BPA, then BPF, with BPS being the least toxic. Various developmental abnormalities occurred following exposure to these bisphenols at various concentrations: 0.50-2.0 mg/L for BPAF, 1.0-12.5 mg/L for BPA, 1.0-35.0 mg/L for BPF, and 10-200 mg/L for BPS (Moreman et al. 2017). All bisphenols caused cardiac edema and craniofacial abnormalities at varying concentrations. In this study, BPAF was the most potent, causing cardiac edema at 1.0 mg/L. Cranial hemorrhage occurred in both BPF and BPA-treated embryos. At 5.0 mg/L, BPA induced several abnormalities while the same concentration of BPF caused fewer abnormalities. However, BPF caused more abnormalities than BPA at and above 10.0 mg/L (Moreman et al. 2017). Both BPA and BPF caused yolk sac deformities at and above 10.0 mg/L. While BPS did so at only 20.0 mg/L, BPAF did not cause yolk sac deformities at any of the concentrations tested (0.50-2.0 mg/L). BPS caused abnormalities at 200 mg/L and was the only bisphenol that caused tail abnormalities (Moreman et al. 2017). However, it should be noted that

low concentrations of BPS were not examined. Several EDCs have a bimodal concentration-response, and there are vast differences in the concentration ranges in this study (Moreman et al. 2017). Therefore, it is not possible to make exact comparisons. Utilization of transgenic zebrafish to observe fluorescence signaling of target tissues revealed that all three bisphenols targeted the heart, liver, and tail tissues, with heart tissues being the most responsive (Moreman et al. 2017). This study concluded that all four bisphenols activated genes through the estrogen response elements in the target tissues via mediating the classical ER pathway and exerted some toxicity (Moreman et al. 2017).

Reproductive and developmental toxicity in turtles

Bisphenols can mimic estrogen, and they may have detrimental effects on both sexes. Certain reptiles such as turtles lack sex chromosomes and rely on temperature and hormones for sexual differentiation in the brain (Supplementary Table 1.3). Thus, it is theorized that reptiles are more sensitive to EDC-induced alterations in reproductive endpoints. Manshack et al. (2016) exposed eggs of painted turtles (*Chrysemys picta*) to BPA (0.01 or 100 µg/mL) at 26ºC (male determining temperature) during the temperature sensitive period. Turtles exposed to a high concentration of BPA (100 µg/mL) traveled less during the late stage of the spatial navigation maze test when compared to other groups (Manshack et al. 2016). However, improvements in spatial navigational learning and memory occurred when compared to the positive (0.02 µg/mL ethinyl estradiol) and negative (no treatment or ethanol) controls, and BPA low concentration (0.01 µg/mL) group (Manshack et al. 2016). This could be due to alterations in brain aromatase activity, resulting in feminization of the brain and possible sex-dependent epigenetic changes (Jandegian et al. 2015; Manshack et al. 2016). Both high (100 µg/µL) and low (0.01 µg/µL) concentrations of BPA disrupted sexual differentiation when eggs were exposed at male determining temperatures

(Jandegian et al. 2015), which resulted in males exhibiting an ovarian-like cortex and a disruption of testicular tubules (Jandegian et al. 2015). Currently, there are no reported studies examining the effects of BPA analogues on sexual differentiation in reptiles. Due to their similar potencies and mechanisms, it is likely that exposure to BPS during the temperature sensitive period would also result in a disruption of sexual differentiation. Further studies using relevant concentrations of BPS are needed to verify this assumption.

Reproductive and developmental toxicity in sheep

To assess the impact of BPA analogues on placental endocrine function, sheep were exposed to 0.05 mg/kg body weight (bw) of either BPS or BPA during mid-gestation (day 30 to day 100 of gestation) as sheep have 152 days of gestation on average (Gingrich et al. 2018). Exposure to BPS (Supplementary Table 1.4) reduced maternal serum concentrations of pregnancy-associated glycoprotein (PAG) 1 and pregnancy-specific protein B (PSPB), suggesting impaired placental endocrine function (Gingrich et al. 2018). Impairment of placental endocrine function by BPS was further supported by a decrease of progesterone during late mid-gestation in BPS-exposed sheep compared to controls. There was also a lower number of binucleated cells, resulting from the reduction in PAG1, PSPB, and progesterone (Gingrich et al. 2018). Under physiological conditions, progesterone should increase through late mid-gestation due to a shift from ovarian to placental production (Harrison and Heap 1978). However, BPS did not alter progesterone during early mid-gestation, further suggesting that BPS acted on the placenta. Interestingly, PAG1 and PSPB concentrations were partially to fully recovered after discontinuation of BPS exposure for 20 days (Gingrich et al. 2018). BPS also reduced the expression of E-cadherin, a protein contributing to the fusogenic ability of the fetus (Kokkinos et al. 2010; Gingrich et al. 2018), which might result in the initiation of an invasive phenotype, irregular cell adhesion, and possibly the

transition of trophoblast cells from epithelial to mesenchymal (Kokkinos et al. 2010). This transition is commonly seen in carcinoma invasiveness examined endpoints when compared to control (Gingrich et al. 2018). This finding supports the conclusion that BPA and BPS have different mechanisms of action as reported by others (Moreman et al. 2017). More importantly, it also suggests that placental endocrine function is more susceptible to BPS than to BPA.

Gingrich et al. (2019) examined the toxicokinetics of BPA, BPS, and BPF in dams and their fetuses. BPS had the shortest half-life in the maternal serum compartment followed by BPA, and lastly by BPF. Although BPS had the shortest maternal half-life, it reached the highest maternal serum concentration (643±29.9 ng/ml), approximately an order of magnitude greater than that of BPA (66.7±1.7 ng/ml) or BPF (48.8±0.2 ng/ml) (Gingrich et al. 2019). When examining the toxicokinetics in the fetal compartment, BPF had the fastest clearance from fetal circulation. In contrast, BPS had the longest half-life, followed by BPA (Gingrich et al. 2019). Together, this demonstrated that the metabolisms of BPA, BPS, and BPF differed from each other, and there existed different mechanisms between maternal and fetal compartments. It also suggested that, due to its longer half-life, BPS might present a greater risk than the other two bisphenols when exposed gestationally. This finding could also partially explain how a greater impairment of placental function in the BPS-exposed sheep was observed compared to BPA treatment.

Reproductive toxicities of bisphenols in rodents and humans (in vitro and in vivo)

Reproductive toxicities in rodents and humans were combined here because one study discussed in the next paragraph used rat, mouse, and human testes and compared their differences (Supplementary Table 1.5). LaPlante et al. (2017) examined the effects of BPS exposure at 2 or 200 µg BPS/kg/day on maternal behaviors and the mammary glands from early gestation (pregnancy day 9) to early or late lactation in mice and revealed that female mice exposed to 200

µg/kg BPS until lactation day 20 had altered mammary gland morphology. Specifically, there were reductions in the number of milk-producing lobules in dams. Interestingly, the low dose (2 µg/kg) BPS caused an increase in prolactin and Esr1 expression, while the 200 µg/kg BPS resulted in decreases. These results suggest a bimodal dose-response curve in terms of gene expression associated with lactation.

Using a culture system (Supplementary Table 1.5), Eladak et al. (2015) examined basal and luteinizing hormone-stimulated testosterone secretion in explanted human, rat, and mouse developing testes following treatment with BPS, BPF, and BPA for up to three days. While human cells were most sensitive to BPA, mice were most sensitive to BPS. In the mouse testes, BPS at and above 100 nM caused a concentration-dependent decrease in basal testosterone secretion for all three days. In human cells, BPS induced the same concentration-dependent decreases as it did in mice; however, human cells were less sensitive with significant decreases observed only at and above 1,000 nM. Testosterone levels showed a bimodal concentration-response curve after exposure to BPF as 10 - 100 nM induced increasing levels, while 1,000 - 10,000 nM induced decreases in mouse testes across the three days, and 10,000 nM induced a significant change on day one. Similarly, only the 1,000 and 10,000 nM concentrations were significant for BPF on days 2 and 3. In human cells, BPF induced the same bimodal concentration-response on days 1 and 2. On day 3, BPF induced a decrease in testosterone secretion in a concentration-dependent manner. Similar to BPF, BPA induced a bimodal concentration-response in human cultures regarding testosterone secretion, exhibiting a significant decrease in basal testosterone secretion at concentrations as low as 10 nM. Mouse testes showed some sensitivity with significant decreases at 100 and 10,000 nM BPA. Although rats exhibited the largest decrease compared to the control, only the 10,000 nM BPA caused a significant decrease.

In summary, both bisphenols can affect the reproductive system of both sexes (Figure 1.2). Based on *C. elegans* and zebrafish studies, both BPS and BPA reduce the number of offspring and increase embryonic lethality. None of the other animal models showed reductions in litter size or increases in fetal lethality; therefore, it is possible that this effect only occurs in these invertebrate and fish species. In species with no sex chromosomes such as turtles, BPA can disrupt sexual differentiation. Additional studies focusing on litter size and survival need to be conducted to confirm these observations that are now based on limited data. In testes cells, both bisphenols altered testosterone secretion. Due to the similarities in sheep and human gestation, it is likely that BPS also alters placental function in humans. Overall, both bisphenols cause reproductive toxicity varying in potency depending on the species and reproductive organs analyzed.

Neurotoxicity of BPA analogues

Behavioral changes in C. elegans

Of the seventeen *C. elegans* studies reviewed, nine used some measure of behavioral change as an endpoint (Supplementary Table 1.6). The most common parameters were the number of body bends and head thrashes observed. BPS reduced the number of head thrashes starting at 1.0 µM and body bends at 10.0 µM (Zhou 2018). When L4 larvae, a later exposure window, were examined, BPS reduced the number of head thrashes, and body bends significantly starting at 0.01 µM following exposure for 24hr (Xiao et al. 2019). These observations suggest that BPS is more toxic at specific exposure windows. Additionally, a multigenerational study showed that *C. elegans* exposed to BPS exhibited a decrease in the number of head thrashes for four generations in a nonlinear response (Xiao et al. 2019). For BPA, exposure to 0.01 µM significantly decreased the number of head thrashes, while a concentration of 0.1 µM significantly decreased body bend rate (Zhou, Yang, Li, Cui, et al. 2016; Zhou, Yang, et al. 2016a, 2016b), while other studies did

not see an effect until 1.0 µM BPA (Supplementary Table 1.6). Similarly, a decrease in head thrashes continued for multiple generations in worms exposed to BPA (Zhou, Yang, et al. 2016a).

Habituation is another popular behavioral parameter in *C. elegans*. One study reported that *C. elegans* have impaired habituations in all the BPA and BPS concentrations (0.1-10 µM) tested (Mersha et al. 2015). When feeding behavior was examined, there were decreases in food attainment at all the time points examined (e.g., 2, 4, 6, and 8 h) for *C. elegans* treated with 0.1, 1.0, and 10.0 µM BPA, although the decrease was not significant for the 0.1 µM (Kohra et al. 2002; Flood 2014). There were no studies that examined feeding behavior in BPS-exposed *C. elegans*. However, since other behavior studies show *C. elegans* to be as sensitive to BPS as they are to BPA, it may be likely that BPS would also cause a decrease in food attainment.

Neurotoxicity in developing and adult zebrafish

Several behavioral studies commonly use zebrafish due to their quick maturation and ease of care. In addition, several of their behavior tests are similar and comparable to rodent tests. Memory recognition was examined (Supplementary Table 1.7) following chronic exposure of adult female zebrafish to 0, 1, 10, or 30 µg/L of BPS (Naderi et al. 2020). When ability to perform several memory tests and the expression of genes and proteins involved in the extracellular signal-regulated protein kinase/cAMP response element binding protein (ERK/CREB) pathway were examined, a bimodal response was observed (Naderi et al. 2020). At the lowest concentration, female zebrafish showed significant improvements in the object placement test compared to control; however, there were no differences in any other behavior test. Consistent with this improved performance, there was an increase in phosphorylation of ERK1/2, indicating a stimulated activity of this protein kinase, as well as upregulations of several genes involved in the ERK/CREB pathway (Naderi et al. 2020).

In developing zebrafish, bisphenol exposure also resulted in behavioral changes and neurotoxicity. Zebrafish exposed to BPA or BPS at a low concentration (0.0068 μM) during neurogenesis exhibited increased locomotor activity, indicative of anxiety-like behaviors (Kinch et al. 2015). Additionally, both BPA and BPS exposures resulted in an increase of neurogenesis in varying brain regions, with BPS exhibiting a much higher effect (Kinch et al. 2015). This study suggests the effects of BPA and BPS on neurogenesis were brain region-specific and had a nonlinear concentration-response relationship. Further studies exposing embryos to various antagonists revealed that both the changes in behaviors and neurogenesis were dependent on aromatase B and androgen receptors, but not on ERs. BPA and BPS activated androgen receptors, which led to the upregulation of aromatase B transcription and an increase in estradiol production (Kinch et al. 2015).

In contrast, Gu et al. (2019) found a concentration-dependent decrease (Supplementary Table 1.7) in locomotor activity and speed in zebrafish exposed to higher concentrations of BPS (0.3 mg/L and 3.0 mg/L) during development. The apparent differences in results between this study and the one discussed in the previous paragraph (Kinch et al. 2015) may be due to both the concentrations and exposure windows, because the exposure in this study occurred after neurogenesis and at a much higher concentration (Gu et al. 2019). In addition, a significant increase in apoptosis in the brain regions and suppressions of six genes involved in neurodevelopment were observed (Gu et al. 2019). Overall, both BPS and BPA were demonstrated to alter the developing brain, resulting in neurotoxicity and changes in behaviors.

Neurotoxicity induced by BPA analogues in rodents

Rodents are the most popular model when examining changes in behaviors and neurotoxicity. EDCs induce detrimental consequences during gestational and lactational periods

of development as these are sensitive windows of exposure. A combined gestational and lactational exposure (Supplementary Table 1.8) to BPS, BPF, or BPA from gestation day 12 to postnatal day 21 was examined in rats (Castro et al. 2015). Rats exposed to a low dose (10 µg/kg) of BPA, BPS or BPF had altered expressions of 5α-R isozymes, in addition to altered transcriptional profiles of genes associated with the dopamine and serotonin systems of the prefrontal cortex (Castro et al. 2015). A change in nursing behaviors was observed in CD-1 dams exposed to BPS (2 or 200 µg/kg) throughout gestation or from gestation to the end of lactation (LaPlante et al. 2017). Dams were observed to be in the high-crouch position, which is associated with maximal milk ejection (Stern et al. 1990). Despite spending a more extended time nursing and the use of the high-crouch position by dams, both doses of BPS led to pups with smaller body weights when compared to control (LaPlante et al. 2017). Pups exposed to the high dose exhibited a longer nursing time during late lactation and were less likely to initiate nursing (LaPlante et al. 2017). There are no reported studies examining nursing behaviors in BPA exposed mice, which for now makes the comparison of potencies impossible.

Daily BPA and BPF exposure at 10 mg/kg in C57BL/6 mice from gestational day 11.5 to 18.5 produced long-term behavioral changes in offspring (Ohtani et al. 2017). These changes included increases in anxiety-like and depression-like behaviors, with higher sensitivity observed in females (Ohtani et al. 2017). It is essential to note that male rodents typically exhibit more anxiety-like behaviors than females and tend to be more sensitive to alterations (Imhof et al. 1993). When comparing behavioral endpoints between sexes, no significant differences were found, suggesting an abolishment of typical sex differences (Ohtani et al. 2017). Although BPF demonstrated weaker estrogenic activity, the effects of BPF on behaviors were more significant than BPA (Ohtani et al. 2017). In contrast, ICR mouse offspring exposed to BPA in utero and

during lactation at 500 µg/kg had a decreased anxiety-like behavior, increased impulsiveness, impaired object recognition, and a decreased working memory (Tian et al. 2010). These behavior test results correlated with dopamine transporter, dopamine receptor 2, and N-methyl-D-aspartate (NMDA) receptor binding. However, it was not possible to tell if there were sex-dependent effects because the report combined both sexes. Nonetheless, the study concluded that BPA changes the NMDA receptor function and impaired cognitive function (Tian et al. 2010). The discrepancy between these two studies in anxiety-like behaviors could be due to different strains used and the fact that BPA often has a bimodal effect. It could also be due to the prolonged exposure, extending to the lactation period, in the latter study.

In summary, these studies demonstrated zebrafish and C. elegans as valid models to study behavioral alterations in addition to rodents (Figure 1.3). Most rodent studies regarding behavioral effects of bisphenols focused on developmental exposure, as this is indeed a sensitive exposure window. However, based on the few studies examining adult exposures of BPS and/or BPA, developmental exposure is not the only window that can cause life-long changes in behaviors. For example, exposure to 100 µg/kg BPS in their drinking water for 10 days during adulthood in male mice resulted in an increased anxiety-index measures (Mornagui et al. 2019). Thus, additional studies need to be done in both sexes in adults for BPA analogues. Although more research regarding behavioral effects of BPA analogues still needs to be done, it is clear that exposure causes long-term changes in behaviors resulting from neurotoxicity regardless of exposure window or species and is therefore likely to cause behavioral deficits in humans.

Immunotoxic effects of BPA and BPS

Type 1 diabetes and immunomodulation in mice

The incidence of type 1 diabetes, an autoimmune disease involving pancreatic β-cell destruction, has increased in adults and children (Chiang et al. 2014). Although there is a genetic predisposition towards type 1 diabetes development, current research suggests that environmental factors can accelerate it (Bodin et al. 2014). It has been reported that BPS might exacerbate the onset of type 1 diabetes (Supplementary Table 1.9) in non-obese diabetic (NOD) mice depending on the sex and diet (Xu, Huang, Guo 2019; Xu, Huang, Nagy, Guo 2019). In NOD males fed a soy-based diet and exposed to BPS at 300 µg/kg, there were significant increases in blood glucose levels during the insulin tolerance test following exposure for one month (Xu, Huang, Guo 2019). Together with an increase in non-fasting blood glucose on days 6 and 13, this finding suggests an increased insulin resistance (Xu, Huang, Guo 2019). However, male mice exposed to a phytoestrogen-free diet and BPS (300 µg/kg) exhibited decreased blood glucose levels at the 15-minute timepoint during the two-month glucose tolerance test and at the 60-minute timepoint during the two-month insulin tolerance test (Xu, Huang, Guo 2019). Additionally, female mice exposed to 30 µg/kg BPS on a soy-based diet exhibited a delayed onset of diabetes, suggesting a potential interaction with phytoestrogens (Xu, Huang, Guo 2019). Because phytoestrogens in soy-based diets attenuate the effects of BPA (Wang J et al. 2014; Bernardo et al. 2015), the same could be true for BPS. Although these results support the notion that phytoestrogens mitigate the effects of BPS similarly to that of BPA, this needs to be further studied in female mice for confirmation. In comparison, exposure to BPA in adulthood accelerated the onset of type 1 diabetes in NOD females, but not in males (Xu, Huang, Nagy, Teng, et al. 2019). Likewise, exposure to BPA in the drinking water (0.1-10 mg/L) of NOD dams from mating to lactation accelerated the development of type 1 diabetes in female offspring (Bodin et al. 2014). These findings were confirmed by

exposing four-week-old NOD female mice to BPA-containing water (1 mg/L) throughout their lifetime (Bodin et al. 2015).

Type 1 diabetes development is associated with changes in the immune system (Malaisé et al. 2020). Pregnant C3H/HeN mice exposed to 5 or 50 µg/kg bw of BPA, BPS, or BPF daily from gestation day 15 to weaning exhibited altered immune profiles. Only exposure to the high dose of BPA (50 µg/kg bw) decreased IgA levels in the feces of the adult offspring. However, both doses of BPS caused an increase in the fecal level of lipocalin, an inflammatory marker (Malaisé et al. 2020), suggesting that the effect of BPS on intestinal immune response may involve a different mechanism from that of BPA. Both BPA (5 µg/kg BW) and BPS (50 µg/kg bw) increased the anti-*E.coli* IgG (Malaisé et al. 2020). Together with the increase in lipocalin levels, these observations support the theory that BPS impaired the intestinal immune barrier. Also, BPA and BPF at the high dose induced increases of T-helper type 1 (Th1) and Th17 cells, which were associated with increased levels of interleukin (IL)-17 and interferon γ (Malaisé et al. 2020). Th17 cells play a critical role in developing chronic inflammation and autoimmunity in mice by secreting IL-17, thus promoting inflammation (Stromnes et al. 2008; Bianchi and Rogge 2019). Because unrestricted IL-17 signaling is associated with autoimmune diseases and cancer progression, both BPA and BPF exposure may lead to carcinogenesis and autoimmune diseases, such as type 1 diabetes development (Hurtado et al. 2018).

Macrophage modulation in vitro

To examine the immunotoxic effects of BPA, BPS and BPAF, Chen et al. (2018) exposed human macrophages differentiated from the cell line U937 to 0.1-100 µM of each bisphenol for 48hr and measured the levels of 30 cytokines and chemokines (Supplementary Table 1.10). While

both BPA and BPAF at a low concentration (0.1 μM) activated the expression of cytokines and chemokines, this was not true for BPS. However, after a principal component analysis, BPS overlapped BPA, but BPAF was separated entirely. At 1.0 μM, BPA decreased IL-3 expression whereas BPS increased this cytokine. Exposure to BPS, BPA, or BPAF at 10 μM majorly impacted the expression of IL-10, vascular endothelial growth factor, macrophage inflammatory protein 1β, IL-8, IL-1RA, and interferon γ (Chen et al. 2018). All three bisphenols were able to up-regulate macrophage inflammatory 1-β and down-regulate IL-10. IL-1RA and IL-1β both increased following exposure to BPAF and BPS, but not BPA (Chen et al. 2018). Interestingly, BPS, but not BPA, decreased vascular endothelial growth factor at 100 μM. Collectively, these results suggest that all three bisphenols induce a pro-inflammatory response. Although BPS exhibited the most significant immunomodulatory effect (e.g., inhibiting IL-10 at 1, 10, and 100 μM), the authors suggested that BPAF is the most toxic because it is cytotoxic, followed by BPA and lastly BPS. Another study using a mouse macrophage cell line saw similar immunomodulatory effects (Zhao et al. 2017). A third study using fish macrophages found exposure to BPS at 100 μg/L increased the expression of IL-10, IL-1β, IL-6, interferon γ, and IL-12 (Qiu, Yang et al. 2018).

Another study compared zebrafish embryos exposed to 0.1, 1, 10, 100, or 1000 μg/L BPS, or BPF or 100 μg/L BPA from four to 120hr post fertilization (Qiu, Shao et al. 2018). At the highest concentration, both BPF and BPS significantly increased the expression of all cytokines and chemokines examined. Compared to BPA, both BPS and BPF increased the expression of IL-6, IL-12α, and interferon γ at or below the same concentration, suggesting similar toxicity. Through the use of an ERα antagonist, Qiu, Shao et al. (2018) confirmed that all three bisphenols use ER-related pathways to exert their immunotoxic effects.

In summary, both *in vivo* and *in vitro* studies have demonstrated that both BPS, BPA, and BPF cause toxicity to the immune system. All three bisphenols altered the expression of cytokines and chemokines. In the NOD mouse model, both BPA and BPS increased the blood glucose level. Given the similar mechanisms of the bisphenols, it is likely that BPF would also increase the risk of type 1 diabetes incidence, but further research is necessary.

Mechanisms of toxicity for BPA and BPS

Epigenetic changes

One of the prominent ways for bisphenols to cause toxicity is through epigenetic changes. When examining changes in RNA profiles in human primary pre-adipocytes, BPA, BPS, and BPF all altered the expression of both coding and non-coding RNAs (Verbanck et al. 2017). The authors compared the differentially expressed RNAs after BPA, BPS, and BPF treatment, and revealed a higher degree of similarity in alterations induced between BPS and BPF but not BPA. Next, the authors hypothesized that BPF requires a higher concentration for it to elicit the same response as BPS (Verbanck et al. 2017). This suggests that BPS is more potent than BPF in terms of altering RNA expression. In addition, bisphenol exposure affected the RNAs for the extracellular matrix, cytoskeletal genes, transcription regulators, and cyclins (Verbanck et al. 2017). This study suggests that, like BPA, BPS and BPF can interfere with of cell metabolism regulation, resulting in metabolic toxicity (Verbanck et al. 2017). More importantly, all three bisphenols altered small nuclear RNAs with C/D motif (Verbanck et al. 2017). These motifs are noncoding RNAs that regulate ribosomal RNA function and assembly, supporting the notion the bisphenols act through epigenetic modifications (Khan and Ahmed 2015; Verbanck et al. 2017).

DNA methylation is another common type of epigenetic modification through which chemicals exert toxic effects. To study methylome-wide DNA alterations caused by exposure to

BPF, BPS and BPA at functional concentrations, investigators utilized breast cancer cell lines MCF-7 and MDA-MB-231 cell lines (Awada et al. 2019). The investigators defined functional concentrations as 10^{-8} M for BPA and BPF, and 10^{-9} M for BPS. All three bisphenols induced ER-dependent increases in cell proliferation, metabolic activity, migration, and S-G2/M cycling proportions. Additionally, BPF and BPS altered the expression of epithelial to mesenchymal transition markers, and BPA increased metastasis. These exposures also induced a trend towards *LINE-1* hypomethylation (Awada et al. 2019), an epigenetic modification associated with cancer development (Barchitta et al. 2014). Investigators also found a genome-wide DNA methylation signature that involved several CpG sites and CpG regions located in promoters and exons (Awada et al. 2019). Of these three bisphenols, BPA had the most potent effect on DNA methylome, followed by BPS and then BPF (Awada et al. 2019). These effects were also ER-dependent. After looking at the gene expression of *DNMT1* and *TET,* researchers concluded changes in signaling pathways without directly affecting enzymatic activities of DNMTs or TETs induced DNA methylation aberrations (Awada et al. 2019). While approximately half of the pathways dysregulated by BPA were ER-dependent, all pathways dysregulated by BPS were ER-dependent (Awada et al. 2019). Interestingly, specific genes were methylated differently by BPS and BPA, both involved in pathways of focal adhesion, cGMPK-PKG, and cancer, e.g., the Wnt signaling pathway (Zhan et al. 2017).

Furthermore, bisphenols enact toxicity through histone acetylation and phosphorylation. Male mice exposed to BPS (0.001-100 µg/kg bw) for eight weeks had impaired spermatozoa development (Řimnáčová et al. 2020). Specifically, mice in the low treatment group (0.001 µg/kg bw) exhibited decreased motile spermatozoa. In comparison, those in the high dose group (100 µg/kg bw) had germ layer cell vacuolization and exhibited an enlarged multinuclear germ cell

phenotype (Řimnáčová et al. 2020). While the high dose group exhibited an increase in DNA damage, the lower dose BPS group exhibited increased acetylation of house-keeping proteins and enzymes, e.g., ATP synthase subunit, hexokinase-1, DNA repair protein (Řimnáčová et al. 2020). This finding suggests that BPS has different system-wide effects at different doses.

Cell signaling pathways

The same study detailed above also determined the effects of BPA, BPS, and BPF on telomerase activity, its expression, and relative telomere length (Awada et al. 2020). At their respective functional concentrations, all three bisphenols increased the activity and expression of telomerase after 24 hours (Awada et al. 2020). Similarly, MDA-MB231, a negative ER cell line, and an ER inhibitor confirmed that the bisphenols' effects were ER-dependent (Awada et al. 2020). Interestingly, only BPS caused a significant increase in relative telomere length. Telomerase activity changes are important as studies suggest a role of telomerase in breast cancer development and metastasis (Collado 2006). In addition, since all the endpoints tested in this study occurred before 48 hours following exposure, the changes in telomerase activity and its expression were not a result of the proliferative effects of these bisphenols (Awada et al. 2019; Awada et al. 2020). Therefore, authors hypothesized that the enzyme telomerase mediated the effects of BPA, BPS, and BPF in ER-positive breast carcinoma, and that telomerase-linked pathways were responsible for the ER-dependent phenotypic changes (Awada et al. 2019; Awada et al. 2020).

To characterize the nongenomic activities of BPS, alterations in the ERK and JNK signaling pathways in rat pituitary cells were examined following exposure to 0.000001-100 nM BPS (Viñas and Watson 2013). BPS had the same capability as E_2 and BPA in initiating phosphorylation of ERK, which occurred in a nonmonotonic concentration-response manner (Viñas and Watson 2013). When it came to JNK, BPS could not activate JNK, but only inhibited

this kinase at 0.01 μM, the highest concentration tested (Viñas and Watson 2013). This simultaneous activation of ERK and inactivation of JNK by BPS could stimulate cell proliferation and decrease cell death, thus magnifying the increase in cell number (Junttila et al. 2008). When combined with E_2, BPS activated JNK at a rate above E_2 alone in a nonmonotonic manner (Viñas and Watson 2013). These authors also used multiple inhibitors and determined that membrane-bound ERα was the predominant receptor used by BPS in regards to JNK and ERK signaling (Viñas and Watson 2013). To confirm the changes in cell signaling pathways, the investigators further examined the activities of caspases 8 and 9 (Viñas and Watson 2013). BPS activated caspase 8 at all concentrations and time points examined, suggesting activation of the extrinsic apoptotic pathway (Viñas and Watson 2013). On the other hand, BPS activated caspase-9 only at the 24-hour time point (Viñas and Watson 2013). Although it was somewhat surprising to see that BPS induced these signaling events at such low (femtomolar to picomolar) concentrations, these studies did suggest that, like BPA, BPS could alter and disrupt E_2 signaling, leading to toxicity.

Use of various receptors

The ability of BPA, BPS, BPF, tetrachloroBPA (TCBPA), and tetrabromoBPA (TBBPA) to interact with various receptors is heavily dependent on their structures. One study using two different cell lines confirmed that BPA, BPF, and BPS all acted as partial agonists for human ERα (hERα) and full agonists for hERβ (Molina-Molina et al. 2013). BPA was the most potent, followed by BPF and, finally BPS for both receptors. Unlike BPA and BPF, BPS induced a higher degree of agonism in the hERβ assay than in the hERα assay. Thus, it is clear that all three bisphenols act as estrogen agonists and exhibit some estrogenic activity. The ability of BPA, BPS, and BPF, but not TCBPA or TBBPA, to act on the two hERs suggests that the distance between para hydroxyl groups and nature substituent of the bridging carbon determine estrogenicity (Perez

et al. 1998; Molina-Molina et al. 2013). Bisphenols with two atoms in the meta position of one aromatic ring, such as TCBPA and TBBPA, demonstrated decreased estrogenic potency (Rivas et al. 2001; Riu et al. 2011; Molina-Molina et al. 2013). Also, ligand polarity modifies the affinity of a compound to ER isoforms (Hillisch et al. 2004). Specifically, substitutions below the D-ring in an estrogen molecule induce an affinity for ERα agonists, whereas substitutions above the B and C rings cause an affinity towards ERβ agonists (Hillisch et al. 2004; Molina-Molina et al. 2013). The higher polarity of BPS may account for its lower estrogenic potency (Molina-Molina et al. 2013).

In terms of androgenic activity, both BPA and BPF acted as full androgen receptor antagonists, while BPA had a stronger affinity (Molina-Molina et al. 2013). BPA and BPS were weak androgen receptor agonists as BPA was more potent, suggesting that these bisphenols are weakly androgenic (Molina-Molina et al. 2013). Although this sounds contradictory, the agonist/antagonist activity of BPA is consistent with reports of other EDCs (Kemppainen and Wilson 1996; Wilson et al. 2002). Only BPA, TCBPA, and TBBPA activated hPXR, a receptor involved in transcriptional control of xenobiotic detoxification (Lamba et al. 2004; Molina-Molina et al. 2013). As EDCs, it is believed that these bisphenols may induce their effects (epigenetic changes, cell signaling, oxidative stress, etc.) through binding to these various receptors.

Oxidative stress and apoptosis

When examining germline apoptosis and the expression of associated genes following BPA exposure in *C. elegans*, results indicated increased germ cell death, as well as induction of three distinct pathways involved in BPA-induced apoptosis in a concentration-dependent manner, with significance starting at 1 μM (Wang Y et al. 2017). These three pathways are the DNA damage response signaling pathway, the ERK or JNK/MAPK pathway, and the IGF-1 signaling pathway

(Wang Y et al. 2017). Although there was an increase in expression of all apoptotic genes examined, the authors concluded that both the *hus-1* and *cep-1* genes were needed for BPA to induce apoptosis using various mutants (Wang Y et al. 2017).

BPS exposure in *C. elegans* during the L4 stage for 24hr caused a significant decrease in lifespan starting at 0.01 µM, possibly due to increased apoptosis and oxidative damage (Xiao et al. 2019). Additionally, *C. elegans* exposed to BPS at concentrations as low as 0.01 µM exhibited a significant increase in reactive oxygen species and a decrease in superoxide dismutase, suggesting increased oxidative damage (Xiao et al. 2019). Qiu, Yang et al. (2018) also showed that BPS exposure induced severe oxidative stress, impaired cellular homeostasis, and apoptosis. Therefore, BPS likely causes an increase in apoptosis by increasing oxidative stress, leading to a decreased lifespan.

Conclusion

Despite the increasing popularity of BPS-containing products that have been marketed as "BPA-free", insinuating that they are safer alternatives, this may not be true. The toxic effects of BPA are well-known, further studies should compare its toxicity with its analogues. In particular, few studies investigate the potential adverse effects of BPS, especially compared to BPA. In addition, there are significant gaps in our knowledge of both bisphenols when it comes to behavioral effects in exposed adults, which may be due to the assumption that EDCs are more detrimental in developmental exposure windows. However, this may not always be the case. Studies with BPA in the exacerbation of type 1 diabetes have identified unique windows of sex-dimorphic susceptibility of type 1 diabetes in NOD mice: adult exposure to BPA accelerated the onset of type 1 diabetes in females, but not in males (Xu, Huang, Nagy, Teng, et al. 2019), while

no exacerbation of type 1 diabetes for either sex for perinatal BPA exposure occurred (Xu, Huang, Nagy, Guo 2019). Future studies should address these discrepancies.

The toxicity potency of each bisphenol depends on the systems and endpoints examined. For example, using *C. elegans*, the LC_{50} of BPS was determined to be 545.59 mg/L, while the LC_{50} of BPA was 327.24 mg/L (Ura et al. 2002; Xiao et al. 2019). After taking into consideration body length, brood size, lifespan, locomotion, total superoxide dismutase, and intestinal reactive oxygen species, *C. elegans* were, however, found to be more sensitive to BPS than BPA (Xiao et al. 2019). Similar to BPA, BPS can exhibit a bimodal concentration-response curve (LaPlante et al. 2017; Eladak et al. 2015; Naderi et al. 2014). In terms of the relevance of these animal models to human health, it is still a debated issue. For example, sheep are one litter species, so they could potentially be the best model for gestational studies. Overall, the fact that bisphenols cause toxic effects in a variety of species makes it likely that there is toxicity in humans. In addition, the species *C. elegans* can be used for ecotoxicological studies and as an indicator of soil health (EFSA 2017; Queirós et al. 2019). Nonetheless, both bisphenols have adverse outcomes and should not be considered safe without further testing. Although there are still several unknown underlying mechanisms through which bisphenols exert their toxic effect, it is apparent that some of the common mechanisms used are epigenetic changes, cell signaling, and apoptosis. Furthermore, these bisphenols seem in some cases to use different mechanisms and receptor specificities, resulting in differences in potencies and even effects. The research in the following chapters aim to address the knowledge gap surrounding BPS's effects on reproduction, behavior, and the immune system and provide meaningful insights about the toxicity of this EDC.

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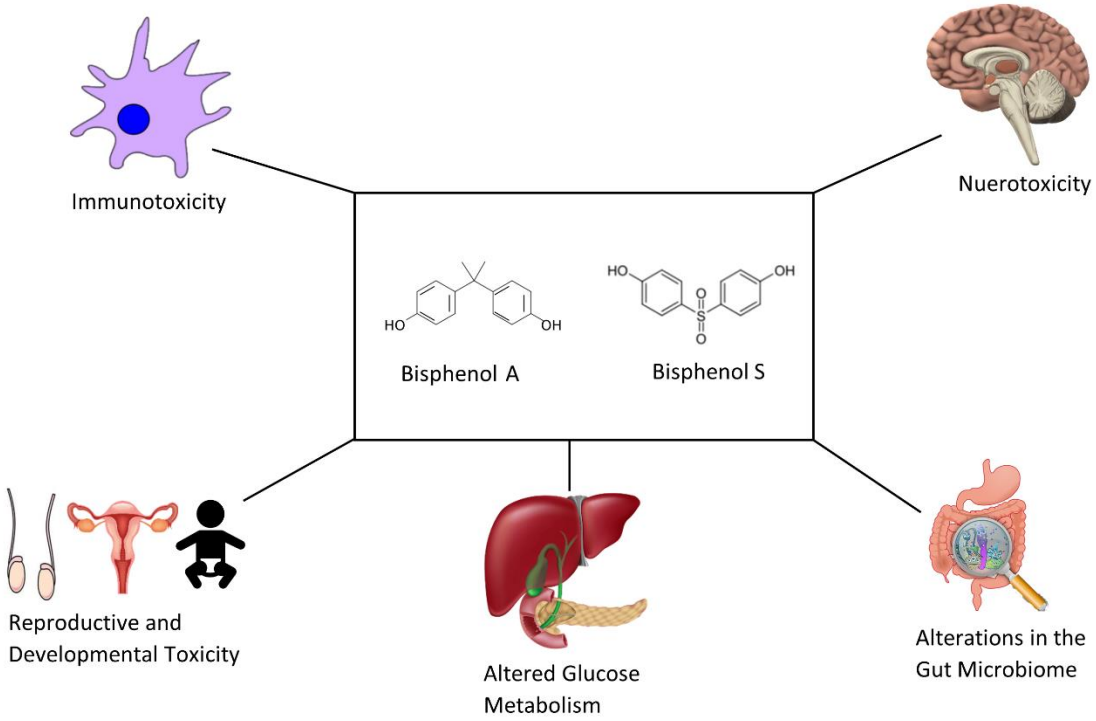

Figure 1.1 Reviewed sensitive organ systems that are potentially affected by Bisphenol A and S.

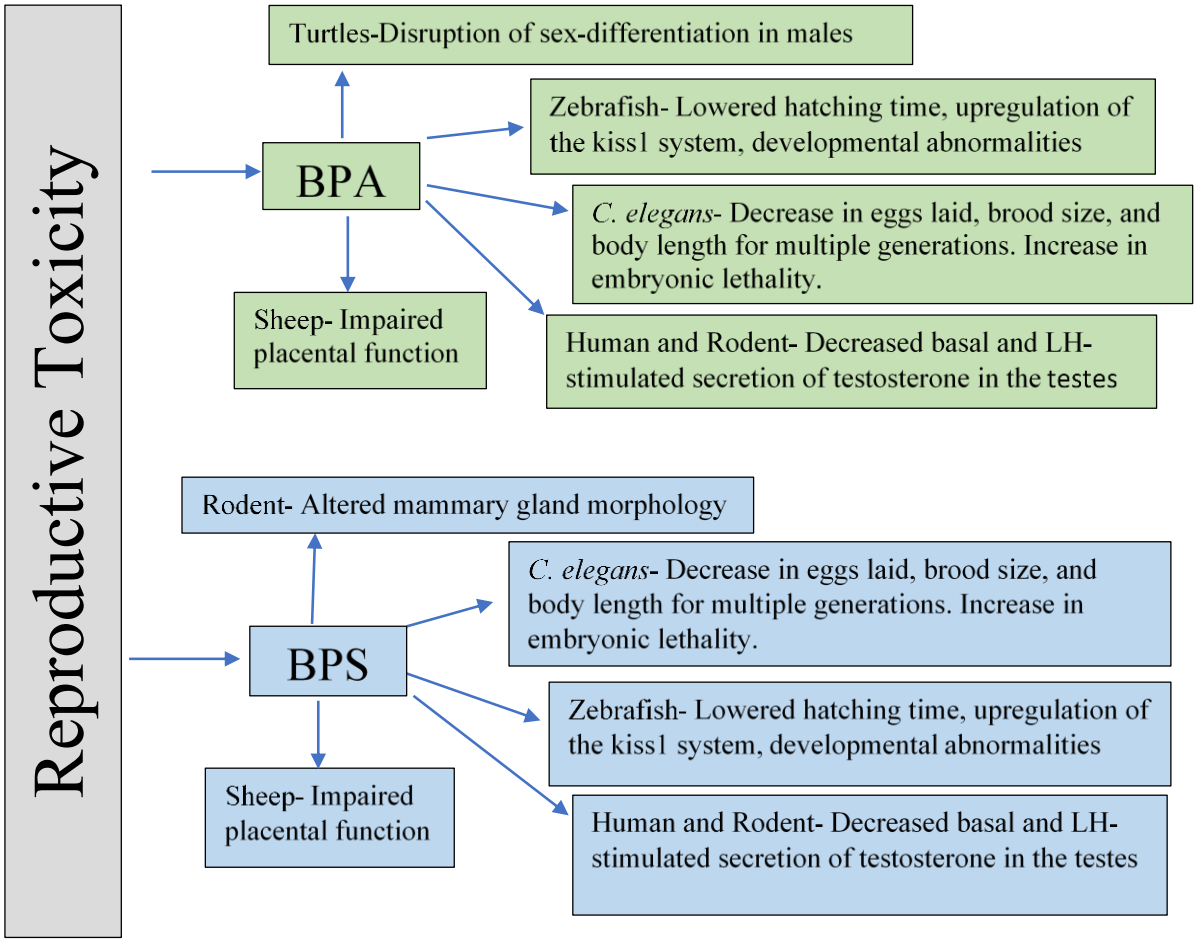

Figure 1.2 Summary of reproductive and developmental toxicity studies of BPA and BPS in different species.

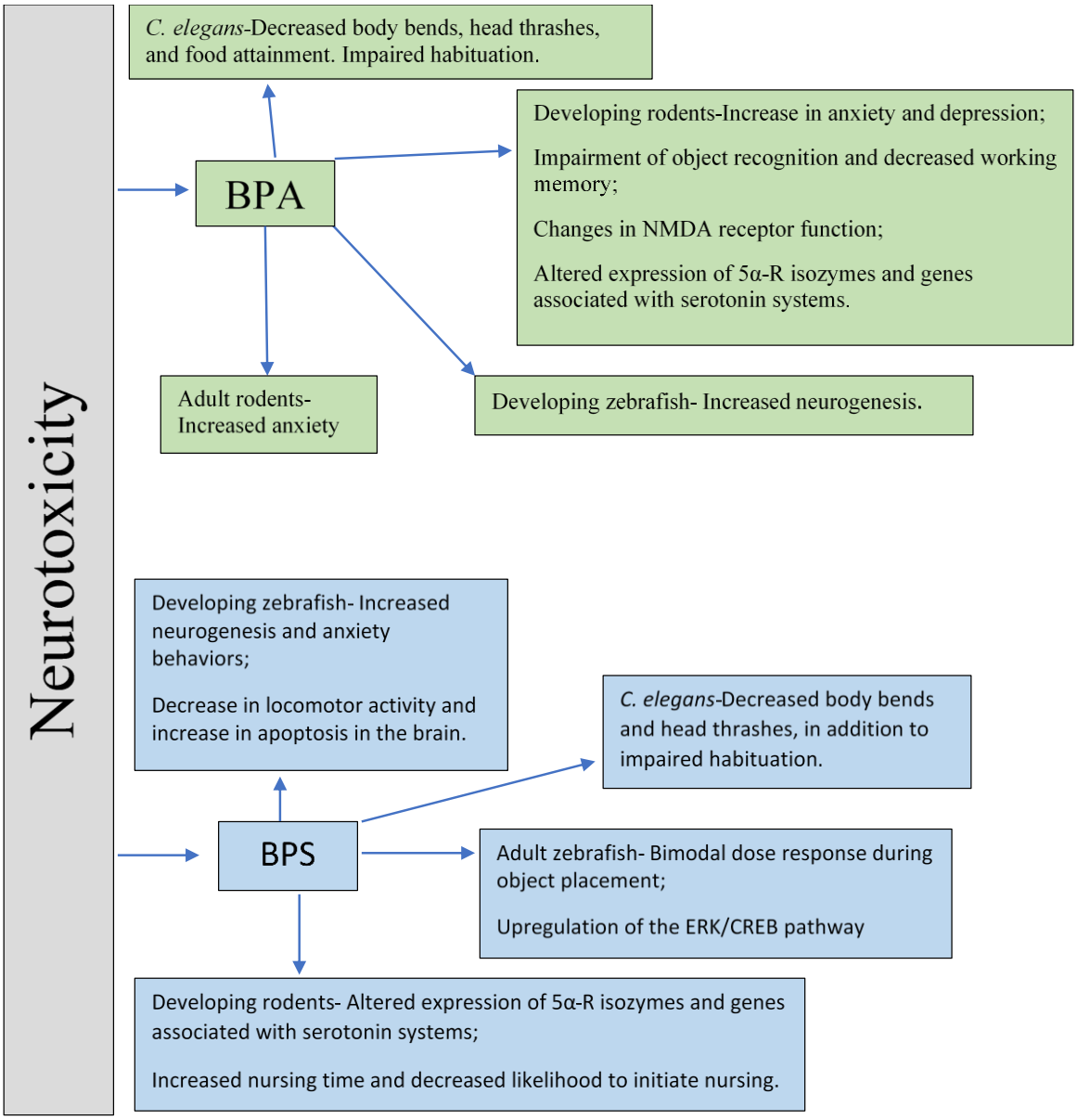

Figure 1.3 Summary of neurotoxicity studies of BPA and BPS in different species.

Supplementary Table 1.1 Summary of reproductive studies on bisphenols in *C. elegans*.

Bisphenols examined	Strains used	Exposure Windows	Concentrations	Exposure method	Reproductive Effects	Study
BPA and BPS	N2	Embryos for 4 hours	0.1-10 µM	Not specified	Decreased egg laying starting at 1.0 µM BPA and 0.5 µM BPS	(Mersha et al. 2015)
BPS	N2	L4 larvae for 24 hours; Continuous for generational studies	0.25, 0.5, 1, 1.5 and 2 mM for lethality; 0.001, 0.01, 0.1,1, 10 and 100 µM for all other tests	Worms in M buffer	Decreased body length and brood size starting at 0.01 µM for all four generations.	(Xiao et. al 2019)
BPA	N2	Eggs to adulthood (96 hours); Same for generational studies	0.001 to 10 µM	Worms in liquid K buffer containing BPA	Decreased brood size and body length in all generations at 1.0 and 10 µM. Decreased brood size starting at G2 for 0.1 µM and 0.01 µM.	(Zhou, Yang, et. al 2016a)
BPS	N2 and TJ375	L1 larvae for 72 hours	0.001, 0.01, 0.1,1.0 and 10.0 µM	Mixed into K buffer	Decreased growth and brood size at 10 µM.	(Zhou 2018)
BPA, BPS, and BPA+BPS	N2, LGI, cep-1, hus-1, LGIII, clk-1, LGIV spo-11, LGV, chk-1	Eggs to adulthood (4 days)	125-500 µM	BPA, BPS, or BPA+BPS added to plates	Decreased number of surviving offspring and increased embryonic lethality at 500 µM BPS, BPA, and BPA+BPS. Increased embryonic lethality at all concentrations of BPS. Reduction in germline size at 500 µM BPA.	(Chen Y. Et al 2016)
BPA	N2	Acute (L4 worms for 24 hours) or prolonged (L1 larvae for 3 days)	0.001, 0.01, 0.1, 1.0 and 10.0 µM	Mixed into K buffer	Prolonged exposure decreased body length starting at 0.01 µM. Decreased brood size at 1 and 10.0 µM for both chronic and acute exposure.	(Zhou, Yang et al. 2016b)
BPA	N2 and TJ375	L4 larvae to day-10 adults (~10 days)	0.0001, 0.001, 0.01, 0.1, 1.0 and 10.0 µM	Mixed into K buffer	Decrease in growth starting at 0.01 µM.	(Zhou et. al 2016)
BPA	N2, *sod-1*, and *old-1*	L1 larvae for 60-65 hours or lifetime for reproduction	0.1 and 1.0 µM	Added to plates	Decreased in body length, body width and worm area in a concentration-dependent manner; Decreased egg production at 1.0 µM. Increase in body length of *old-1* mutants exposed to 0.1 µM. Decreased length for both mutants exposed to 1.0 µM	(Flood 2014)

Supplementary Table 1.2 Summary of reproductive studies on bisphenols in zebrafish

Bisphenols examined	Strains used	Exposure Windows	Concentrations	Exposure method	Reproductive Effects	Study
BPA and BPS	Brass GnRH3-EMD transgenic zebrafish	2 hour post fertilization; Embryos for 25 or 120 hours post fertilization	0.1, 1, 10, 100, or 1,000 µg/L BPA or 100 µg/L BPS	Exposed on E3 medium in petri dishes	BPA at 1 and 10 µg/L induced a higher hatching rate at 48 and 55 hours post fertilization. Increased nuerogensis and upregulation of the kiss1 system at 10 and 100 µg/L BPA and 100 µg/L BPS. BPA and BPS at 100 µg/L increased the levels of ERα Treatment of embryos with inhibitors inhibited the stimulatory effects of BPA and BPS on gene expression.	(Qui et al. 2016)
BPA, BPAF, BPF, and BPS	Wild type, WIK, and transgenic zebrafish	Eggs 1 hour post fertilization until 120 hours post fertilization	0.5-200 mg/L BPA, BPAF, BPF, or BPS	In water	BPAF had the lowest LD50 followed by BPA, then BPF, then BPS. All bisphenols delayed hatching at 72 hpf. At or above 5.0 mg/L, BPA exposure caused cardiac edema and craniofacial abnormalities. BPF at and above 10 mg/L caused cardiac edema and craniofacial abnormality, and caused spinal malformation, cranial hemorrhage and yok sac deformity at and above 20 mg/L. BPAF caused cardiac edema at 1.0 mg/L. BPS only caused deformities at 200 mg/L. The use of transgenic fish showed that all bisphenols target the same tissue, with the heart being the most responsive.	(Morem an et al. 2017)

Supplementary Table 1.3. Summary of reproductive studies on bisphenols in turtles

Bisphenol examined	Exposure Windows	Concentrations	Exposure method	Reproductive Effects	Study
BPA	Eggs incubated at male-determining temperatures during the sensitive period	0.01 or 100 µg/mL BPA, or 0.2 µg/mL ethinyl estradiol	Pipetted onto egg	Feminization of the brain as seen with changes in spatial navigation.	(Manshack et al. 2016)
BPA	Eggs incubated at male-determining temperatures during the sensitive period (stage 17)	0.01, 1.0, or 100 µg/g egg	Pipetted onto egg	Eggs exposed to BPA exhibited disrupted sex development with no full sex-reversal. The BPA treated group also had males with an ovarian-like cortex and various levels of disorganization of the testicular tubules.	(Jandegian et al. 2015)

Supplementary Table 1.4 Summary of reproductive studies on bisphenols in sheep

Bisphenols examined	Strain used	Exposure Windows	Dose	Exposure method	Reproductive Effects	Study
BPA and BPS	Polypay x Dorset cross-bred sheep	Day 30 to day 100 of gestation	0.05 mg/kg bw	Subcutaneous injection	BPS exposed dams had: (1) decreased maternal serum concentrations of PAG1 and PSPB, (2) decreased progesterone during late mid-gestation, and (3) decreased binucleated cells and expression of e-cadherin.	(Gringrich et al. 2018)
BPS and BPA/BPS/BPF mixture	Polypay x Dorset cross-bred sheep	A single dose at mid-pregnancy (Gestational period = 121.8 ± 0.8 days). Samples collected 0-72 hours after exposure	0.5 mg/kg bw	One-time subcutaneous injection	BPS reached maternal circulation one order of magnitude higher than the others and had the shortest half-life. BPF had a long half-life in maternal circulation but cleared from fetal circulation faster than BPS and BPA. BPS had the longest half-life in the fetal compartment.	(Gringrich et al. 2019)

Supplementary Table 1.5 Summary of reproductive studies on bisphenols in humans and rodents

Bisphenols examined	Strains used	Exposure Windows	Dose/ Concentration	Exposure method	Reproductive Effects	Study
BPS	Adult pregnant CD-1 mice	Gestation day 9 to lactation day 1 or 20	2 or 200 µg/kg	BPS in a wafer	Reduced volume fraction of lobules in the mammary gland and increased volume fractin of adipose tissue at 200 µg/kg. Increased prolactin and Esr1 expression at 2 µg/kg. Decreased Esr1 and prolactin expression at 200 µg/kg.	(LaPlante et al. 2017)
BPA, BPS, and BPF	Mouse, rat (BPA only) and human testes in a fetal testis assay without LH	Mouse = 12.5 days post coception, Rat = 14.5 days post-conception, Human = 6.3-11.1 gestational weeks for 3 days	0.001-10,000 nM	In medium	Decreased basal testosterone secretion in human cells at and above 10 nM BPA. Mouse cells were more sensitive to BPS and BPF than BPA. BPS at and above 100 nM caused a concentration-dependent decrease in basal testosterone secretion in mouse testes.	(Eladak et al. 2015)

Supplementary Table 1.6 Summary of neurological studies on bisphenols in *C. elegans*

Bisphenols examined	Strain used	Exposure Windows	Concentrations	Exposure method	Neurological Effects	Study
BPS	N2 and TJ375	L1 larvae for 72 hours	0.001, 0.01, 0.1, 1.0 and 10.0 µM	Mixed into K buffer	Decreased head thrashes above 1.0 µM and body bends at 10 µM only.	(Zhou 2018)
BPS	N2	L4 larvae for 24 hours. Continuously for generational studies	0.25, 0.5, 1, 1.5 and 2 mM for lethality; 0.001, 0.01, 0.1, 1, 10 and 100 µM for all other tests	Worms in M buffer	Concentrations above 0.01 µM decreased the number of head thrashes for all 4 generations. Concentrations at and above 1 µM decreased the number of body bends for 3 generations.	(Xiao X et al. 2019)
BPA	N2	Eggs to adulthood (96 hours). Same for generational	0.001 to 10 µM	Worms in liquid K solution containing BPA	Decreased locomotion for all generations at concentrations of 0.1 µM and above.	(Zhou, Yang, et al. 2016a)
BPA	N2	Acute (L4 worms for 24 hours) or prolonged (L1 larvae for 3 days)	0.001, 0.01, 0.1,1.0 and 10.0 µM	Mixed into K buffer	Decreased number of head thrashes at concentrations of 0.01 µM and above. Decreased body bends at 1.0 and 10.0 µM.	(Zhou, Yang, et al. 2016b)
BPA	N2 and TJ375	L4 larvae to day-10 adults (~10 days)	0.0001, 0.001, 0.01, 0.1,1.0 and 10.0 µM	Mixed into K buffer solution	Decrease head thrashes starting at 0.01 µM, decrease body bends starting at 0.1 µM.	(Zhou, Yang, Li, Cui et al. 2016)
BPA and BPS	N2	Embryos for 4 hours	0.1-10 µM	Not specified	Increased stimuli needed for habituation at all levels of BPA and BPS	(Mersha et al. 2015)
BPA	N2, *sod-1* and *old-1*	L1 larvae for 60-65 hours or lifetime for reproduction	0.1 and 1.0 µM	Added to plates	Decreased number of average reversals in a dose-dependent manner. Decreased speed at 1.0 µM and increased speed at 0.1 µM. Decreased food attainment at 4 and 6 hours at 0.1 µM. Decreased attainment at 1.0 µM at 2,4,6,8, and 24 hours.	(Flood et al. 2014)
BPA	N2	2-day old worms exposed for 46 hours	0.1 and 10 µM	Added to plates	Decreased food attainment at all time points for 10 µM. Decreased food attainment at 2, 4, 6, and 8-hour time point for 0.1 µM	(Kohra et al. 2002)

Supplementary Table 1.7 Summary of neurological studies on bisphenols in zebrafish

Bisphenols examined	Strain used	Exposure Windows	Concentrations	Exposure method	Neurological Effects	Study
BPS	9 month old female wild-type (AB) zebrafish	Adults for 120 days	1, 10, or 30 µg/L	In water, replaced daily	Slight improvement of object placement memory in the 1 µg /L group. Impairment of long-term recognition memory in the 10 and 30 µg/L groups. The 1 µg/L exposure increased ERK phosphorylation and genes associated with the ERK/CREB pathway, while 30 µg/L decreased ERK and CREB phosphorylation.	(Naderi et al. 2020)
BPA and BPS	Wild-type Zebrafish	Just before 10-16 hours post fertilization, at 10-16 hours post fertilization, and 16-24 hours post fertilization.	0.0068, 0.1 or 1.0 µM	Embryos were immersed in solution	Increased neurogenesis in the hypothalamus and appearance of hyperactive behaviors in BPA and BPS exposed zebrafish.	(Kinch et al. 2015)
BPS	AB zebrafish	2-6 days post fertilization	For LC_{50} 150-400 mg/L, for other tests 0.3-3.0 mg/L	Embryos were immersed in solution	Decreased locomotor activity and speed. Increase in oxidative stress leading to apoptosis. The apoptosis was system-wide, but highest in the brain-region. Neurodevelopment genes were suppressed.	(Gu et al. 2019)

Supplementary Table 1.8 Summary of neurological studies on bisphenols in rodents

Bisphenol examined	Strain used	Exposure Windows	Dose	Exposure method	Neurological Effects	Study
BPA, BPS, and BPF	Wistar rats	Gestation day 12 to parturition and then again 1-21 days post natal	10 µg/kg BPS, BPA, or BPF	Daily subcutaneous injection	A total of 25 genes were significantly regulated by BPA, 56 genes by BPF, and 24 genes by BPS.	(Castro et al. 2015)
BPS	Adult pregnant CD-1 mice	Gestation day 9 to lactation day 1 or 20	2 or 200 µg/kg	BPS in a wafer	Decreased ability to initate nursing and increased time spent nursing. Decreased serum concentrations of 17β-estradiol than control. Decreased pup weight at post natal day 14.	(LaPlante et al. 2017)
BPF and BPA	C57BL/6 NCrSlc mice	Gestation days 11.5 to 18.5	10 mg/kg BPF or BPA	Oral gavage	Both BPF and BPA mice exhibited increased anxiety-like behaviors in both sexes. Increase in depression-like behaviors in BPF-treated female mice.	(Ohtani et al. 2017)
BPA	ICR mice	Prenatal day 7 to 21 then postnatal day 22 to 36	100 or 500 µg/kg	Oral administration	Increase in central locomotion, D2 receptor binding, and impairement of object recognition at 100 µg/kg. Decreased anxiety-like behaviors 500 µg/kg. Decrease DAT and NMDA binding, increased impulsiveness, and reduction in working memory.	(Tian et al. 2010)

Supplementary Table 1.9 Summary of immunotoxic studies on bisphenols in rodents

Bisphenol examined	Strain used	Exposure Windows	Dose	Exposure method	Effects	Study
BPA	NOD mice	Adults females for 198 days; Offspring of both sexes exposed during gestation and lactation	300 µg/kg	Oral pipette; BPA dissolved in corn oil	Protective effect in the female offspring, no effect on male offspring. Adult females had an increase in pro-inflammatory gut microbiota and an increased risk of type 1 diabetes.	(Xu, Huang, Nagy, et al. 2019)
BPS	NOD mice	Mice 8-15 weeks old for 90-150 days	0, 3, 30, 150, or 300 µg/kg	Oral pipette; BPS dissolved in corn oil	Females fed a soy-based diet were protected against diabetes with no alteration to immunity. Increased insulin resitance in males fed a soy-based diet.	(Xu, Huang, Guo 2019)
BPA	NOD mice	Mice 8-12 weeks old for a month	0, 30, or 300 µg/kg	Oral pipette; BPA dissolved in corn oil	Increased diabetes incidence and pro-inflammatory cytokines in adult females.	(Xu, Huang, Nagy, Teng et al. 2019)
BPA	NOD mice	Start of mating until weaning	0,0.1,1, or 10 mg/L	In drinking water	Increased diabetes incidence in female offspring at 1 and 10 mg/L. Increased number of regulatory T cells in pancreatic islets, increased number of apoptotic cells, and decreased number of tissure resident macrophages.	(Bodin et. al. 2014)
BPA, BPS, BPF	C3H/HeN mice Female offspring	Gestational day 15 to weaning	0,5, or 50 µg/kg	Oral administration dissolved in corn oil	Decreased IgA levels at 50 µg/kg BPA. All bisphenols decreased plasmatic IgG levels. Increased lipocalin levels at both concentrations of BPS. Increased Th1 and Th17 cells accosiated with increased IL-17 and interferon γ at 50 µg/kg BPA and BPF.	Malaisé et al. 2020)

Supplementary Table 1.10 Summary of immunotoxic studies on bisphenols in cells

Bisphenol examined	Cell type	Exposure Windows	Concentration	Effects	Study
BPA, BPAF, BPS	U937-derived macrophage	Differentiated macrophages for 48 hours	0.1-100 µM	BPA exhibited an inverted concentration-response curve. All three bisphenols down-regulated IL-10. IL-1RA was decreased by 1.0 µM BPA and BPS. IL-3 was oppositely modulated by BPA and BPS at low concentrations (0.1-1.0 µM).	(Chen et al. 2018)
BPA, BPF, BPS	Zebrafish embryos	4 hours post fertilization to 120 hours post fertilization	100 µg/L BPA, 0.1, 1, 10, 100, 1000 µg/L BPS or BPF	BPA, BPS and BPF increased all expression of all cytokienes and chemokines examined. BPS was able to increase expression of *il-6* and *il-12a* starting at 1 µg/L. Use of ERα antagonist confirmed use of ER-related pathways.	(Qui, Shao et al. 2018)

CHAPTER 2

BEHAVIORAL CHANGES AND HYPERGLYCEMIA IN NODEF MICE FOLLOWING BISPHENOL S EXPOSURE ARE AFFECTED BY DIETS[2]

[2]McDonough Callie M, Xu Joella, and Tai L. Guo "Behavioral changes and hyperglycemia in NODEF mice following bisphenol S exposure are affected by diets" submitted to *Neurotoxicology* March 30, 2021.

Abstract

Bisphenol S (BPS), an analogue of the controversial bisphenol A (BPA) that is found in epoxy-resins and plastics, is a potential endocrine-disrupting chemical that can mimic endogenous hormone signaling. However, little is known about the behavioral or immunologic effects of BPS. The purpose of this study was to examine the impact of diets in BPS-treated mice in relation to hyperglycemia, development of type 1 diabetes, immunomodulation, and behavioral changes. Adult male and female nonobese diabetic excluded flora (NODEF) mice were exposed to environmentally relevant doses of BPS (VH, 30, or 300 μg/kg BW) and fed either a soy-based diet, a phytoestrogen-free diet, or a Western diet. NODEF male mice fed a soy-based diet exhibited a decreased curiosity/desire to explore, and possibly increased anxiety-like behavior and decreased short-term memory when exposed to BPS (300 μg/kg BW). In addition, these mice had a significant increase in non-fasting blood glucose levels along with increased insulin sensitivity, impaired glucose tolerance, resistance to fasting and proinflammation. Although BPS had little effect on the glucose parameters in NODEF male mice fed a Western diet, there was a decrease in %$CD24^+CD5^+$ and %$B220^+CD40L^-$ cell populations, as well as an increase in distance traveled during the novel object test, suggesting hyperactivity. NODEF females fed a phytoestrogen-free diet exhibited a slight decrease in time spent immobile during the tail suspension test in both the 30 and 300 μg/kg BW dose groups along with an increase in %$CD4^+CD8^+$ and %$Mac3^+CD45R^+$ cell populations, signifying increased hyperactivity and anxiety-like behavior. In conclusion, BPS-exposed NODEF mice exhibited sex-dependent changes in hyperglycemia, behaviors, and immune endpoints, which were affected by diets.

Introduction

Endocrine-disrupting chemicals (EDCs) are a class of compounds that possess agonistic or antagonistic properties for various hormones in the body. A prime example of EDCs is a group of

chemicals known as bisphenols. Bisphenols are found in plastics and epoxy resins, and at least sixteen bisphenols have been commercially applied, with BPA being the most common (Chen D et al. 2016). Due to increased public concerns with bisphenol A (BPA), several manufacturers have begun marketing "BPA-free" products that contain the BPA analogue, bisphenol S (BPS). This has resulted in widespread exposures to BPS with continuously increased buildup in the environment (Usman and Ahmad 2016; Wu et al. 2018). In the body, both BPA and BPS may mimic endogenous hormone signaling to cause disruption of the endocrine system, leading to adverse biological effects (Siracusa et al. 2018). Because estrogen also plays an important role in insulin signaling, BPA has been shown to modulate the development of both type 1 (T1D) and type 2 diabetes (Xu et al. 2016) and reduce plasma insulin levels (Zhao et al. 2018). Additional animal studies also suggest that exposure to BPA may impair brain development, cognitive functions, and related behaviors, resulting in mental disorders, including autism (Tian et al. 2010; Castro et al. 2015; Kinch et al. 2015; Johnson et al. 2016; Thongkorn et al. 2021). Of particular concern, some studies have shown that BPS can disrupt immune responses, e.g., altering macrophage cytokine production (Zhao et al. 2017; Chen Y et al. 2018), as chronic inflammation has been the etiology of many diseases across the life span. However, the adverse effects of BPS on T1D, immunomodulation, and behavioral changes are poorly understood because of the paucity of studies.

Previous studies have shown that BPA and BPS could induce sex- and diet-specific toxicity in the non-obese diabetic (NOD) mice that spontaneously developed type 1 diabetes. Specifically, NOD male mice fed a soy-based diet and exposed to 300 μg/kg body weight (BW) BPS exhibited an increase in insulin resistance (Xu, Huang, Guo 2019). In contrast, NOD females on the same soy-based diet exposed to 30 μg/kg BW BPS showed a delayed onset of T1D, suggesting sex-

specific effects. Further study in NOD male mice fed a phytoestrogen-free diet and exposed to 300 µg/kg BW BPS demonstrated an improved glucose tolerance and decreased insulin resistance (Xu, Huang, Guo 2019), suggesting a potential interaction with phytoestrogens. Similar observations have been reported for BPA (Wang J et al. 2014; Bernardo et al. 2015). However, there are currently no studies examining the effects of BPS in females fed a phytoestrogen-free diet. The Western diet, which is characterized by a high intake of red meat, refined sugars and saturated fat, but little fiber, is increasingly consumed across the world (Statovci et al. 2017). It is a major driver of chronic, low-grade, metabolic inflammation that contributes to many health issues including obesity, metabolic syndrome and cardiovascular diseases (Statovci et al. 2017). The purpose of this study was to examine the impact of diets (e.g., the soy-based diet, phytoestrogen-free diet, and Western diet) in BPS-treated mice in relation to hyperglycemia, T1D development, immunomodulation, and behavioral changes.

NOD mice are no longer offered by Taconic due to their low incidence of T1D. Instead, they now offer nonobese diabetic excluded flora (NODEF) mice, which have an excluded flora that is protective against T1D. Due to the differences in these strains, e.g., NOD and NODEF, NODEF males fed a soy-based diet were included in this study to serve as a baseline and comparison to previous studies (Xu, Huang, Guo 2019). Moreover, the effects of different diets in BPS-treated mice have not been studied in terms of behavioral effects, and inclusion of a soy-based diet would also add new knowledge of behavioral differences. Doses of 30 and 300 µg/kg body weight (BW) BPS for the female mice and a 300 µg/kg BW dose for male mice were chosen to be consistent with previous studies (Xu, Huang, Guo 2019). Two different doses of BPS were used for female NODEF mice because females are more sensitive to estrogen mimics (Lauretta et al. 2018) and to examine the possibility of a bimodal dose-response. The 30 µg/kg BW dose is

within estimated human exposure levels, and the 300 μg/kg BW is also relevant to human exposure based on median human blood levels of BPA (Calafat et al. 2005; Taylor et al. 2011). It was hypothesized that BPS could have sex-specific and diet-specific effects on hyperglycemia, T1D development, immunomodulation, and behavioral changes in NODEF mice.

Materials and Methods

Animal husbandry and BPS exposure

NODEF mice were initially obtained from Taconic Biosciences (Germantown, NY). A breeding colony was established and housed in Coverdell animal facility at the University of Georgia (UGA) in polysulfone cages with irradiated laboratory animal bedding and Bed-r'Nest for enrichment (The Andersons Inc., Maumee, Ohio). Negligible amounts of BPA have been reported to leach from new or used polysulfone cages maintained at room temperature (Delclos et al. 2016; Johnson et al. 2016). They were kept at 22-25°C with a relative humidity of 50±20 and a 12hr light/dark cycle. Filtered water was provided *ad libitum* through the animal facility's automatic watering system. Food was provided *ad libitum*. All animals were treated humanely and with regard to alleviating animal suffering. An approved animal protocol by the UGA Institutional Animal Care and Use Committee (IACUC) was followed for all procedures.

BPS was obtained from Sigma (St. Louis, MO), and it was dissolved in 100% ethanol and added to corn oil at a final concentration of 0.05% ethanol. The vehicle (VH) mice received the same volume of corn oil with 0.05% ethanol. For each study, mice were randomly assigned to either the treatment or the control groups, and there were no significant differences in the initial BWs among groups. Mice were dosed daily via oral pipette based on the average BW of the mice in the prior week. Non-fasting blood glucose levels (BGLs) and BWs were measured and recorded

weekly. After mice were euthanized, organs, including GI tracts, livers, spleens, thymus, kidneys, heart and lungs, and pancreas, were dissected and weighed.

NODEF males fed a soy-based diet

Adult male mice at approximately 10 weeks in age were divided into a vehicle group or a treatment group and were dosed with either VH or 300 µg BPS/kg BW (6/group). The dose was chosen because similar amounts of BPA were previously shown to alter the immunity in mice and provided levels of unconjugated BPA in mice closer to what was found in humans (Yoshino et al. 2003; Taylor et al. 2011). These mice were fed a soy-based PicoLab diet (LabDiet, St. Louis, MO). This diet consisted of 24.7% protein, 62.1% carbohydrate, and 13.2% fat (Huang et al. 2017). BPA exposure was found to have sex dimorphic effects on T1D when NOD mice were maintained on this diet (Xu, Huang, Nagy, Teng, et al. 2019). BGLs and BWs were measured and recorded weekly. At the two, three, four, five, and six-month timepoints, both insulin tolerance tests (ITT) and glucose tolerance tests (GTT) were performed. Before euthanizing, the behavioral assays including Y-maze, tail suspension, open field and novel object tests were conducted. All males were euthanized at the end of the study, which was approximately eight months (e.g., 239 days) of treatment.

NODEF males fed a Western diet

A second study using adult male mice was similarly conducted. However, to mimic the high-fat and high-sucrose diet typical in western society, mice were given a Western diet high in fat and sucrose, obtained from Research Diets Inc. (D12079B, New Brunswick, NJ). This diet consisted of 17% protein, 40% fat, and 43% carbohydrate (Chen Y et al. 2020). Food intake was measured by weighing the amount of food given and the amount left each week. BGLs and BWs were measured and recorded weekly. At the two, three, and five-month timepoints, the ITT and

GTT were performed. Prior to euthanizing, Y-maze, tail suspension, open field and novel object tests were conducted. All males were euthanized at the end of the study since none of these males developed T1D, which was approximately six months (e.g., 188 days) of treatment.

NODEF females fed a phytoestrogen-free diet

Adult female mice at approximately 10 weeks in age were divided into 3 groups (10/group) and dosed with 0, 30, or 300 µg/kg BW BPS. These females were fed the phytoestrogen-free 5K96 diet (TestDiet, St. Louis, MO). This diet consisted of 22.1% protein, 66.6% carbohydrate and 11.3% fat (Huang et al. 2017). BGLs and BWs were measured and recorded weekly. At the one and two-month timepoints, the ITT and GTT were performed. Prior to euthanizing, Y-maze, tail suspension, open field, and novel object tests were conducted. Females were euthanized after approximately three months (e.g., 93 days) of treatment due to the high onset of diabetes in mice fed the phytoestrogen-free diet.

Body weight, blood glucose measurement, diabetic incidence, GTT, and ITT

BWs were measured weekly using a Sartorius scale (TE1502S; Denver Instrument, Bohemia, NY). BGLs were measured using a Prodigy Autocode Blood Glucose Meter (Charlotte, NC) by nicking the tail of each mouse to allow for collection of a small sample of venous blood. Mice with a BGL higher than 250 mg/dL for two consecutive weeks were considered diabetic (Guo et al. 2014). If non-fasting BGLs were 600 mg/dL or higher for two consecutive weeks, the mice were euthanized humanely using CO_2 asphyxiation followed by dislocation of the cervical vertebrae. All remaining mice were euthanized using this method at the end of the study.

For the GTT (Susiarjo et al. 2015), mice were fasted overnight (approximately 16hr), and then had their BWs and BGLs measured. Based on their respective weights, each mouse was injected intraperitoneally with 2 g/kg BW of glucose (Sigma). BGLs were measured 15, 30, 60, and 120 min after injection. A similar method was used for ITT (Cui et al. 2015). Baseline BWs

and non-fasting BGLs are obtained, followed by an intraperitoneal injection of 1.5 IU/kg BW insulin (Sigma). BGLs were then measured 15, 30, 60 and 120 min after injection.

Behavior tests

Y-maze test

The Y-maze is an apparatus consisting of three arms of equal lengths that converge into a "Y" shape. The purpose of this test is to assess working memory by observing if the mouse can remember which arm it has already explored. In theory, mice should enter each arm without repeating a previous arm. A mouse was placed in one arm of the maze and recorded for 10 min. Distance traveled in each arm, number of entries into each arm, number of spontaneous alternations, sequential order of arm entries, and total number of arm entries were analyzed using ANYmaze (Stoeling, Wood Dale, IL). A spontaneous alternation is defined by the occurrence of a mouse entering a different arm of the maze in each of three consecutive arm entries (Miedel et. al 2017). It was calculated using the equation:

$$\%\ Spontaneous\ Alterations = \frac{\#\ spontaneous\ alterations}{total\ number\ of\ arm\ enteries - 2} * 100$$

Tail suspension test

The tail suspension test was used to evaluate depression-related behaviors in mice (Can et al. 2012). In brief, mice were hung by their tails with medical tape for six min, and the amount of time mobile/immobile was calculated. Mice that spent more time mobile exhibited decreased depression-like behaviors.

Open field test

The open field test measures locomotion and anxiety-like behavior. Different sizes of open field apparatuses starting from 21x21 cm have been used, with most studies typically using an apparatus of 30-45 cm in size (Fukui et al. 2007; Ohtani et al. 2017). In our studies, a 25x25 cm

apparatus was used for the studies of male mice on the western diet and female mice on the phytoestrogen-free diet, while a 45x45 cm apparatus was used for the study male mice on the soy-based diet. Mice were placed in the center of the apparatus and allowed to wander freely for 10 min. The percentage of time spent in the center, corners, and periphery, along with the distance traveled, were calculated using ANYmaze. Mice who are anxious would spend more time in the corners and edges than the center.

Novel object test

The novel object test has been used to assess short term memory, which was conducted in 3 intervals (Wang C et al. 2016). In brief, during the first interval, the habituation period, mice were placed in the same apparatus as the open field test and allowed to freely explore for 10 min. The purpose of this procedure was to allow the mice to get familiarized with the apparatus. This interval also served as our open field test. 24 hours following the habituation period, mice were placed in the center of same apparatus. Inside the apparatus were two identical objects (familiar objects) placed in adjacent corners, far enough from the corners to allow mice to examine the objects from all sides. To prevent moving of the objects, mounting putty was placed on the bottom. Mice were allowed to explore the familiar objects for 10 min (this is often referred to as the learning period). The final interval tests short-term memory. Approximately 1.5hr following the learning period, mice were placed in the same apparatus, however, one of the familiar objects had been replaced with a new object (novel object). Again, mice were allowed to explore the apparatus and objects freely for 10min. This session was recorded, the time spent exploring each object was calculated, and the object bias index scores were calculated for the novel object using the equation:

$$Index = \frac{Time\ spent\ exploring\ the\ novel\ object}{Time\ spent\ exploring\ familar\ object + Time\ spent\ exploring\ novel\ object}$$

A 25x25 cm apparatus was used for the male western diet study and the female phytoestrogen-free diet study, with two dice being used as the familiar objects and a marble as the novel object. In the soy-based diet male study, a 45x45 cm apparatus was used, with two wooden blocks being the familiar objects and a ping pong ball the novel object.

Flow cytometric analysis

Following euthanasia, spleens were mashed in 3 mL PBS solution on ice. Flow cytometric analysis was performed to quantify leukocyte populations with different combinations of fluorochrome-labeled antibodies (diluted 1:80; BD PharMingen, San Diego, CA) including cluster of differentiation (CD) 40L-B220 (PE-FITC), CD5-CD24 (PE-FITC), CD44-CD40 (PE-FITC) and CD4-CD8-CD25-Mac3-CD45R (V450-APCH7-APCA-FITC-PE) along with isotype-matched irrelevant antibodies for controls. After antibody addition, cells were incubated in the dark for 30 min at 4°C, and then, washed and enumerated with a Becton Dickinson LSRII Flow Cytometer (BD Biosciences, San Jose, CA) in which log fluorescence intensity was read. Red blood cells were eliminated by using a high forward scatter threshold, and each sample had ten thousand cells counted. Analysis was done using FlowJo software (FlowJo LLC).

Statistical analysis

The rate of T1D development and total T1D incidence over time were analyzed with Likelihood ratio and Logrank test, respectively. For all other data sets, Dunnett's test (VH as the reference group) was used for homogeneous data and Wilcoxon test for non-homogeneous data, determined by unequal variances analysis using the Bartlett's test. Correlational analysis was conducted using Spearman's correlation test. A group was considered statistically significant if $p < 0.05$. JMP Pro 13 (SAS Inc., Cary, NC) and GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA) were used for statistical analysis and data visualization, respectively.

Results

BPS exposure in NODEF males fed a soy-based diet

There were no significant differences in BWs between the treatment and vehicle groups of mice fed a soy-based diet (Figure 2.1A). Out of the total 12 mice, only one mouse in the control group developed T1D on day 120 of the approximately eight-month study. There were significant increases of the non-fasting BGLs in non-diabetic mice on days 190, 217, 225, 232 and 238 during the study (Figure 2.1B). Mice in the BPS group had significantly smaller GI tracts, averaging 3.41±0.25g, compared to the control group averaging 4.38±0.25g. In addition, the livers and spleens were also significantly smaller (Figure 2.1C). When the organ weights were compared as a % of body weight, similar significant differences were observed. Four behavioral tests, including the tail suspension test, open field test, novel object test and Y-maze, were conducted on days 208-214 to determine possible neurological changes. There were no significant differences in % spontaneous alterations during the Y-maze test following 208 days of exposure (Supplementary Figure 2.1A). In addition, there were no significant differences in the open field test (e.g., distance traveled, and time spent in each section; Supplementary Figure 2.1B-C) and tail suspension test (e.g., immobile time; Figure 2.1D), suggesting the BPS did not significantly cause hyperactivity or anxiety-like behaviors. Although there was no significant difference in novel object index (Figure 2.1E), mice in the BPS group traveled significantly less (Figure 2.1F) when compared to the control group during the novel object test, suggesting a decreased curiosity and desire to explore.

Although no significant differences were found for the 2- (Figure 2.2A), 3- (Supplementary Figure 2.1D), 4- (Figure 2.2B) and 5-month GTTs (Figure 2.2C), the 6-month GTT showed increases in BGLs with significances observed at 0, 30 and 60-min time points (Figure 2.2D). For

the ITT, there was a significant decrease in BGLs at 30 min following 2 months of BPS exposure (Figure 2.2E). During the 4-month ITT, there was a significant decrease in BGLs at the 15-minute time point followed by a significant increase at the 30-min time point in the BPS exposed mice (Figure 2.2F), suggesting an increased insulin sensitivity that was overcome quickly. In contrast, there was a significant decrease in BGLs at the 30-min time point in the BPS group during the 5-month ITT (Figure 2.2G), confirming an increase in insulin sensitivity. However, there was no significant differences at any time points during the 3- (Supplementary Figure 2.1E) and 6-month (Figure 2.2H) ITTs. This could signify that the BPS group was able to overcome their insulin sensitivity.

The decrease in spleen size could be a result of altered leukocyte populations. Therefore, flow cytometric analysis was performed on the spleens. There was a significant increase in $\%CD4^{+}CD25^{-}$ and $\%CD40^{+}CD44^{+}$ cell populations in the treated group when compared to control (Table 2.1). In terms of mean fluorescent intensity (MFI), there was a significant increase in CD44 MFI by $CD40^{-}CD44^{+}$ cells (Figure 2.3A), CD40 MFI by $CD40^{+}CD44^{+}$cells (Figure 2.3B), and CD40L MFI by $B220^{-}CD40L^{+}$cells (Figure 2.3C) when compared to control. In addition, there was a significant decrease in CD8 MFI by $CD4^{+}CD8^{-}$ cells and CD8 MFI by $CD8^{-}CD25^{+}$ cells in the treated group (data not shown). Correlation analyses suggested that the increase in $\%CD40^{+}CD44^{+}$ was significantly correlated with the distance traveled during novel object test (Figure 2.3D), and its association with the novel object index was nearly significant (p=0.057; Supplementary Figure 2.1F).

BPS exposure in NODEF males fed a Western diet

Similar to the male mice fed a soy-based diet, NODEF males fed a Western diet had no significant differences in body weights at any time points between VH and BPS treatment groups

(Figure 2.4A). In addition, there was no onset of T1D in any of the mice during this study, and food intake was not significantly different between the groups (data not shown). Overall, there were no changes in weekly non-fasting BGLs, although there was a significant increase in mice exposed to BPS on day 38 (Figure 2.4B). When the weights of organs were compared, there was a significant difference in the weight of spleens between the two groups (Figure 2.4C). When the organ weights were compared as a % of body weight, similar significant differences were observed (data not shown). There were no significant changes in other organs, including liver, thymus, kidneys, heart, lungs, pancreas, and GI tract. In terms of behavioral changes, there were no significant differences for any endpoints of the Y-maze test or tail suspension test (Supplementary Figure 2.2A-B), suggesting that BPS did not alter working memory or induce depression- like behaviors. Although there was no significant difference in the novel object indexes (Figure 2.4D), mice treated with BPS traveled further during the novel object test (Figure 2.4E) when compared to control, suggesting possible hyperactivity. Although not significant, there was an increase in the distance traveled during the open field test as well (Figure 2.4F), supporting that BPS treatment might increase hyperactivity when mice were maintained on the Western diet. However, there were no differences in time spent in each section during the open field test (Supplementary Figure 2.2C).

Both GTT and ITT were conducted during the study at 2- (Figure 2.5A, 2.5D), 3- (Figure 2.5B, 2.5E) and 5-month (Figure 2.5C, 2.5F) time points, and there was no significant difference at any time point, suggesting neither glucose metabolism nor insulin sensitivity was impacted by BPS exposure in these mice fed a Western diet.

The increase in spleen size could be due to altered leukocyte populations. Flow cytometric analysis suggested that there was a significant decrease in %$CD24^{+}CD5^{+}$ and %$B220^{+}CD40L^{-}$ cell

populations in treated mice when compared to control (Table 2.2). Additionally, BPS treatment increased %$CD44^{-}CD40L^{-}$ and %$B220^{-}CD40L^{-}$ cell populations. There was also a significant decrease in the MFI of B220 by $B220^{+}CD40L^{-}$ (Figure 2.6A), CD40L by $B220^{-}CD40L^{+}$ (Figure 2.6B), CD5 by $CD24^{-}CD5^{+}$ (Figure 2.6C), CD5 by $CD24^{+}CD5^{+}$ (Figure 2.6D), CD24 by $CD24^{+}CD5^{-}$ (Figure 2.6E), and CD40L by $B220^{+}CD40L^{-}$ (data not shown). A significant correlation was observed between the distance traveled during the novel object test and CD5 MFI by $CD24^{-}CD5^{+}$ spleen cells (Figure 2.6F).

BPS exposure in NODEF females fed a phytoestrogen-free diet

The differential responses to BPS in the NODEF male studies prompted us to examine the effects of BPS in NODEF female mice fed a phytoestrogen-free diet. Female mice were fed the phytoestrogen-free 5K96 diet and dosed with VH, 30, or 300 µg/kg BW BPS. Although not statistically significant, there were changes in T1D development in both BPS treatment groups when compared to control, with the 30 µg/kg BW group having the highest incidence toward the end of the study (Figure 2.7A), suggesting that BPS might exhibit a bimodal dose-response relationship. When the weekly non-fasting BGLs were compared in nondiabetic mice (Figure 2.7B), there was a significant difference between the 300 µg/kg BPS group and the 30 µg/kg or control on day 11. There was also a significant difference between the two treatment groups on day 18. In addition, there was a significant increase of BGLs in the low dose group when compared to the control group on day 60 (Figure 2.7B). However, there were no significant differences in either the one-month GTT (Supplementary Figure 2.3A) or ITT (Supplementary Figure 2.3B). In contrast, BPS-treated mice had higher BGLs than the control mice at 30-, 60-, and 120-min time points in the two-month GTT (Figure 2.7C), although they did not reach the level of statistical significance. BGLs at the 30-min time point was significantly increased in the BPS groups when

they were compared to the control group separately. In addition, there was a significant decrease in the BGLs at the 120-min time point during the two-month ITT in the 300 µg/kg group when compared to control (Figure 2.7D), suggesting an increased sensitivity to insulin.

Exposure to BPS did not affect the weekly BWs, except for the 300 µg/kg dose group, which had a significantly increased body weight when compared to the vehicle on day 71 (Figure 2.8A). No differences were observed for organ weights (data not shown). In terms of behavioral changes, there was no significant difference in the Y-maze performance for either BPS group when compared to the control (Supplementary Figure 2.3C). In addition, neither the open field (e.g., distance, time spent in each area) nor the novel object test showed any differences (Supplementary Figures 2.3D-F). However, there was a slight decrease in time immobile during the tail suspension test for both the 30 µg/kg group (p=0.0612) and 300 µg/kg group (p=0.0564) when compared to the control (Figure 2.8C), suggesting possible hyperactivity. When both treatment groups were combined and compared to control, there was a significant decrease in time immobile (p=0.0180; Figure 2.8D), further supporting the notion that BPS might cause hyperactivity.

Flow cytometric analysis suggested that %$CD4^+CD25^+$ and $CD8^+CD25^-$ splenic cell populations were significantly increased in the 30 µg/kg BPS group when compared to the control (Table 2.3). There was also a significant increase in %$CD3^+CD45R^+$, %$CD3^-CD45R^+$, and %$CD40^+CD44^+$ cell populations for the 300 µg/kg BPS group when compared to control (Table 2.3). Furthermore, %$CD4^+CD8^+$ and %$Mac3^+CD45R^+$ cell populations were also increased in both treatment groups when compared to the control (Table 2.3). In terms of mean fluorescence intensity there was a decrease in Mac3 MFI by $CD45R^-Mac3^+$ cells (p=0.0242; Figure 2.8B). Taken together these results suggest a potentially increased pro-inflammatory response mediated by activation of T-cells.

Discussion

In this study, we examined the interactions among BPS, diets, and sex in NODEF mice in relation to hypoglycemia, T1D development, immunomodulation, and behavioral changes. Consistent with the previous report (Xu, Huang, Guo 2019), our studies demonstrated that exposure to BPS had varying adverse outcomes depending on the diets. In our study, only one out of twelve NODEF male mice fed a soy-based diet and none of the twelve NODEF male mice fed the Western diet developed T1D in a study period of 6 - 8 months. In contrast, none of the male NOD mice (approximately 30) on either the soy-based diet or the phytoestrogen-free diet became diabetic (Xu et. al 2019). Several studies using NOD male mice have reported a diabetic incidence of 0-30%, with 10% being the most common (Huang et al. 2017; Huang et al. 2018; Xu, Huang, Guo 2019; Xu, Huang, Nagy, Guo 2019; Xu, Huang, Nagy, Teng, et al. 2019). In our study, 15 out 30 female NODEF mice became diabetic when fed a phytoestrogen-free diet, which was consistent with the female NOD mouse studies that have a diabetic incidence of 20-90% depending on the age of the mice and the duration of exposure (Huang et al. 2017; Huang et al. 2018; Xu, Huang, Guo 2019; Xu, Huang, Nagy, Guo 2019; Xu, Huang, Nagy, Teng, et al. 2019). Overall, our studies suggest that NODEF mice do not have an increased incidence of T1D when compared to NOD mice.

In NODEF male mice fed a soy-based diet, there was a significant increase in weekly non-fasting BGLs following exposure to BPS in nondiabetic animals, suggesting a prediabetic state. This was further supported by the decreased glucose absorption or metabolism exhibited during the 6-month GTT and resistance to fasting evidenced by an increase in fasting BGLs (e.g., 0-min time point). In terms of ITTs, BPS-treated mice had significantly lower BGLs during the 2, 4, and 5-month ITTs with no differences during the 6-month ITT. These findings could signify a shift

from a state of increased insulin sensitivity to impaired glucose tolerance. However, our results differed from those of Xu et al (2019), who found that their NOD males on a soy-based diet exhibited insulin resistance. This discrepancy is likely due to the different strains used. It would be interesting to further determine if these differential responses were related to the alteration of microbiome. In our study with NODEF males exposed to BPS and fed a Western diet, there were no significant changes in BGLs during any ITTs, GTTs, or weekly non-fasting BGLs. However, heightened BGLs were seen in the control mice fed the Western diet when compared to those fed a soy-based diet (Figure 2.2 vs. Figure 2.4). Therefore, it was possible that the Western diet masked the effects of BPS. To the best of our knowledge, this is the first study to examine the impact a Western diet on BPS-induced changes in glucose homeostasis.

NODEF female mice fed a phytoestrogen-free diet were studied because there were previous studies examining the effect of BPS in male NOD mice on a phytoestrogen-free diet (Xu, Huang, Guo 2019). NODEF females fed a phytoestrogen-free diet exhibited decreased BGLs at the 120-min timepoint during the 2-month ITT following exposure to 300 µg/kg BW BPS, suggesting a decreased insulin resistance. However, the 2-month GTT also showed some increase in BGLs in both groups exposed to BPS when compared to control. It is possible that simultaneous decrease in insulin resistance and glucose tolerance would lead to overall non-significant effects on the T1D incidence (five in control group, six in the 30 µg/kg BPS group, and four in the 300 µg/kg BPS group) and weekly non-fasting BGLs in these NODEF female mice. Interestingly, NODEF males on the soy-based diet also showed decreased insulin resistance and glucose tolerance simultaneously in this study. In addition, similar to our results, male NOD mice fed the phytoestrogen-free diet also had a significant decrease in BGLs during the 2-month ITT (Xu, Huang, Guo 2019). Overall, these studies suggest that the decreased insulin resistance observed in

BPS-treated mice may not be affected by sex or diet. Further studies are needed to confirm these findings using different models.

The lack of significant differences in the % spontaneous alterations during the Y-maze test in all three studies suggests that exposure to BPS does not cause impairment of working memory. However, NODEF males fed the soy-based diet had a significant decrease in the distance traveled during the novel object test following BPS exposure, suggesting a decreased curiosity and a possible increase in anxiety-like behaviors. Decreased insulin sensitivity can be associated with anxiety (Shomaker et al. 2010; Wu et al. 2012). Moreover, there was a slight decrease in time spent immobile during the tail suspension test in both the BPS-treated females on the phytoestrogen-free diet and the BPS exposed males on the soy-based diet, further supporting that BPS exposure might increase hyperactivity and anxiety-like behavior. Consistent with an increased hyperactivity, BPS also induced a significant increase in the distance traveled during the novel object test in NODEF male mice fed the Western diet. To our knowledge, there are no previous studies examining behavioral changes from adult exposure to BPS using NOD, NODEF or other mice as a model. Although the different responses between diets could be a result of BPS interacting with diet components, e.g., fat in the Western diet or phytoestrogens in the soy-based diet, it might also be related to the size of the apparatus used (25x25 cm vs. 45x45 cm). We chose to use a larger apparatus to increase accuracy. However, when comparing the amount of time spent in the periphery vs center there was a significant difference in both soy-based groups, suggesting that the larger apparatus increased anxiety-like behaviors.

Consumption of a western diet can significantly alter brain function, e.g., emotions, learning, memory and motivation, and a growing body of preclinical and experimental evidence have shown that the Western diet can induce chronic, low-grade, metabolic inflammation (Christ

et al. 2019; Stevenson et al. 2020). In this study, BPS exposure caused an increase in the distance traveled during the novel object test in male NODEF mice fed the Western diet. This increased hyperactivity correlated with the decreased CD5 MFI of $CD24^{-}CD5^{+}$ cells. In addition, BPS exposure in male NODEF mice on the Western diet led to a decreased $\%CD24^{+}CD5^{+}$ cells. It has been reported that $CD5^{+}$ cells were negatively associated with pro-inflammatory status (Lundell et al. 2014; Baglaenko et al. 2015). In addition, both BPS groups of female NODEF mice fed the phytoestrogen-free diet exhibited an increase in $CD4^{+}CD8^{+}$ T cells, a typical population of autoreactive T cells that promote inflammation and increase the risk of autoimmune disease development (Parel and Chizzolini 2004; Abo et al. 2012; Devarajan and Chen 2013). Additionally, male NODEF mice on the soy-based diet exhibited an increase in $\%CD4^{+}CD25^{-}$ and $CD40^{+}CD44^{+}$ proinflammatory cells, with the latter negatively correlated with the distance traveled during novel object test. Similarly, female NODEF mice on the phytoestrogen-free diet also exhibited an increase in $CD40^{+}CD44^{+}$ cells. CD40 expression is associated with development of T1D (Vaitaitis et al. 2017). Studies examining BPS exposed human, or zebrafish macrophages also saw a proinflammatory response (Chen Y et al. 2018; Qiu et al. 2018). In addition, some studies have demonstrated a correlation between increased proinflammatory markers and anxiety-related disorders (Felger 2018). Taken together, it is possible that BPS exposure caused a proinflammatory response that might be related to behavioral changes and increased risk of T1D. It should be noted that our leukocyte yield was lower than other studies (Malaisé et al. 2020; Malaisé et al. 2021; Sawai et al. 2003). This is likely due to a difference in flow cytometry protocol, such as use of a lysing agent.

Due to the wide use of BPA and BPS and their detection in humans with urine concentrations of 0.37 µg/L and 1.24 µg/L, respectively (Lehmler et al. 2018), insights surrounding

the toxicity of these bisphenols are critical because they would help determine the safety levels. It has been reported that BPA exposure can cause increased anxiety-like behaviors and impairments in working memory and object recognition (Tian et al. 2010; Ohtani et al. 2017). Although the observed effects of BPS in our study seemed less severe when compared to BPA, BPS was still able to alter the immune system and caused some changes in behaviors. Both BPS exposed male mice and female mice exhibited increased anxiety-like behavior and hyperactivity. More research should be conducted to determine if BPS is indeed neurotoxic and should be regulated similarly to BPA.

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Table 2.1. Percentages of splenic immune cell populations in male NODEF mice on a soy-based diet following exposure to 300 μg/kg BPS. Data are presented as mean ± SEM. *, $p < 0.05$ as compared to the respective vehicle control group. VHM, vehicle males. N = 5-6.

Antibodies	Group	+/- (%)	+/+ (%)	-/+ (%)	-/- (%)
CD3/CD45R	VHM	11.06±1.04	2.67±0.16	18.70±2.08	64.56±2.96
	300	12.05±0.44	2.43±0.35	20.87±1.08	64.67±1.10
CD4/CD8	VHM	9.99±0.59	0.99±0.07	5.61±0.32	83.42±0.79
	300	11.33±0.90	1.06±0.13	5.36±0.39	82.25±1.14
CD4/CD25	VHM	6.45±0.49	1.88±0.12	3.09±0.39	88.58±0.86
	300	7.86±0.36*	1.74±0.14	2.68±0.34	87.70±0.72
CD8/CD25	VHM	10.89±0.85	1.63±0.23	1.11±0.11	86.36±0.83
	300	11.03±0.55	1.44±0.15	1.02±0.10	86.52±0.61
CD24/CD5	VHM	26.68±2.08	3.26±0.34	19.94±0.75	50.10±1.97
	300	17.90±3.36	3.70±0.78	22.48±1.47	55.92±5.01
CD40/CD44	VHM	3.50±0.85	24.68±2.15	34.16±1.54	37.76±2.48
	300	2.92±0.15	31.85±1.90*	34.07±0.83	31.28±1.87
B220/CD40L	VHM	20.34±1.21	2.35±0.43	2.22±0.10	75.08±1.58
	300	24.57±2.20	5.20±1.33	8.05±3.52	62.18±6.84
Mac3/CD45R	VHM	4.91±0.46	5.36±0.37	15.78±1.83	73.96±2.57
	300	7.07±1.56	6.01±0.72	16.92±1.24	70.02±1.53

Table 2.2. Percentages of splenic immune cell populations in male NODEF mice on a Western diet following exposure to 300 μg/kg BPS. Data are presented as mean ± SEM. *, $p < 0.05$ as compared to the respective vehicle control group. VHM, vehicle males. N = 6.

Antibodies	Group	+/- (%)	+/+ (%)	-/+ (%)	-/- (%)
CD3/CD45R	VHM	9.87±0.84	0.81±0.10	16.98±1.73	72.35±2.53
	300	10.32±0.81	0.98±0.06	19.42±1.50	69.23±2.18
CD4/CD8	VHM	8.71±0.69	1.12±0.16	4.05±0.41	86.17±1.16
	300	10.07±0.39	1.18±0.09	4.47±0.46	84.30±0.68
CD4/CD25	VHM	7.15±0.69	1.19±0.14	0.93±0.10	90.73±0.86
	300	8.38±0.46	1.20±0.12	0.93±0.06	89.48±0.61
CD8/CD25	VHM	4.96±0.55	0.65±0.07	1.51±0.16	92.88±0.74
	300	5.53±0.45	0.65±0.06	1.53±0.12	92.30±0.59
CD24/CD5	VHM	37.06±8.64	3.79±1.08	30.90±2.94	20.45±0.76
	300	44.87±4.29	1.63±0.29*	26.70±1.20	34.55±9.90
CD40/CD44	VHM	3.80±0.58	30.02±2.59	38.50±1.59	27.80±1.85
	300	2.98±0.36	25.37±1.26	38.23±1.35	33.50±1.68*
B220/CD40L	VHM	25.53±2.14	2.66±0.56	4.86±1.46	66.93±3.95
	300	20.22±0.89*	1.34±0.18	1.47±0.47	76.98±0.90*
Mac3/CD45R	VHM	9.02±2.35	3.48±0.26	13.35±1.98	74.18±0.62
	300	7.10±2.15	3.30±0.13	15.60±1.55	73.98±1.21

Table 2.3. Percentages of splenic immune cell populations in female NODEF mice on a phytoestrogen-free diet following exposure to 30 or 300 μg/kg BPS. Data are presented as mean ± SEM. *, $p < 0.05$ **, $p<0.001$ as compared to the respective vehicle control group. #, $p < 0.05$ as compared to the 30 μg/kg BW group. N = 8. VHF, vehicle females.

Antibodies	Group	+/- (%)	+/+ (%)	-/+ (%)	-/- (%)
CD3/CD45R	VHF	15.68±0.61	1.88±0.17	27.55±0.91	54.93±1.36
	30	15.65±0.92	2.65±0.32	29.46±1.11	52.29±1.96
	300	14.99±0.84	2.39±0.10*	33.21±1.00*#	49.46±1.49*
CD4/CD8	VHF	13.90±0.51	1.31±0.14	7.50±0.47	77.28±0.68
	30	14.53±0.69	1.97±0.15*	8.97±0.73	74.53±1.42
	300	14.53±0.25	1.85±0.17*	8.55±0.33	75.08±0.53*
CD4/CD25	VHF	13.51±0.49	1.30±0.04	1.73±0.50	83.46±0.91
	30	14.73±0.72	1.55±0.09*	1.73±0.17	82.03±0.93
	300	14.63±0.44	1.42±0.05	1.63±0.11	82.33±0.47
CD8/CD25	VHF	7.12±0.53	0.90±0.37	1.04±0.09	90.94±0.49
	30	9.14±0.78*	0.82±0.09	1.18±0.06	88.86±0.80*
	300	8.83±0.41	0.70±0.07	1.20±0.04	89.28±0.49
CD24/CD5	VHF	45.74±2.49	1.91±0.09	27.28±1.31	28.04±3.73
	30	42.27±2.13	1.93±0.10	28.69±0.60	27.13±1.84
	300	42.20±1.39	2.10±0.12	28.11±0.62	27.60±1.33
CD40/CD44	VHF	1.09±0.12	29.06±1.32	39.3±1.69	30.54±1.68
	30	1.10±0.15	31.29±0.76	41.59±0.98	26.03±1.21
	300	1.04±0.11	34.51±1.06*	38.78±1.39	25.68±1.39*
B220/CD40L	VHF	23.89±1.25	1.37±0.14	2.36±0.19	72.36±1.44
	30	26.44±1.49	1.43±0.14	3.28±0.53	68.84±1.34
	300	26.63±2.74	1.76±0.38	4.35±1.05	67.25±2.00
Mac3/CD45R	VHF	2.67±0.26	4.31±0.22	21.93±1.01	71.08±1.06
	30	3.30±0.53	5.85±0.48*	22.25±0.99	68.6±1.06
	300	4.11±0.66	6.40±0.40**	23.40±1.75	66.10±1.46*

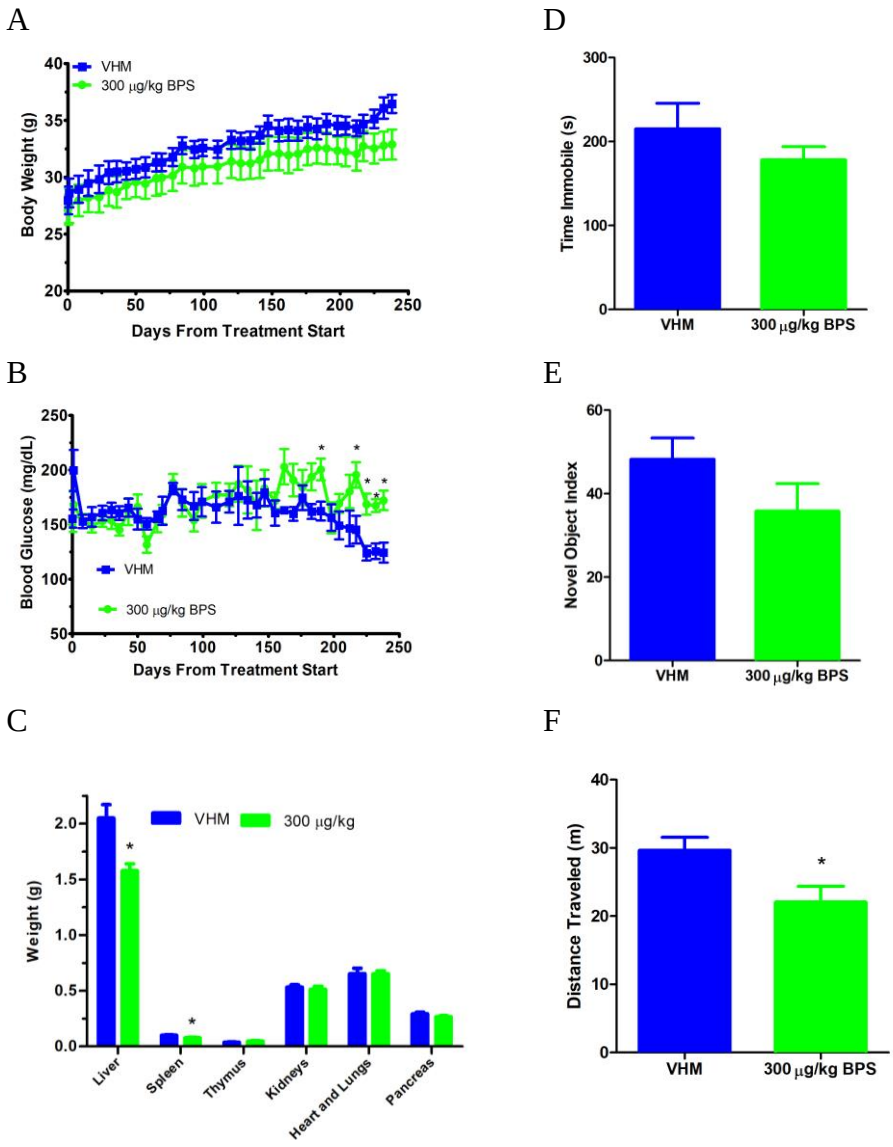


Figure 2.1. Weekly body weights, blood glucose levels, organ weights and behavior tests in male NODEF mice on a soy-based diet following exposure to 300 µg/kg BW BPS. (A) Time course for body weights; (B) Time course of non-fasting blood glucose levels for non-diabetic mice; (C) Organ weights; (D) Time spent immobile during the tail suspension test; (E) Novel object index and (F) distance traveled during the novel object test using a 45x45 cm apparatus. The values are presented as mean ± SEM. *, $p < 0.05$. N =5-6. VHM, vehicle males.

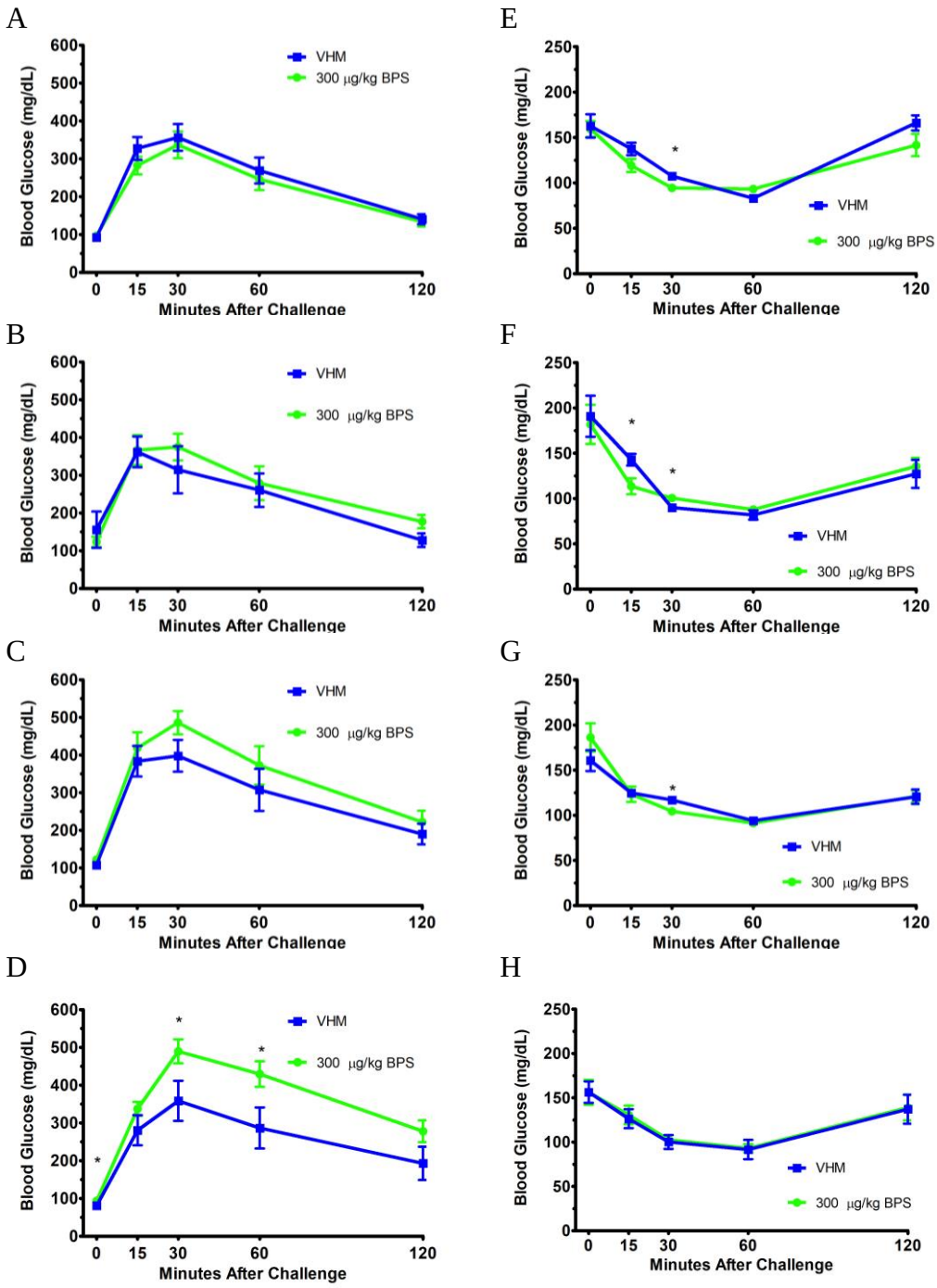


Figure 2.2. Tolerance tests in male NODEF mice on a soy-based diet following exposure to 300 µg/kg BPS. Glucose tolerance tests after 2 months (A), 4 months (B), 5 months (C), and 6 months (D) of exposure are shown in the left column. ITTs after 2 months (E), 4 months (F), 5 months

(G), and 6 months (H) of exposure are shown in the right column. The values are presented as mean ± SEM. *, $p < 0.05$. N =5-6. VHM, vehicle males.

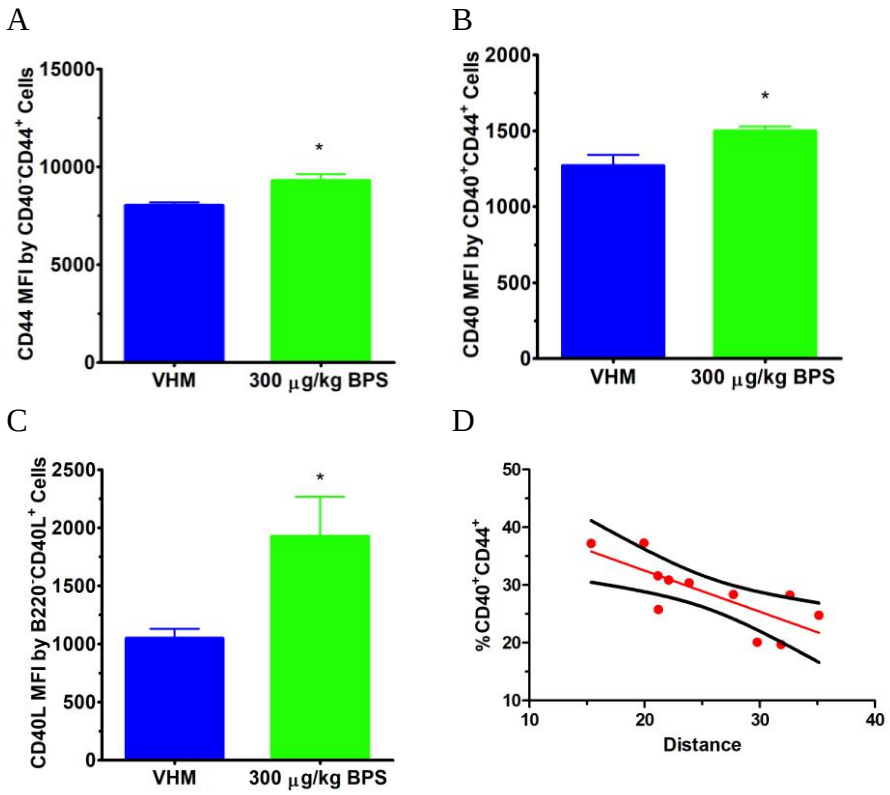


Figure 2.3. Mean fluorescence intensity (MFI) of splenic leukocyte populations in male NODEF mice on the soy-based diet following exposure to 300 µg/kg BPS. (A) CD44 MFI by $CD40^{-}CD44^{+}$. (B) CD40 MFI by $CD40^{+}CD44^{+}$. (C) CD40L MFI by $B200^{-}CD40L^{+}$. (D) Correlation analysis between $\%CD40^{+}CD44^{+}$ and the distance traveled during novel object test. The values are presented as mean ± SEM. *, $p < 0.05$ for the BPS vs vehicle (VHM) groups. N = 5-6.

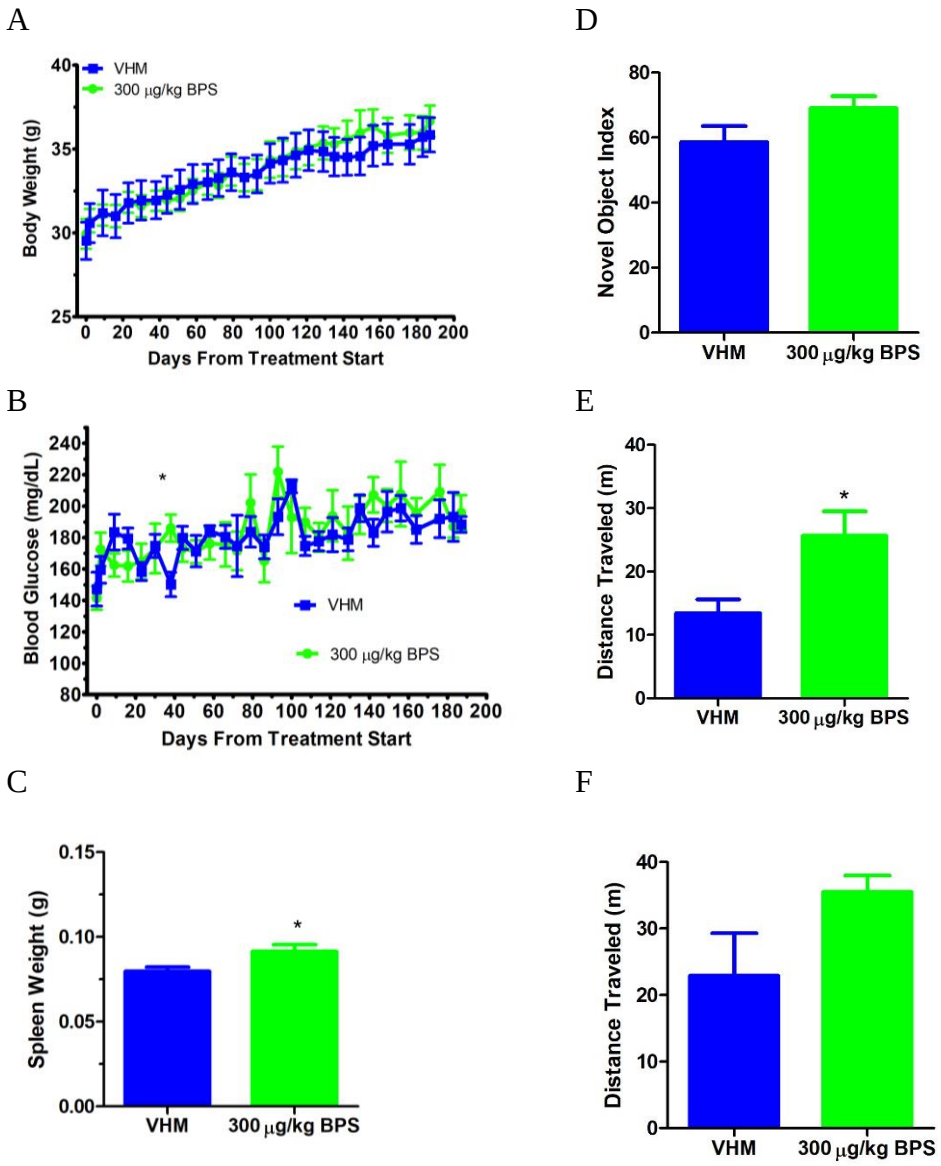


Figure 2.4. Weekly body weights, blood glucose levels, organ weights and behavior tests in male NODEF mice on the Western diet following exposure to 300 μg/kg BPS. (A) Weekly body weights, (B) weekly non-fasting blood glucose levels, (C) spleen weights, (D) novel object index, (E) distance traveled during the novel object test, and (F) distance traveled during the open field test using a 25x25 cm apparatus. VHM = vehicle male mice. N=6. The values are presented as mean ± SEM. *, $p < 0.05$.

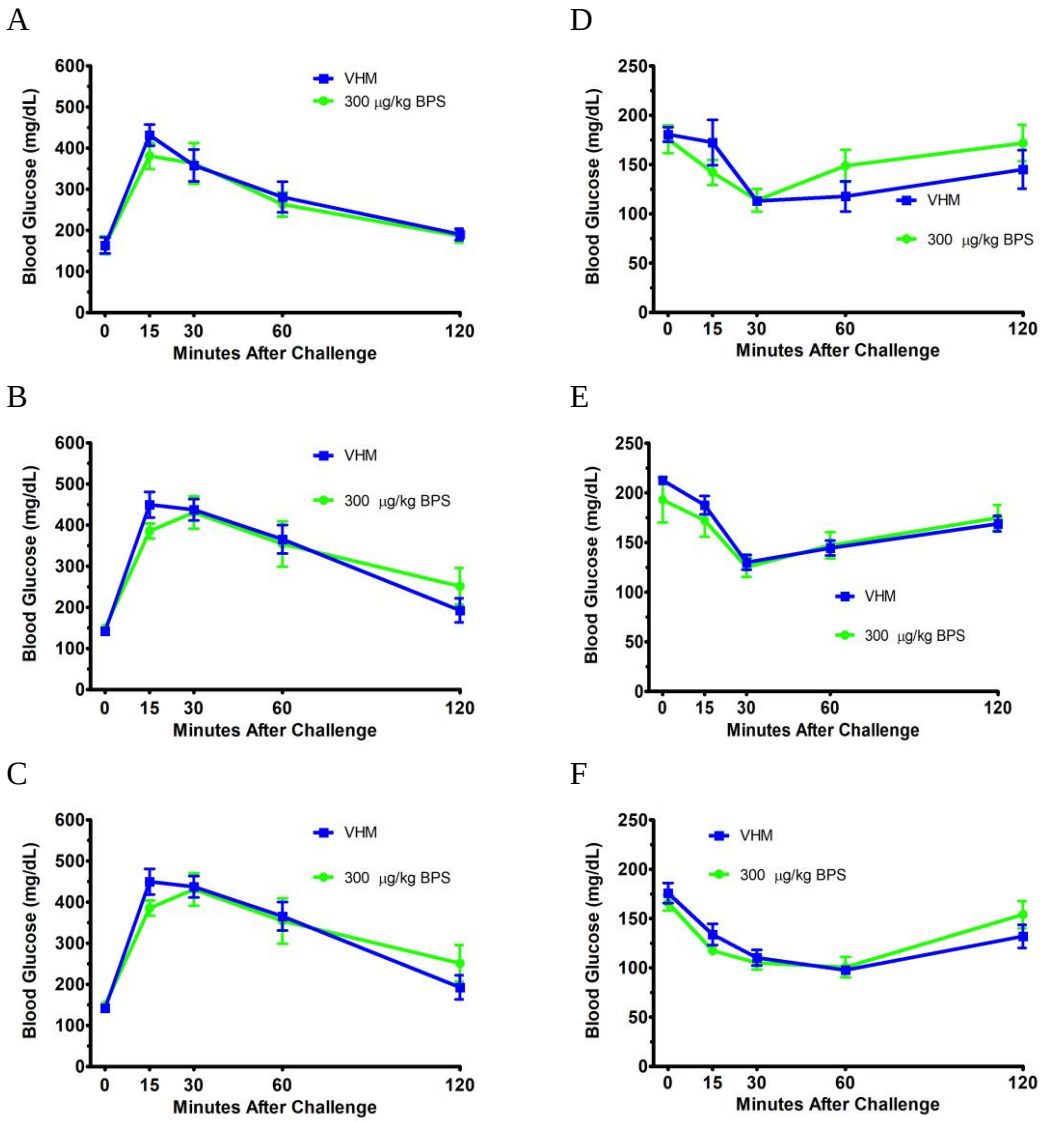


Figure 2.5. Glucose tolerance tests (GTT) and insulin tolerance tests (ITT) in male NODEF mice fed a Western diet following exposure to 300 µg/kg BPS. GTTs after 2 months (A), 3 months (B), and 5 months (C) of exposure were shown in the left column. ITTs after 2 months (D), 3 months. (E), and 5 months (F) of exposure are shown in the right column. The values are presented as mean ± SEM. VHM = vehicle male mice. Statistical analysis was conducted as described in the method.

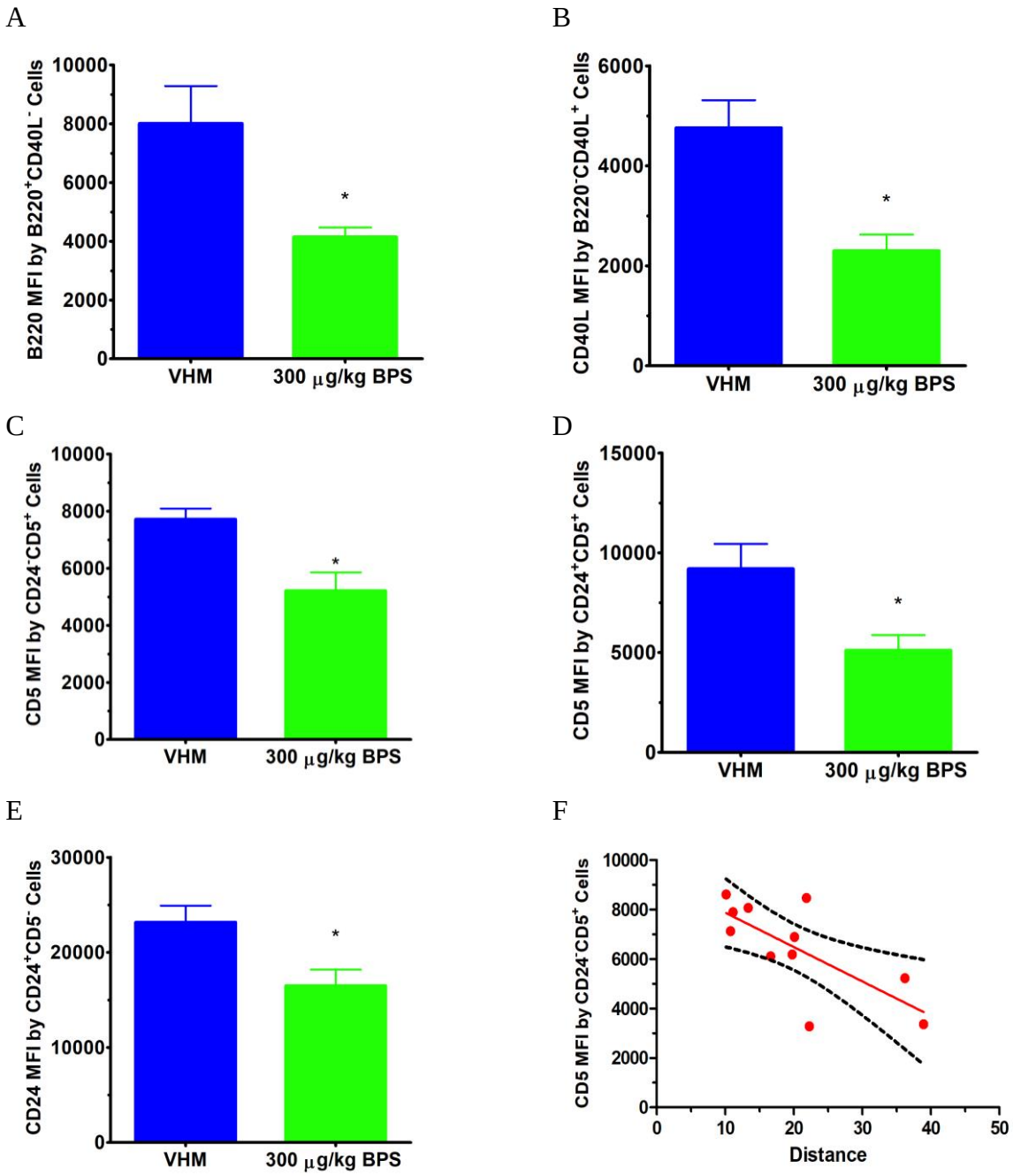


Figure 2.6. Mean fluorescence intensity (MFI) of splenic leukocyte populations in male NODEF mice on the Western diet following exposure to 300 µg/kg BPS. (A) MFI B220 by B220+CD40L-. (B) MFI CD40L by $B220^{-}CD40L^{+}$. (C) MFI CD5 by $CD24^{-}CD5^{+}$. (D) MFI CD5 by $CD24^{+}CD5^{+}$. (E) MFI CD24 by $CD24^{+}CD5^{-}$. (F) Correlation analysis between the distance traveled during the

novel object test and CD5 MFI by $CD24^{-}CD5^{+}$ spleen cells. VHM = vehicle male mice. The values are presented as mean ± SEM. *, $p< 0.05$

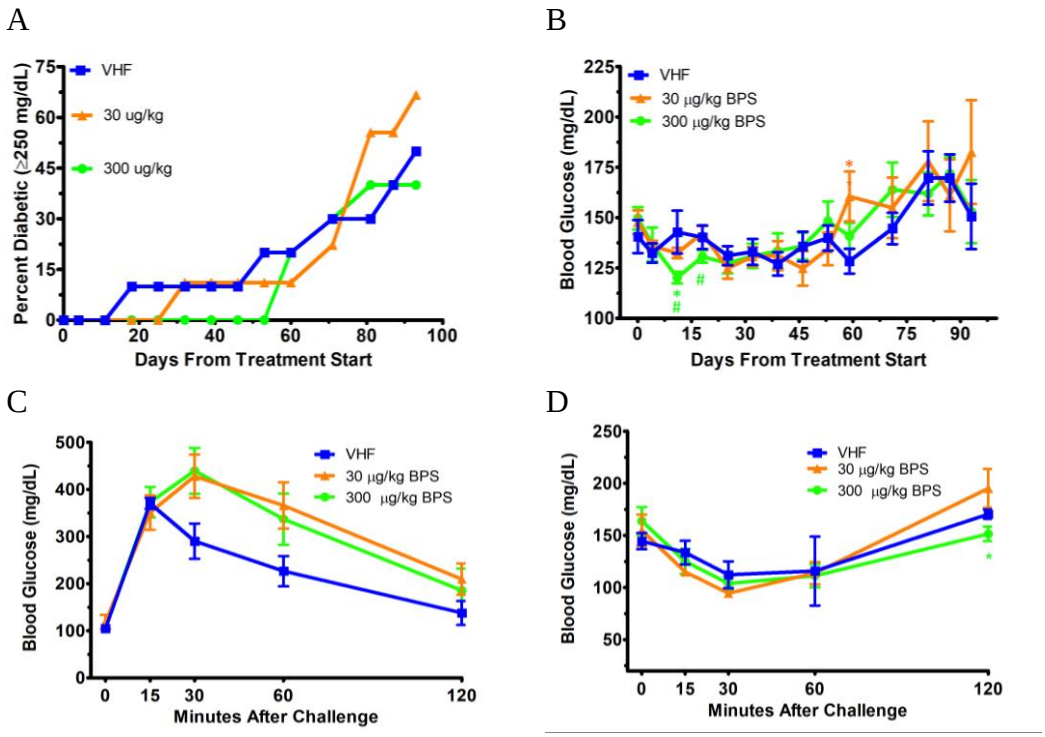


Figure 2.7. Diabetic incidence, nondiabetic blood glucose levels (BGLs), and glucose and insulin tolerance tests in female NODEF mice on the phytoestrogen-free diet following exposure to 30 and 300 µg/kg BW BPS. (A) T1D incidence (N = 10). A mouse with a blood glucose level ≥250 mg/dL was considered diabetic. (B) Time course of non-fasting BGLs in nondiabetic mice. GTTs after 2 months of BPS exposure (C; N=8-10), and ITTs after 2 months of BPS exposure (D; N = 6-9) are also shown. The values are presented as mean ± SEM. *, $p< 0.05$, vs control, # $p<0.05$ compared to 30 µg/kg BW BPS. VHF, vehicle females.

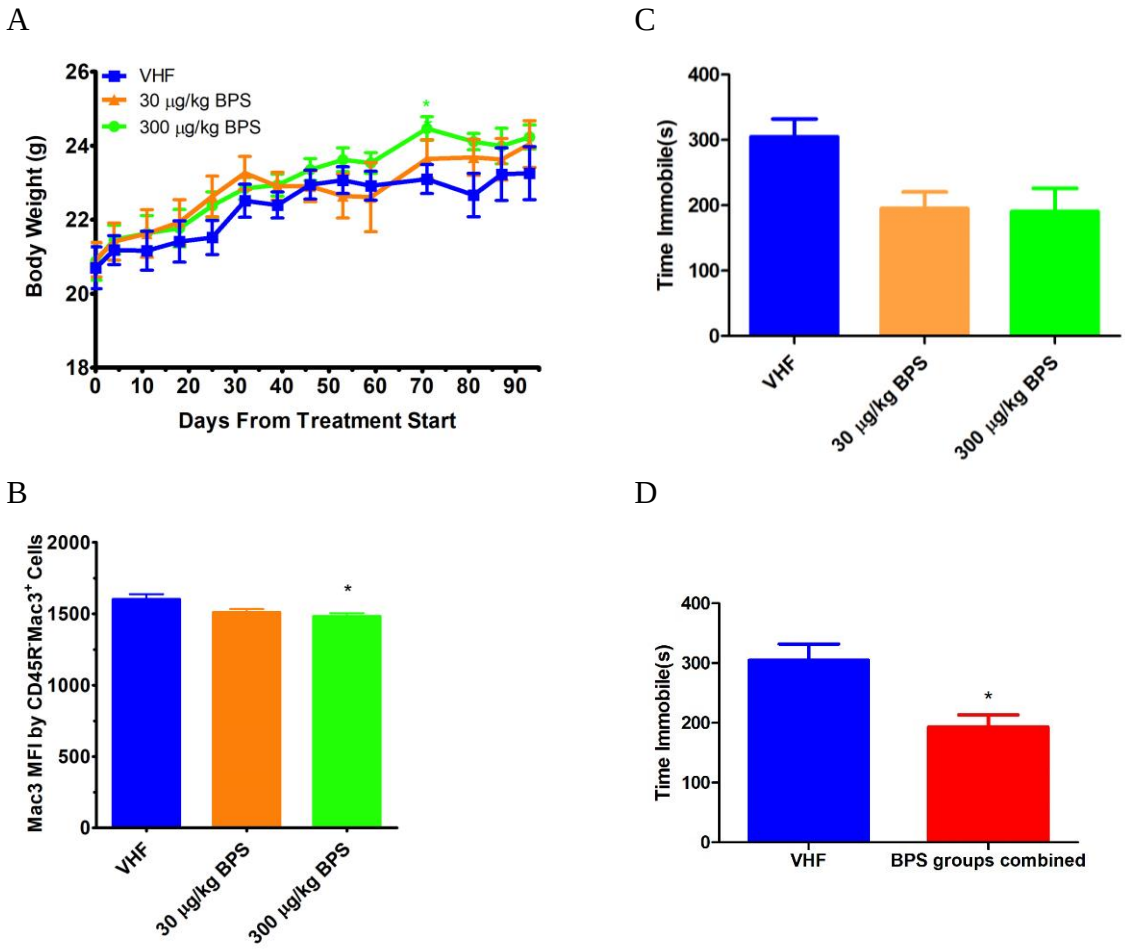
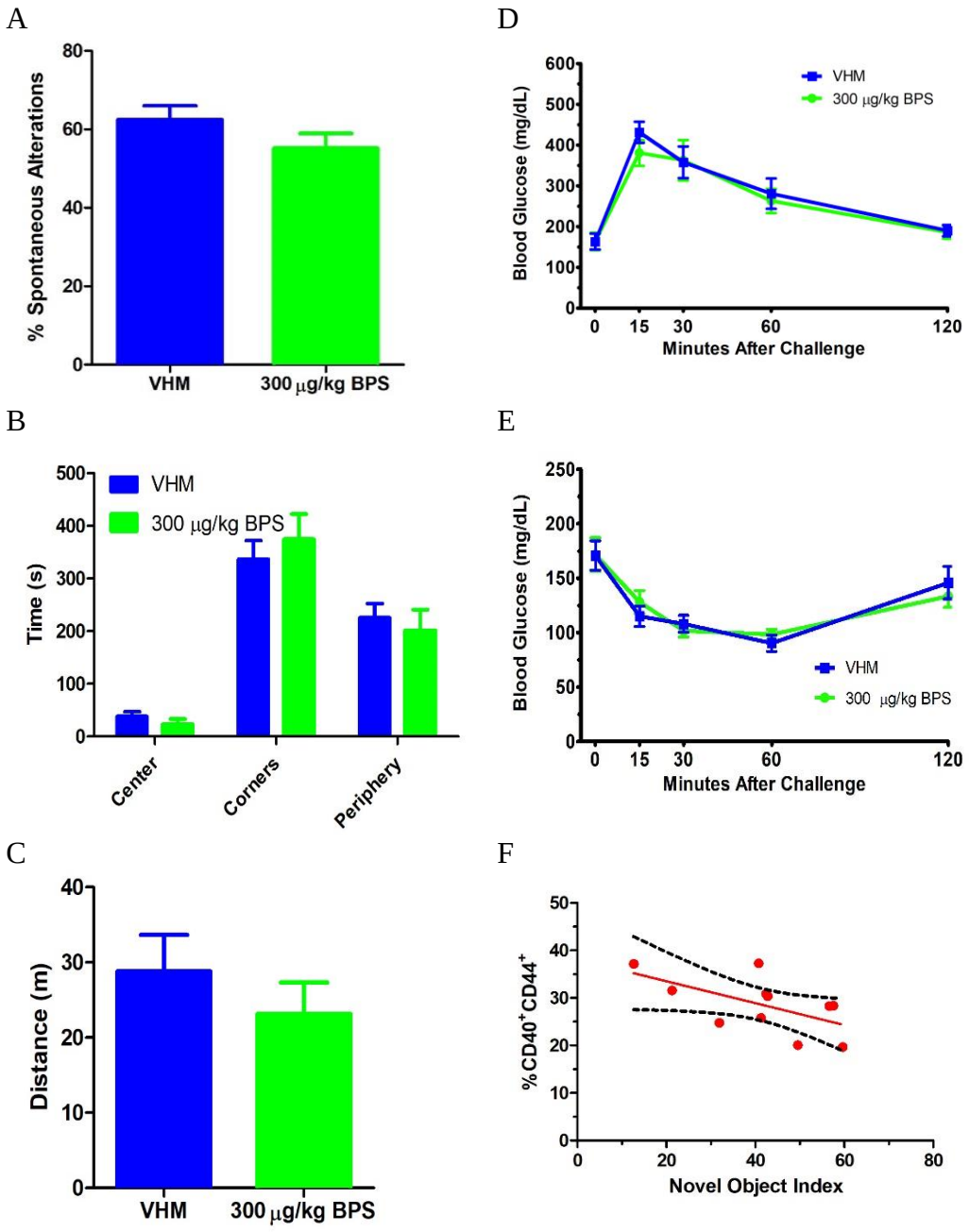
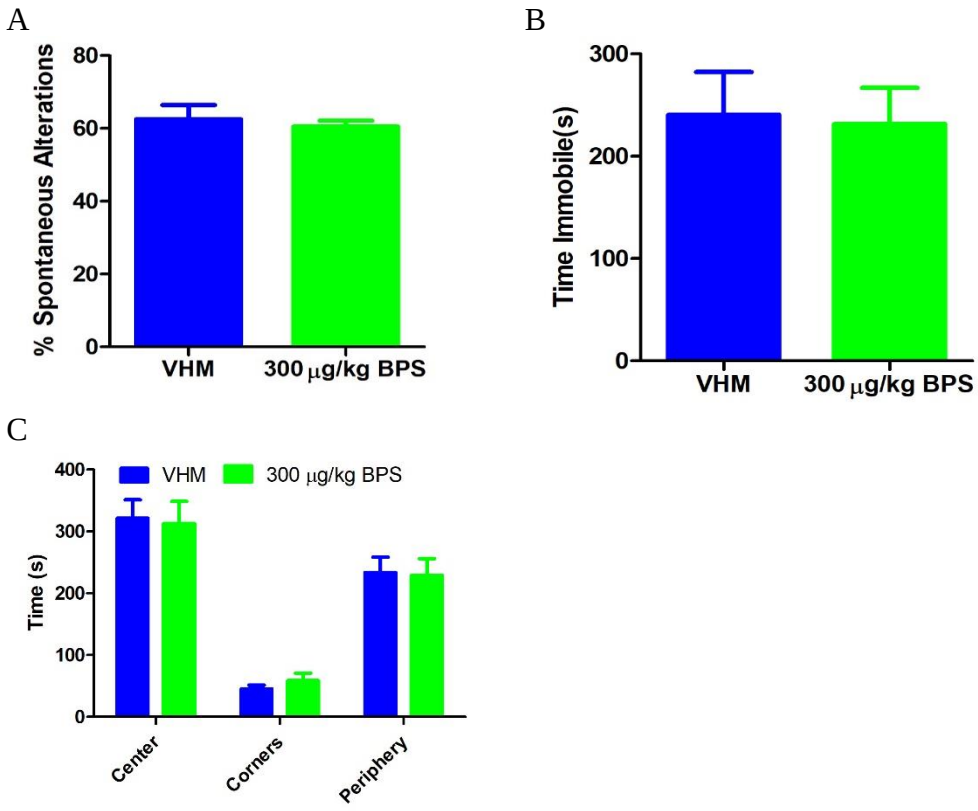


Figure 2.8. Weekly body weights, behavioral tests, and flow cytometric analysis in female NODEF mice on the phytoestrogen-free diet following exposure to 30 and 300 µg/kg BPS. (A) Changes in body weight over time (N = 8-10). (B) Mean fluorescence intensity (MFI) for Mac3 by splenic $CD45R^{-}Mac3^{+}$ cells (N=5-6). (C) Time immobile during the tail suspension test. (D) Time immobile during the tail suspension test with both treatment groups combined. The values are presented as mean ± SEM. *, $p< 0.05$. VHF, vehicle females.

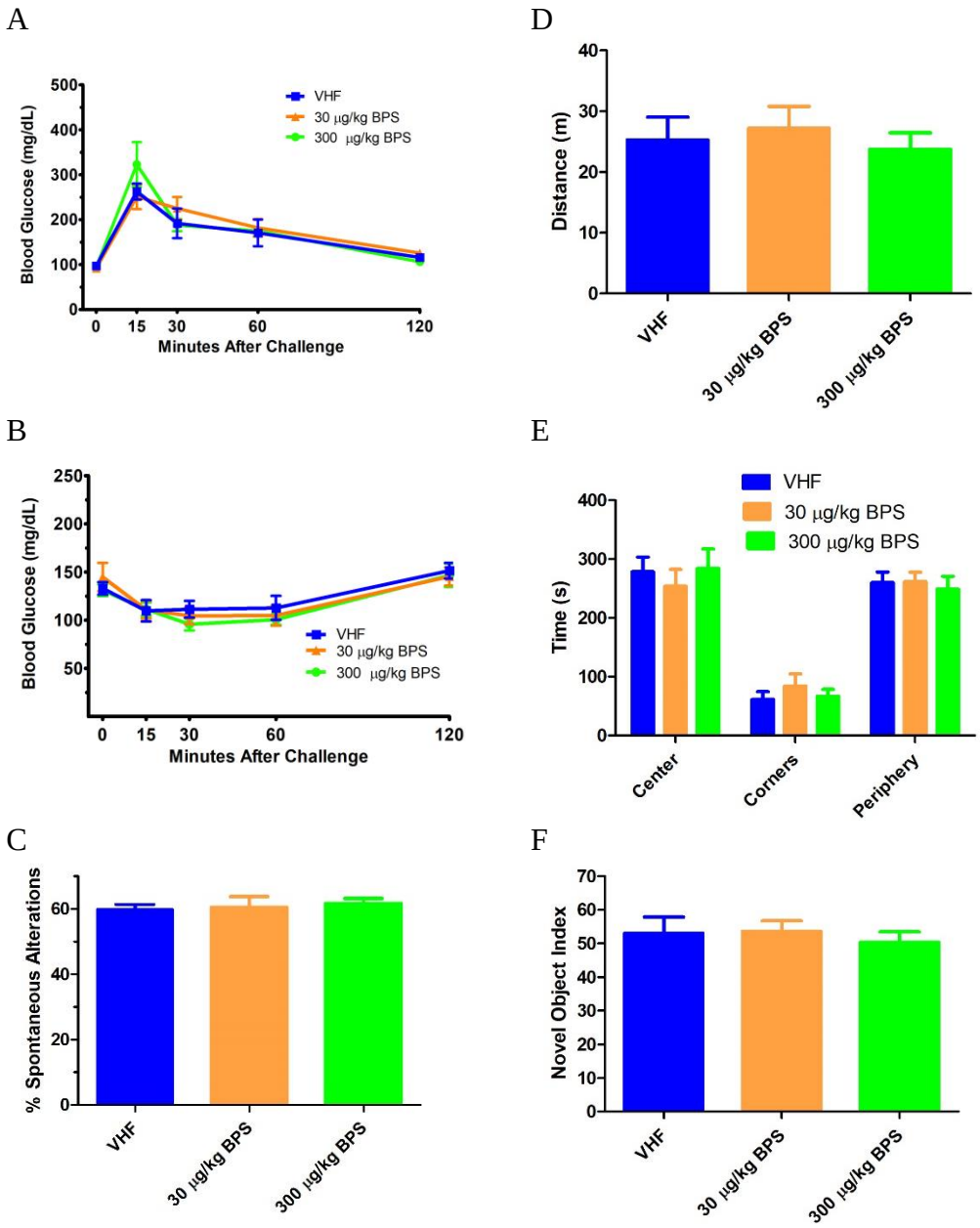


Supplementary Figure 2.1. Insulin and glucose tolerance tests and behavior tests in male NODEF mice on a soy-based diet following exposure to 300 μg/kg BW BPS. (A) % spontaneous alteration during the Y-maze test. (B) Time spent in each area and (C) distance traveled during the open field test using a 45x45 cm apparatus. (D) 3-month GTT and (E) ITT. (F) Correlation analysis between

%$CD40^{+}CD44^{+}$ and the novel object index during novel object test (p=0.057). The values are presented as mean ± SEM. Statistical analysis was conducted as described in the method. N=5-6. VHM, vehicle males.



Supplementary Figure 2.2. Behavior tests in male NODEF mice fed a Western diet. (A) % Spontaneous alterations during the Y-maze. (B) Time immobile during the tail suspension test. (C) Time spent in each section during the open field test using a 25x25 cm apparatus. N = 6. The values are presented as mean ± SEM. Statistical analysis was conducted as described.



Supplemental Figure 2.3. GTT, ITT and behavioral tests in female NODEF mice fed a phytoestrogen-free diet. 1-month GTT (A) and ITT(B). (C) % Spontaneous alterations during the Y-maze. (D) Distance traveled during the open field test. (E)Time spent in each section during the open field test using a 25x25 cm apparatus. (F) Novel object bias index. N = 8. The values are presented as mean ± SEM. Statistical analysis was conducted as described in the methods.

CHAPTER 3

REPRODUCTIVE TOXICITY AND NEUROTOXICITY OF BISPHENOL S IN *C. ELEGANS* AND NOFEF MICE FOLLOWING DEVELOPMENTAL EXPOSURE[3]

[3]McDonough Callie M and Tai L. Guo "Reproductive toxicity and neurotoxicity of bisphenol S in *C. elegans* and NODEF mice following developmental exposure" to be submitted to *Neurotoxicology*.

Abstract

There is growing concern surrounding bisphenol A (BPA), leading to increased industrial production and application of its analog bisphenol S (BPS). The goals of the study were: (1) To investigate the generational reproductive effects of BPS in *C. elegans* by exposing eggs to BPS for 48hr for one or multiple generations, and (2) To examine type 1 diabetes incidence and neurological effects in NODEF mouse pups exposed to BPS *in utero* throughout gestation. In this study, we first examined the developmental effects of BPS (0.1, 1.0, 5.0 and 10.0 μM) on the lifespan and fertility of the nematode *C. elegans* for up to three generations. To determine the effects of BPS following a single period of exposure, worms were exposed to BPS for 48hr and then propagated without BPS for 2 additional generations. Worms exposed to 0.1 and 1.0 μM BPS had a decreased lifespan and decreased number of progenies after one generation of exposure with an ability to recover without further exposures in subsequent generations. In contrast, worms exposed to 5.0 or 10.0 μM BPS exhibited a generational effect, with the offspring of worms treated for one generation displaying a decreased lifespan and reduced number of progenies in the second generation. Only worms exposed to 10.0 μM BPS during the first generation continued to have a significant effect (e.g., decreased lifespan) in the third generation. To examine the effects of exposure for multiple generations, worms were exposed to BPS for 48hrs at each generation for three generations. There was an accumulative effect in worms treated with 0.1 or 1.0 μM BPS for two generations, but not for three generations, suggesting a threshold effect. Worms exposed to either 5.0 or 10.0 μM BPS for two or three generations demonstrated accumulative effects. When the developmental effects of BPS were studied in NODEF mice, pups exposed gestationally to 3 μg/kg BW BPS exhibited behavioral deficits at the age of 12 weeks old, but not at the age of 3 weeks old. Specifically, female pups had decreases in working and short-term memories while

male pups showed increases in hyperactivity and anxiety-like behavior. In summary, this study demonstrates the sex-dependent effects of BPS in NODEF mice exposed *in utero*, along with the generational effects observed in *C. elegans.*

Introduction

The growth of fetuses and infants depends on the production and secretion of several tightly regulated hormones during the periods of gestation and early development, making them susceptible to the exposure of endocrine disrupting chemicals (EDCs) (Gore et al. 2015). Bisphenol A (BPA) is a member of the chemical class known as bisphenols often found in epoxy-resins. It can bind to estrogen receptors (ERs), both ER alpha and beta, to potentially cause system-wide toxicity (Xu et al. 2016), including, but are not limited to, exacerbation of type 1 diabetes (T1D), reproductive toxicity, developmental toxicity and neurological deficits (Castro et al. 2015; Jandegian et al. 2015; Mersha et al. 2015; Manshack et al. 2016; Qiu et al. 2016; Schirmer et al. 2021). The growing public concerns over BPA has led to a shift towards "BPA-free" products, which contain bisphenol analogues, with bisphenol S (BPS) being the most common. Due to the structural similarities between BPA and BPS, recent studies have shown that BPS also binds to ERs, resulting in similar toxicities (Naderi et al. 2014; Castro et al. 2015; Mersha et al. 2015; Qiu et al. 2016; LaPlante et al. 2017; Zhao et al. 2017; Gingrich et al. 2018; Gu et al. 2019; Xu, Huang, Guo 2019; Naderi et al. 2020). However, detailed studies on the reproductive and neurological toxicities of BPS are still lacking.

The nematode *C. elegans* is a common model used to study reproductive endpoints and lifespan. To date there are a limited number of studies examining the reproductive effects of BPS in *C. elegans,* including only one study spanning multiple generations (Xiao et al. 2019). In addition, the only study examining more than one generation employed a continuous exposure

regimen, and thus the effects of BPS in subsequent generations after a single exposure are unknown. One goal of this study was to investigate the reproductive effects of BPS in *C. elegans* by exposing eggs to BPS for 48hr, a period prior to when worms reach maturity and are able to lay eggs (e.g., the L_4 stage) for one or multiple generations. We chose the period of 48hr to ensure worms in the lifespan assay would have enough time to absorb the FUDR and to ensure all offspring of the worms used in the fertility assay were counted. Although *C. elegans* are excellent research models there are limitations, especially when examining neurological changes. For this reason, we chose to utilize NODEF mice in addition to *C. elegans.* After examining the effects in C. *elegans*, we determined the 3 µg/kg BW BPS was the most appropriate concentration for exposure in NODEF mice. The goal of our mouse study was to examine T1D incidence and neurological toxicities in mouse pups exposed *in utero.*

Previous studies have shown that exposure to BPA at an external concentration of 1 mM (e.g., in NGM) resulted in an uptake of approximately 2 µg/g worm extract (2 ppm) of this chemical in *C. elegans* (Allard and Colaiacovo 2011). Based on these results and taking into the consideration of BPS doses used by Xu et al. (2019) in their mouse studies, we chose environmentally relevant concentrations and doses for gestational and behavioral studies in *C. elegans* (0, 0.1, 1.0, 5.0 or 10.0 µM) and mice (0 or 3 µg/kg BW), respectively. The 3 µg/kg BW dose used in our gestational mouse study is equivalent to an external exposure of 1.5 µM in *C. elegans* according to the measurements taken previously by Allard et al. (2011). NODEF mice, which have an excluded flora that is protective against diabetes, are promoted by Taconic to replace NOD mice, a strain previously used as a model for T1D, with the hope of increasing the rate of T1D incidence. Based on the previous studies in *C. elegans* and mice, we hypothesized that worms exposed to BPS for one generation would exhibit a decreased lifespan and lowered fertility

in the subsequent generations, while worms exposed for multiple generations would have accumulative decreases. In addition, mice exposed to 3.0 µg/kg BW BPS would have a sex-dependent effect on the incidence of diabetes, glucose sensitivity, and behavioral changes.

Methods

C. elegans Model

Preparation of nematode growth medium (NGM) petri plates and BPS exposure. BPS obtained from Sigma (St. Louis, MO) was dissolved in ethanol and further diluted with K buffer (3.04 g/L NaCl, 2.39 g/L KCl) to make a 100 mM stock solution containing 10% ethanol as previously described by Mersha et al. (2018). NGM agar (2.3 g/L NaCl, 15 g/L agar, 20 g/L peptone, 1 mM KH_2PO_4 [pH=7.0]) was prepared and autoclaved according to the WormBook (Stiernagle). Once the agar cooled to 55°C, cholesterol (1 mM), $CaCl_2$ (1 mM), and $MgSO_4$ (1mM) were added and mixed into the agar along with BPS at various concentrations to prepare plates with BPS at final concentrations of 0, 0.1, 1.0, 5.0, and 10.0 µM. Plates were then seeded with *E. coli* OP50 and incubated at 37°C overnight. There were no apparent differences between control and BPS-containing plates in terms of *E. coli* growth.

Age-synchronization and treatment of *C. elegans.* The *C. elegans* strain N2 was obtained from Dr. Lili Tang (Dept. of Environmental Health Science at the University of Georgia), who originally procured it from the Caenorhabditis Genetics Center (Minneapolis, MN; funded by the NIH National Center for Research Resource, USA). Once the NGM plates had sufficient *C. elegans* growth they were age-synchronized using the bleaching method (Stiernagle). Briefly, a worm pellet was collected in a 15 mL conical tube and then exposed to a hypochlorite solution (1N) containing 5% household bleach. Tubes were vortexed until all worms appeared dead. The pellet was then rinsed with K buffer and centrifuged. The intact eggs remaining were then transferred to

seeded NGM plates containing 0, 0.1, 1.0, 5.0, or 10.0 µM BPS and allowed to hatch and grow for 48hr as described below for generational studies.

Generational exposure of *C. elegans*. To study the generational effects, worms were age-synchronized as described above and eggs were allowed to hatch and grow for 48hr on NGM plates without (control) or with BPS (Figure 3.1). Worms only treated for one generation are referred to as $F_{gen\#a}$, while worms treated for multiple generations are referred to as $F_{gen\#b}$. To ensure worms from all generations (F_1, F_2 and F_3) were only treated for 48hr during each generation, the synchronized L4 larvae were transferred to fresh NGM plates without BPS after 48hr of exposure and allowed to mature into adult larvae and lay eggs for an additional 48hr. Worms from these plates were then age-synchronized, and eggs were transferred to either NGM plates with or without BPS for multigenerational or generational studies, respectively. This was done to examine the effects of worms treated for only one generation (F_1, F_{2a}, and F_{3a}) or multiple generations (F_{2b} and F_{3b}) . All eggs were allowed to grow on the new NGM plates with or without BPS for 48hr. They were then used for the fertility and lifespan assay (Described below) or transferred to fresh NGM plates for egg laying to precure next generation (Figure 3.1).

Lifespan assay. The reproduction inhibitor 5-Fluoro-2′-deoxyuridine (FUDR) was obtained from MP Biomedicals, LLC (Irvine, CA) and dissolved in filtered DI water to create a 150 mM stock. Because FUDR needs time to be absorbed to inhibit egg laying, and to make the lifespan and fertility assays comparable, age-synchronized eggs were exposed to 0, 0.1, 1.0, 5.0, or 10.0 µM BPS by culturing them on BPS-containing NGM plates for 48 hours. Following BPS exposure, 5-25 worms were transferred to each well of 8-32 wells in a 96-well plate that contained 2 µL FUDR, 5 µL K buffer and 0.5 µL OP50-seeded LB broth (10 g/L Tryptone, 10 g/L NaCl, 5 g/L yeast extract, 1mM NaOH [pH=7]) per well. This resulted in approximately 100-200 worms per

treatment. Twenty-four hours after transfer, worms were assessed for viability (dead or alive) with any eggs/offspring removed using a pipette tip. This was repeated daily until all worms had died. The seeded LB broth was replenished as needed. Each study was repeated 3 times to ensure accuracy.

Fertility assay. Worms typically start laying eggs at the L4 or adult stage, e.g., 60-72hr after the eggs are laid and hatched (the time spent as an egg after being laid is ~12hr). As described earlier, we exposed eggs/worms to BPS for 48 hours prior to transferring them to ensure all offspring were recorded. Specifically, worms exposed to 0, 0.1, 1.0, 5.0, or 10.0 µM BPS for 48hr were transferred to 96-well plates containing 5 µL K buffer and 0.5 µL concentrated OP50 suspended in LB broth. Each row of the 96-well plate had 2 worms on opposite sides. Twenty-four hours after transfer, each worm was examined for egg laying. Worms which had started laying eggs were transferred to the adjacent well. This was repeated daily until all worms had finished laying. Total offspring, number of unhatched eggs, number of L1 (hatched) offspring and lethality rates (number of unhatched eggs/total offspring x 100) were measured and recorded daily along with total rates. Worms that failed to lay eggs, possibly males, were excluded from study. Each study was repeated 3 times to ensure accuracy.

NODEF Mouse Study

Animal husbandry and BPS exposure. NODEF mice were initially obtained from Taconic Biosciences (Germantown, NY). A breeding colony was established and housed in Coverdell animal facility at the University of Georgia (UGA) in polysulfone cages with irradiated laboratory animal bedding and Bed-r'Nest for enrichment (The Andersons Inc., Maumee, Ohio). Negligible amounts of BPA have been reported to leach from new or used polysulfone cages maintained at room temperature (Delclos et al. 2016; Johnson et al. 2016). They were kept at 22-25°C with a

relative humidity of 50±20% and a 12hr light/dark cycle. Filtered water was provided *ad libitum* through the animal facility's automatic watering system. Breeder Chow (PicoLab Rodent Diet 5058) or a regular growth diet (PicoLab Rodent Diet 5053) was provided *ad libitum*. All animals were treated humanely and with regard to alleviating animal suffering. An approved animal protocol by the UGA Institutional Animal Care and Use Committee (IACUC) was followed for all procedures. Prior to starting, the initial body weights (BWs) and non-fasting blood glucose levels (BGLs) were obtained on eleven female NODEF ranging from 8-16 weeks of age. Based off this data mice were then divided into the treatment (n=6) or vehicle (n=5) group and ANOVA was preformed to ensure there were no significant differences between the two groups. Each female mouse was housed with one male NODEF male for five days to allow for breeding. This period was referred to as the breeding period. After 5 days males were removed, and female mice were housed individually. These female mice were dosed with either 0 (vehicle) or 3 µg/kg BW BPS from breeding day one to parturition to ensure exposure for the entire gestational period. Body weights (BW) and non-fasting blood glucose levels (BGLs) were measured and recorded weekly.

Pups exposed *in utero.* Offspring from both control and BPS-exposed dams were weaned at postnatal day 21. On this day, BW and BGLs were measured, and pups were sexed and distributed to cages at random. To eliminate litter effect, each cage had pups from each litter. The Y-maze, open field, and novel object tests, as described below, were then performed. Thereafter, weekly BWs and BGLs were measured and recorded. At 12 weeks of age, behavior tests were repeated, with the addition of the tail suspension test. The insulin tolerance and glucose tolerance tests were performed when pups were 17 weeks old. Female offspring were euthanized at 22 weeks of age and male offspring at 25 weeks of age. Females were euthanized earlier due to diabetes development in the control group. According to Taconic (https://www.taconic.com/mouse-

model/nod), NOD mice diabetes occurrence should reach 50% incidence in both sexes at 13-15 weeks of age. We chose to go beyond that timepoint to ensure maximal diabetes incidence was reached. However, males in our control and treatment groups did not reach above 30% diabetes incidence. We therefore decided to focus our paper on neurotoxicity.

Body weight, blood glucose measurement, and diabetic incidence. BWs were measured weekly using a scale (TE1502S; Denver Instrument; Bohemia, NY) and the average body weight of each group was used to determine the following week's dose. BGLs were measured using a Prodigy Autocode Blood Glucose Meter (Charlotte, NC) by nicking the tail of each mouse to allow for collection of a small sample of venous blood. Mice with a BGL higher than 250 mg/dL for two consecutive weeks were considered diabetic (Guo et al. 2014). If non-fasting BGLs were 600 mg/dL or higher for two consecutive weeks, the mice were euthanized humanely using CO_2 asphyxiation followed by dislocation of the cervical vertebrae. All remaining mice were euthanized using this method at the end of the study.

Glucose tolerance and insulin tolerance test. At 17 weeks of age a glucose tolerance test (GTT) and an insulin tolerance test (ITT) were performed on one random female and male pup from each litter. For the GTT (Susiarjo et al. 2015), mice were fasted overnight (approximately 16hr), and then had their BWs and BGLs measured. Based on their respective weights, each mouse was injected intraperitoneally with 2 g/kg BW of glucose (Sigma). BGLs were measured 15, 30, 60, and 120min after injection. A similar method was used for ITT (Cui et al. 2015). Baseline BWs and non-fasting BGLs are obtained, followed by an intraperitoneal injection of 1.5 IU/kg BW insulin (Sigma). BGLs were then measured at 15, 30, 60, and 120min after injection.

Behavior tests

Y-maze test. The Y-maze is an apparatus consisting of three arms of equal lengths that converge into a "Y" shape. The purpose of this test is to assess working memory by observing if the mouse can remember which arm it has already explored. In theory, mice should enter each arm without repeating a previous arm. A mouse was placed in one arm of the maze and recorded for 10min. Distance traveled in each arm, number of entries into each arm, number of spontaneous alternations, sequential order of arm entries, and total number of arm entries were analyzed using ANYmaze (Stoeling, Wood Dale, IL). A spontaneous alternation is defined by the occurrence of a mouse entering a different arm of the maze in each of three consecutive arm entries (Miedel et. al 2017). It is calculated using the equation:

$$\%\, Spontaneous\ Alterations\ = \frac{\#\ spontaneous\ alterations}{total\ number\ of\ arm\ enteries - 2} * 100$$

Tail suspension test. The tail suspension test was used to evaluate depression-related behaviors in mice (Can et al. 2012). In brief, mice were hung by their tails with medical tape for six minutes, and the amount of time mobile/immobile was calculated. Mice that spend more time mobile were considered to have fewer depression-related behaviors.

Open field test. The open field test measures anxiety-like behavior and locomotion. Different sizes of open-field apparatus starting from 21x21 cm have been used, with most studies typically use an apparatus 30-45 cm (Fukui et al. 2007; Ohtani et al. 2017). In our studies, a 25x25 cm apparatus was used for the 3-week-old mice. When retested at 12 weeks, a 45x45 cm apparatus was used. Mice were placed in the center of the apparatus and allowed to wander freely for 10min. Distance traveled, time spent in the corners, time spent in the edges, and time spent in the center were measured. The percentage of time spent in the center, corners, and periphery, along with the distance traveled, were calculated using ANYmaze (Stoeling, Wood Dale, IL). Mice who are anxious would spend more time in the corners and edges than in the center.

Novel object test. The novel object test has been used to assess short term memory, which is conducted in 3 intervals (Wang C et al 2016). During the first interval, the habituation period, mice were placed in the same apparatus as the open field test and allowed to freely explore for ten minutes. The purpose of this procedure was to allow the mice to get familiarized with the apparatus. This interval also served as our open field test. 24hr following the habituation period, mice were placed in the center of the same apparatus. Inside the apparatus were two identical objects (familiar objects) placed in adjacent corners, far enough from the corners to allow mice to examine the object from all sides. To prevent moving of the objects, mounting putty was placed on the bottom. Mice were allowed to explore the familiar objects for 10min (this is often referred to as the learning period). The final interval tests short-term memory. Approximately 1.5hr following the learning period, mice were placed in the same apparatus, however one of the objects had been replaced with a new object (novel object). Again, mice were allowed to explore the apparatus and objects freely for 10min. The time spent exploring each object was calculated, and the object bias index scores were calculated for the novel object using the equation:

$$Index = \frac{Time\ spent\ exploring\ the\ novel\ object}{Time\ spent\ exploring\ familar\ object + Time\ spent\ exploring\ novel\ object}$$

A 25 x 25 cm apparatus was used for 3 weeks old mice and a 45 x 45 cm was used when mice reached 12 weeks of age. The familiar objects were dice for the 3 weeks old mice and wooden blocks for the 12-week-old mice. The novel object was a marble for 3-week-old mice and a ping pong ball for 12-week-old mice.

Statistics

The rate of diabetes development and total diabetes incidence over time were analyzed with Likelihood ratio and Logrank test, respectively. For all other data sets (e.g., behavior tests, weekly non-fasting BGLs, and weekly BWs in mice, average lifespan and time spent laying, total

number of progenies in *C. elegans*), Dunnett's test (VH as the reference group) was used for homogeneous data and Wilcoxon test for non-homogeneous data; determined by unequal variances analysis using Bartlett's test. A group was considered statistically significant if $p < 0.05$. JMP Pro 13 (SAS Inc., Cary, NC) and GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA) were used for statistical analysis and data visualization.

Results

C. elegans- Lifespan

BPS exposure in the F_1 generation produced a significant decrease in the average lifespan of the F_1 worms at all concentrations tested (Figure 3.2A). When compared to control, BPS exposure at 0.1 µM for one generation did not induce significant generational effects on the average lifespans in the next generations (F_{2a}, F_{3a}), suggesting a reversible effect after halting treatment for one generation (Figure 3.2B). The significant increase in lifespan in the F_{3a} generation when compared to F_1 generation supported this finding. In contrast, worms treated for multiple generations (F_{2b} and F_{3b}) demonstrated a significant reduction in average lifespan when compared to any other generation (control, F_1, F_{2a} and F_{3a}), suggesting an accumulative effect (Figure 2B). However, there were no significant differences in lifespan when worms treated for two generations (F_{2b}) were compared to those treated for three generations (F_{3b}), suggesting a threshold effect.

Similar to 0.1 µM BPS exposure, worms treated with 1.0 µM BPS for only one generation did not exhibit any significant differences in the subsequent generations (F_{2a} and F_{3a} compared to control; Figure 3.2C). The significant increase in average lifespan in both F_{2a} and F_{3a} when compared to F_1 supported this notion. In addition, there was a significant increase in lifespan when the third generation F_{3a} was compared to the second generation F_{2a}, suggesting a washout effect.

In addition, worms treated with 1.0 µM BPS for two (F_{2b}) or three (F_{3b}) generations had a significantly reduced lifespan when compared to the control, F_1, or the generations only exposed during F_1 only (F_{2a} and F_{3a}). Similar to 0.1 µM BPS treatment, there was no significant difference in lifespan between the F_{2b} and F_{3b} worms, suggesting the toxicity reached a threshold after treatment for 2 generations.

Worms exposed to 5.0 µM BPS for one generation continued to have a significant reduction in average lifespan in the second (F_{2a}) generation, but not the third (F_{3a}) generation when compared to control (Figure 3.2D). In addition, both F_{2a} and F_{3a} generations exhibited a significant increase in lifespan when compared to F_1 suggesting a reversible effect. Moreover, worms treated with 5.0 µM BPS for two (F_{2b}) or three (F_{3b}) generations had a significantly decreased lifespans when compared to the control or any other treatments (Figure 3.2D), suggesting an accumulative effect. For 10.0 µM BPS exposure (Figure 3.2E), similar observations were made as the 5.0 µM-BPS treatment except for the third generation of worms only exposed during F_1 (F_{3a}) had a decreased lifespan when compared to control, although there was some recovery when compared to the second generation of worms exposed for one generation (F_{2a}).

***C. elegans*-Fertility**

At 1.0, 5.0, and 10.0 µM there was a significant decrease in the number of offspring hatched following treatment for one generation (F_1; data not shown). There was no significant effect observed regarding fertility in any generation of worm only exposed to 0.1 µM for one generation (F_1, F_{2a}, F_{3a}) when compared to control (Figure 3.3A). However, worms treated for two (F_{2b}) or three (F_{3b}) generations had a significant decrease in number of offspring hatched when compared to control or F_1, suggesting an accumulative effect. Worms exposed to 0.1 µM for three generations (F_{3b}) had a decrease in total offspring hatched when compared to control or any other generation

(F_1, $F_{2a,}$ F_{2b}, F_{3a}, F_{3b}), further supporting an accumulative effect. In contrast, there was no significant difference between worms treated for two generations (F_{2b}) and the F_{2a} and F_{3a} groups.

Worms treated for one generation with 1.0 µM BPS had no significant difference in number of offspring hatched when compared to control for either subsequent generation (F_{2a} and F_{3a}), suggesting an ability to recover (Figure 3.3B). In addition, this endpoint was significantly increased in both generations (F_{2a} and F_{3a}) when compared to F_1, suggesting a reversible effect and ability to recover. Worms treated with 1.0 µM BPS for two (F_{2b}) or three (F_{3b}) exhibited a reduction in total offspring hatched when compared to control or any generation of worms only treated for one generation (F_1, F_{2a}, F_{3a}), suggesting an accumulative effect. However, there was no significant difference in the number of offspring hatched F_{2b} and F_{3b} when compared to each other, suggesting a threshold was reached.

Worms exposed to 5.0 µM BPS had a decrease in offspring hatched in the subsequent generation (F_{2a}) when compared to control (Figure 3.3C), suggesting a long-term effect. However, when the next generation (F_{3a}) was compared to control there was no difference in number of offspring hatched, suggesting an ability to recover. When the F_{2a} and F_{3a} generations were compared to F_1 there was a significant increase in number off offspring hatched, further supporting this notion of recovery. Worms exposed to 5.0 µM for two (F_{2b}) or three (F_{3b}) generations had a significant decrease in offspring when compared to the control or any other generation (F_1, F_{2a}, F_{3a}), suggesting an accumulative effect. However, similar to 1.0 µM, when the F_{2b} and F_{3b} generations were compared there was no significant difference in number of offspring hatched between the two, suggesting a threshold or maximum toxicity.

Worms exposed to 10.0 µM BPS for one generation (F_1) or the subsequent generation (F_{2a}) had a significant decrease in the number of offspring hatched when compared to control (Figure

3.3D). However, the next generation (F_{3a}) exhibited no significant difference in the number of offspring hatched when compared to the control, suggesting an eventual ability to recover. Interestingly, there was no significant difference between worms exposed to 10.0 µM BPS for one generation, the following generation (F_{2a}) or worms exposed for two generations (F_{2b}), suggesting there was no accumulative effect and threshold was reached. In contrast worms the F_{3a} generation worms had a significant increase in offspring hatched when compared to F_1, suggesting a reduction in toxicity and an ability to recover. Worms treated for three generations exhibited a reduction in number of offspring hatched when compared to control and other generations (F_1, $F_{2a,}$ F_{2b}, F_{3a}), suggesting an accumulative effect.

Both embryonic lethality and the number of offspring hatched per day showed no difference between treatments or generations (data not shown). Worms exposed to 0.1 µM BPS for 2 generations (F_{2b}) exhibited a significant increase in their age when laying started when compared to the control (Figure 3.4A), suggesting a possible delay in maturation. There was a decrease in number of days laying in the worms exposed to 1.0 µM BPS for one (F_1) or two (F_{2b}) generations when compared to control (Figure 3.4B), suggesting a decrease in fertility. There was a decrease in both the number of days spent laying eggs in the worms exposed to 5.0 µM BPS for one (F_1) or three (F_{3b}) when compared to control (Figure 3.4C), suggesting reduced fertility. Worms treated for two (F_{2b}) or three (F_{3b}) generations exhibited an increase in the average age of worm when laying started, suggesting delayed maturation. Worms exposed to 10.0 µM BPS for one generation also exhibited a shortened laying period (Figure 3.4D). These shortened laying periods suggest a decrease in fertility. To the best of our knowledge no other study has examined the number of days spent laying or the age of worms when egg laying started.

Gestational NODEF Study

There was no significant difference in body weight or blood glucose levels in female pups at weaning time (Figure 3.5A and C). However, there was a significant decrease in both pup BW (p=0.0012; Figure 3.5B) and BGLs (p=0.0420 Figure 3.5D) in the BPS exposed males when compared to control. At the time of weaning, behavior tests (open field, novel object, and Y-maze) were preformed, however none were significant (Supplementary Figures 3.1-2). Still, when mice were retested at 12 weeks of age there was significance (Figures 3.6-7). There was a significant decrease (p=0.0274) in spontaneous alterations in the BPS exposed female mice when compared to control (Figure 3.6A), suggesting an impairment in working memory. In terms of the open field test there was a decrease in distance traveled in the male BPS treated group (p=0.0791; Figure 3.6D) when compared to control. Although not significant this could indicate an increase in anxiety-like behaviors. When both sexes were combined BPS treated mice spent significantly less (p=0.0451) time in the center than control mice (Figure 3.6G). When only comparing the treated male pups to the controls, a decrease in time spent in the center was observed but it was not significant (p=0.0764; Figure 6F). The results of the open field suggest that males were more affected and exhibited signs of increased anxiety-like behaviors. Like the open field test, there was no significant difference in the distance traveled between groups during the 12-week novel object test (Figure 3.7A-B). The BPS treated males had a decreased novel object index score (Figure 3.7D) when compared to the control group, but it was not significant (p=0.0757). Similarly, BPS exposed females also had a decreased novel object index score (Figure 3.7C), however theirs was significant (p=0.0370). Because there was no difference between sexes, they were combined. BPS exposed pups had a significantly (p=0.0119) decreased novel object index score when compared to control (data not shown). This suggests that BPS exposure leads to an impairment in memory and object recognition, with females being impacted to a greater extent. The tail suspension test

showed an increase in time spent immobile (p=0.0448; Figure 3.8F) in the male exposed mice, suggesting an increase in depression-related behavior. In contrast, there was no difference in time spent immobile in the female exposed mice (Figure 3.7E).

Neither the GTT nor ITT were significant at any time point for any group (data not shown). There was no significant difference in weekly BGLs between BPS exposed and control female pups at any time point (Figure 3.5C). Males exposed to BPS had a significantly lower BGLs at 3 weeks of age (p=0.0420), and 12 weeks of age (p=0.0398) when compared to control (Figure 3.5D), suggesting a possible protective effect against diabetes. There was a significant increase in the body weights of the BPS female pup group when compared to control at 18 weeks of age (p=0.0153; Figure 3.5A). There was no significant difference in diabetes incidence at any time point between the treatment groups in either the male or female pups (Figure 3.5E-F). In terms of organ weight there were no significant differences in male or female pups when compared to control.

Discussion and Conclusion

In this study developmental exposure to BPS resulted in a reduced lifespan and reproductive toxicity in *C. elegans* and neurological toxicity in NODEF mice. Generational studies of exposed *C. elegans* demonstrated that high concentrations of BPS can reduce average lifespan and offspring numbers for one generation after treatment (F_{2a}), exhibiting a long-term effect. However, only worms exposed to 10 µM during the first generation exhibited a continued significant decrease in average lifespan when compared to control in the third generation (F_{3a}), suggesting an ability to recover in the other concentrations of treated worms. In addition, worms exposed for multiple generations exhibited an accumulative effect until threshold was reached. The results of our studies are similar to that of Xiao et al. (2019) who continuously exposed *C.*

elegans to 0-100 µM BPS. To the best of our knowledge this is the first study to examine the effects of BPS in *C. elegans* only treated for one generation.

Although BPS exposure did not affect BGLs or diabetes incidence in exposed pups it did cause impaired memory in both sexes as seen by the novel object test. Male pups exposed to BPS had significantly lower BWs and BGLs when weaned, suggesting a protective effect against diabetes and obesity. In contrast gestational exposure to BPA increased the risk of type 1 diabetes, while there was no effect on the BGLs or BW of male offspring (Xu, Huang, Nagy, et al. 2019). This suggests that both BPA and BPS have sex-dependent effects and more importantly that they exert different effects and likely use different mechanisms. However, in the BPA study, researchers used a high dose of BPA (300 µg/kg BW) while we used a low dose (3 µg/kg BW). Because EDCs are known to create a bimodal dose-response curve further research utilizing both high and low concentrations of BPA and BPS are needed to compare their differences.

Female mice exhibited an impairment in both working and short-term memory, as shown by the lower novel object index and % spontaneous alterations in the Y-maze while males exhibited an increase in depression-like behaviors/ anxiety-like behaviors, indicated by the tail suspension test. When the results of the open field tests for both sexes were combined there was a significant decrease in the time spent in the center of the apparatus, suggesting an increase in anxiety-like behavior. Developing zebrafish exposed to a low concentration of BPS also exhibited an increase in anxiety-like behavior and neurogenesis dependent on androgen receptors (Kinch et al. 2015). It is possible that BPS implores a similar mechanism in mice however further research is needed to determine the underlying mechanisms. Adult female zebrafish exposed BPS at low and high doses exhibited improved short-term memory and increased phosphorylation of the ERK at low doses and impaired short-term memory and decreased ERK phosphorylation at higher doses

(Naderi et al. 2020). This upregulation CREB/ERK pathway could be another possible mechanism BPS uses to cause neurotoxicity. Again, further research is needed to confirm this. Another possible mechanism BPS uses to cause behavioral deficits is altering expression of 5α-R isozymes and genes associated with serotonin systems (Castro et al. 2015).

To the best of our knowledge there are only two gestational studies examining the effects of BPS on behavior and the brain in rodents and no adult studies (Castro et al. 2015; LaPlante et al. 2017). In addition, neither of the two studies utilized behavioral tests related to memory, depression-like behaviors, or anxiety-like behaviors. Similar to BPS, BPA exposure can cause an increase in anxiety-like behaviors and depression-like behavior (Ohtani et al. 2017) and an impairment in working and short-term memory with higher sensitivity in females (Tian et al. 2010). This further supports our observation that BPS induces increased anxiety-like behaviors and depression-like behavior along with an impairment of working and short-term memory in gestationally exposed mice.

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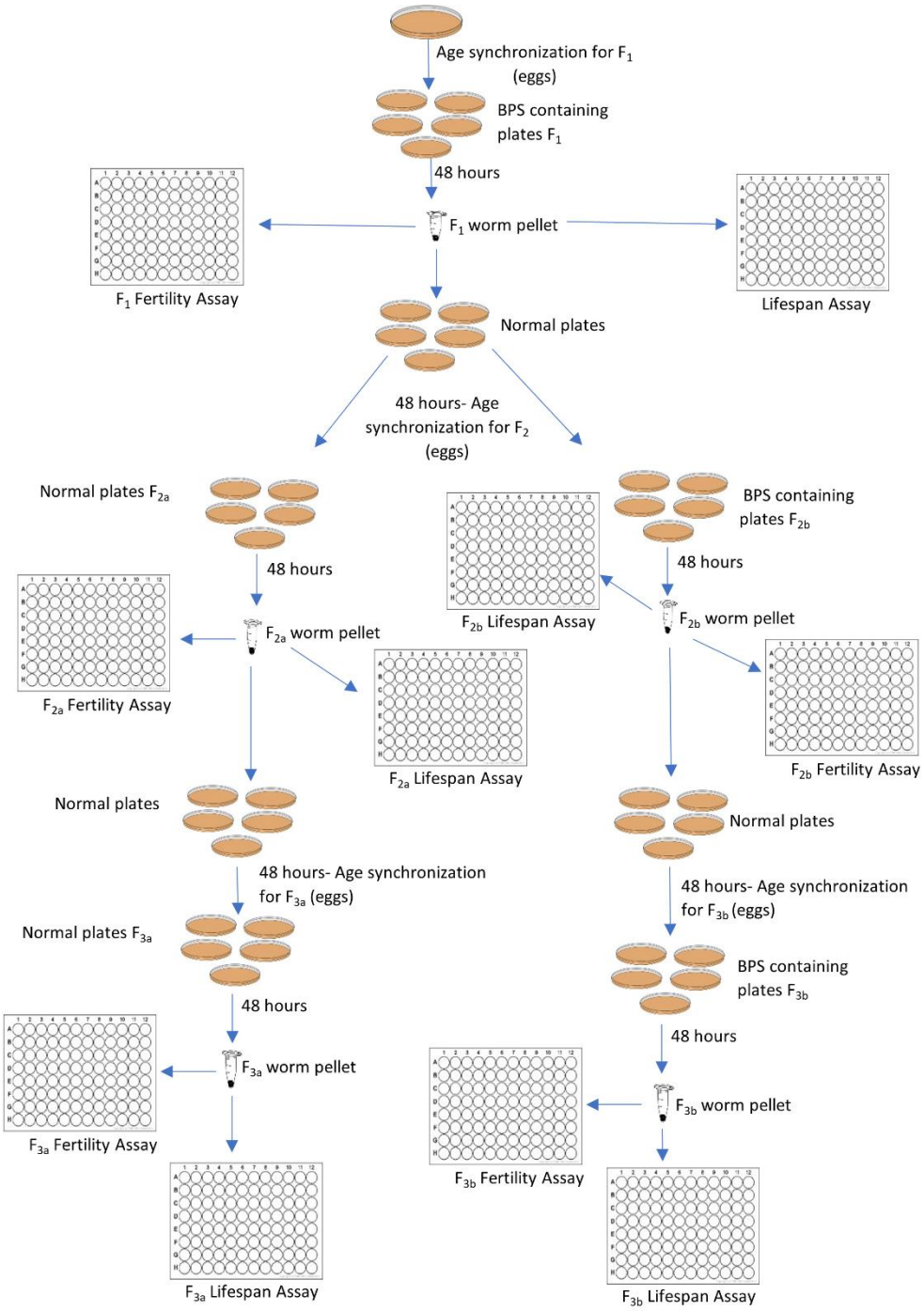
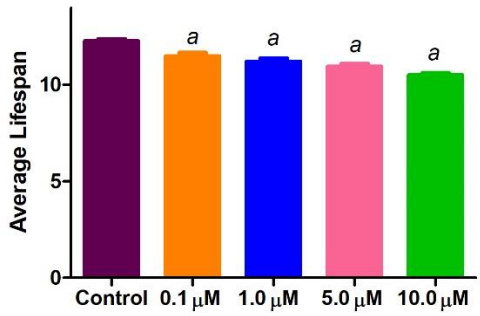
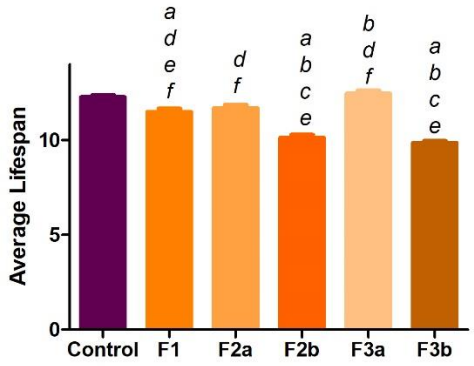


Figure 3.1. Outlines for generational studies. To ensure there were no effects from additional bleaching, each generation of the study had an untreated control (e.g., without BPS). Since there were no differences among them for the parameters measured, they were combined and presented as the control in Figures 3.2-4.

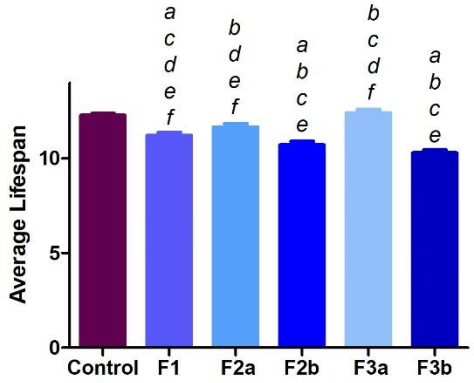
A



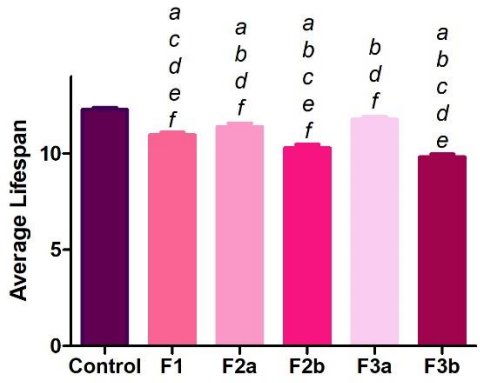
B (0.1 µM BPS)



C (1.0 µM BPS)



D (5.0 µM BPS)



E (10.0 µM BPS)

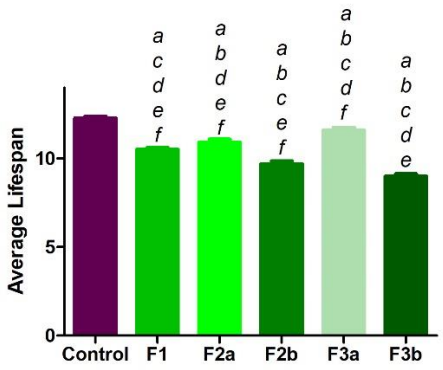
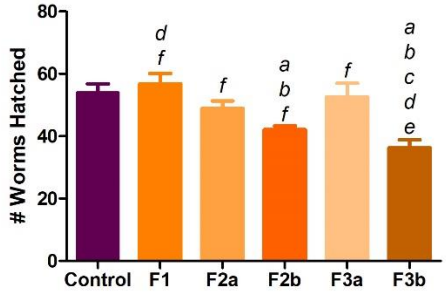


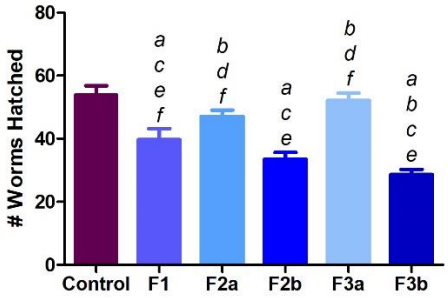
Figure 3.2. Lifespan study for *C. elegans.* Average lifespan for worms treated for 1 generation (A), for 1, 2, or 3 generations at 0.1 µM (B), 1.0 µM (C), 5.0 µM (D), and 10.0 µM (E). The values

are presented as mean ± SEM. a = significant compared to control, b = significant compared to F_1, c = significant compared to F_{2a}, d = significant compared to F_{2b}, e = significant compared to F_{3a}, f= significant compared to F_{3b}, $p< 0.05$. N = 350-500 worms. F_1, F_{2a}, F_{2b} F_{3a}, and F_{3b} were described in Figure 3.1.

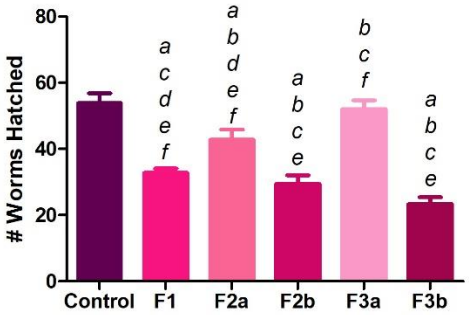
A (0.1 µM BPS)



B (1.0 µM BPS)



C (5.0 µM BPS)



D (10.0 µM BPS)

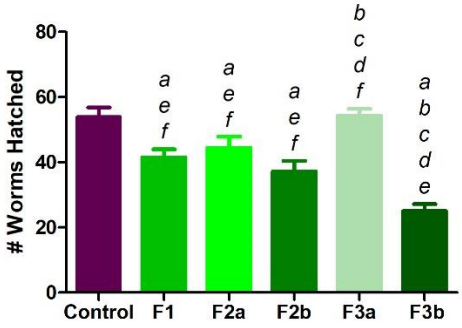
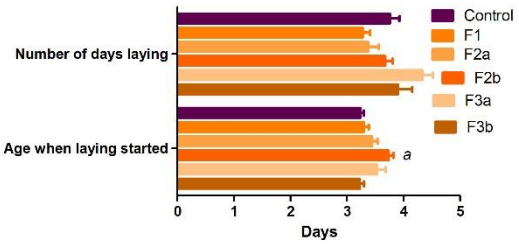
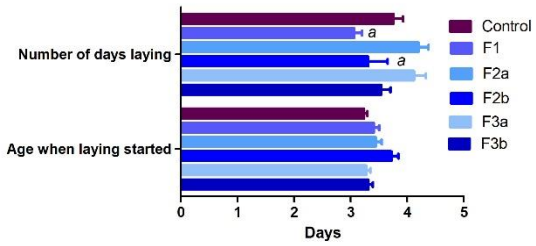


Figure 3.3. Total Offspring for *C. elegans.* Average number of surviving offspring of *C. elegans* treated for 1, 2, or 3 generations at 0.1 µM (A), 1.0 µM (B), 5.0 µM (C), and 10.0 µM (D). The values are presented as mean ± SEM. a = significance compared to control, b = significance compared to F_1, c = significance compared to F_{2a}, d = significance compared to F_{2b}, e = significance compared to F_{3a}, f= significance compared to F_{3b} $p< 0.05$. N=30-50 worms.

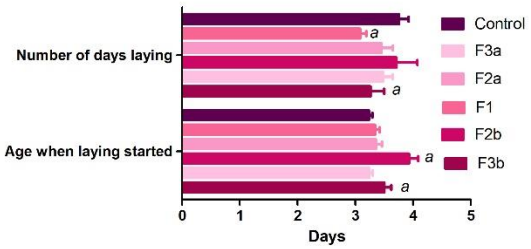
A (01. µM BPS)



B (1.0 µM BPS)



C (5.0 µM BPS)



D (10.0 µM BPS)

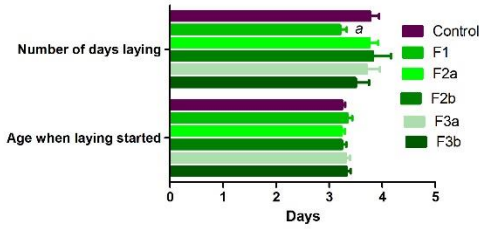


Figure 3.4. Age of *C. elegans* when laying started and number of days spent laying for *C. elegans* treated for 1,2, or 3 generations at 0.1 µM (A), 1.0 µM (B), 5.0 µM (C), and 10.0 µM (D). The values are presented as mean ± SEM. a= significance compared to control, b= significance compared to F_1, c= significance compared to F_{2a} 2a, d=significance compared to F_{2b}, e=significance compared to F_{3a}, f= significance compared to F_{3b} $p< 0.05$. N=30-50 worms.

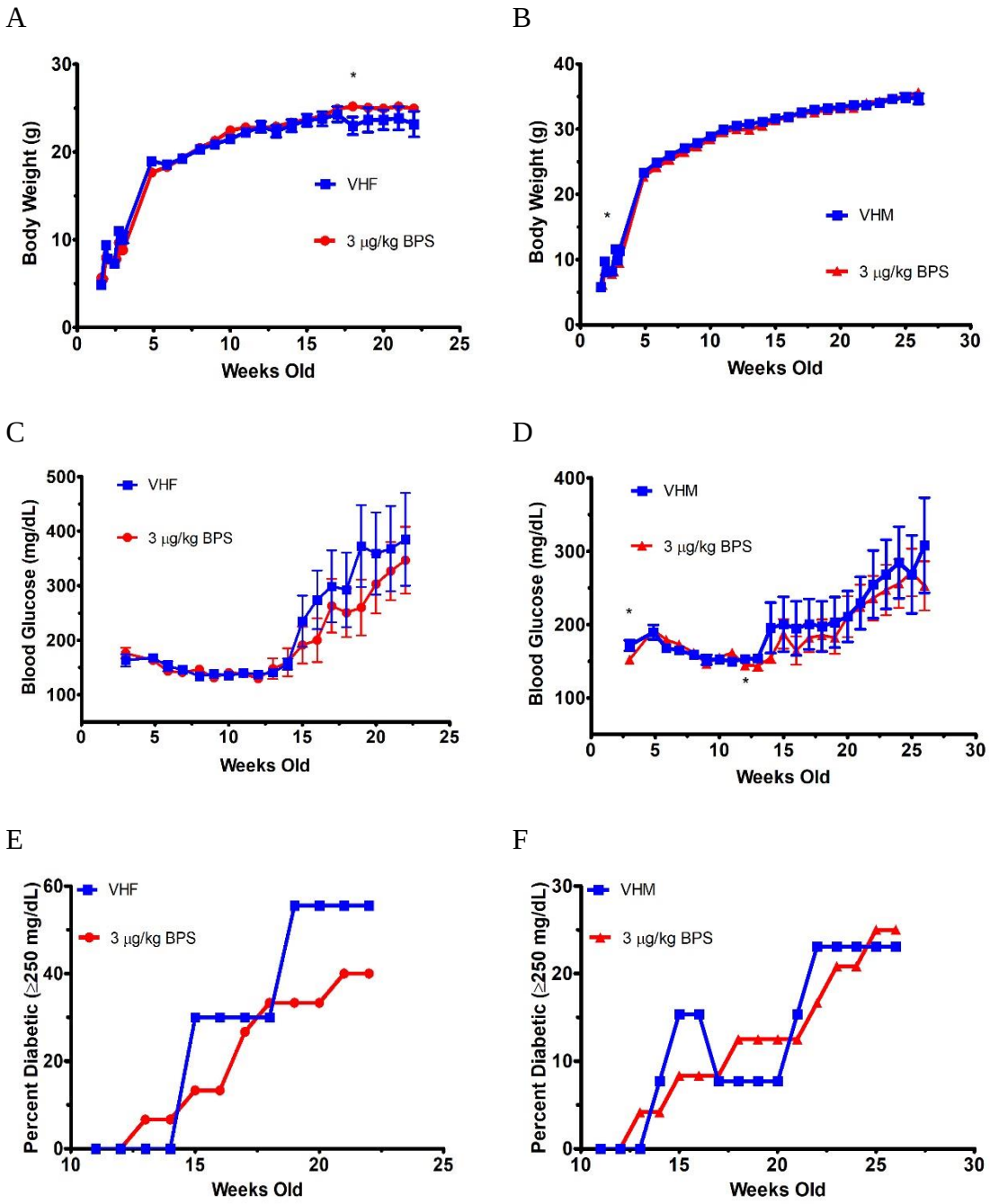


Figure 3.5. Weekly body weights, blood glucose levels, and diabetes incidence. Weekly BW for female (A) and male (B) pups. Weekly blood glucose levels for female (C) and male (D) pups. Diabetes incidence for female (E) and male (F) pups. VHM, vehicle males. VHF, vehicle females. N = 4-6. The values are presented as mean ± SEM. *, $p < 0.05$.

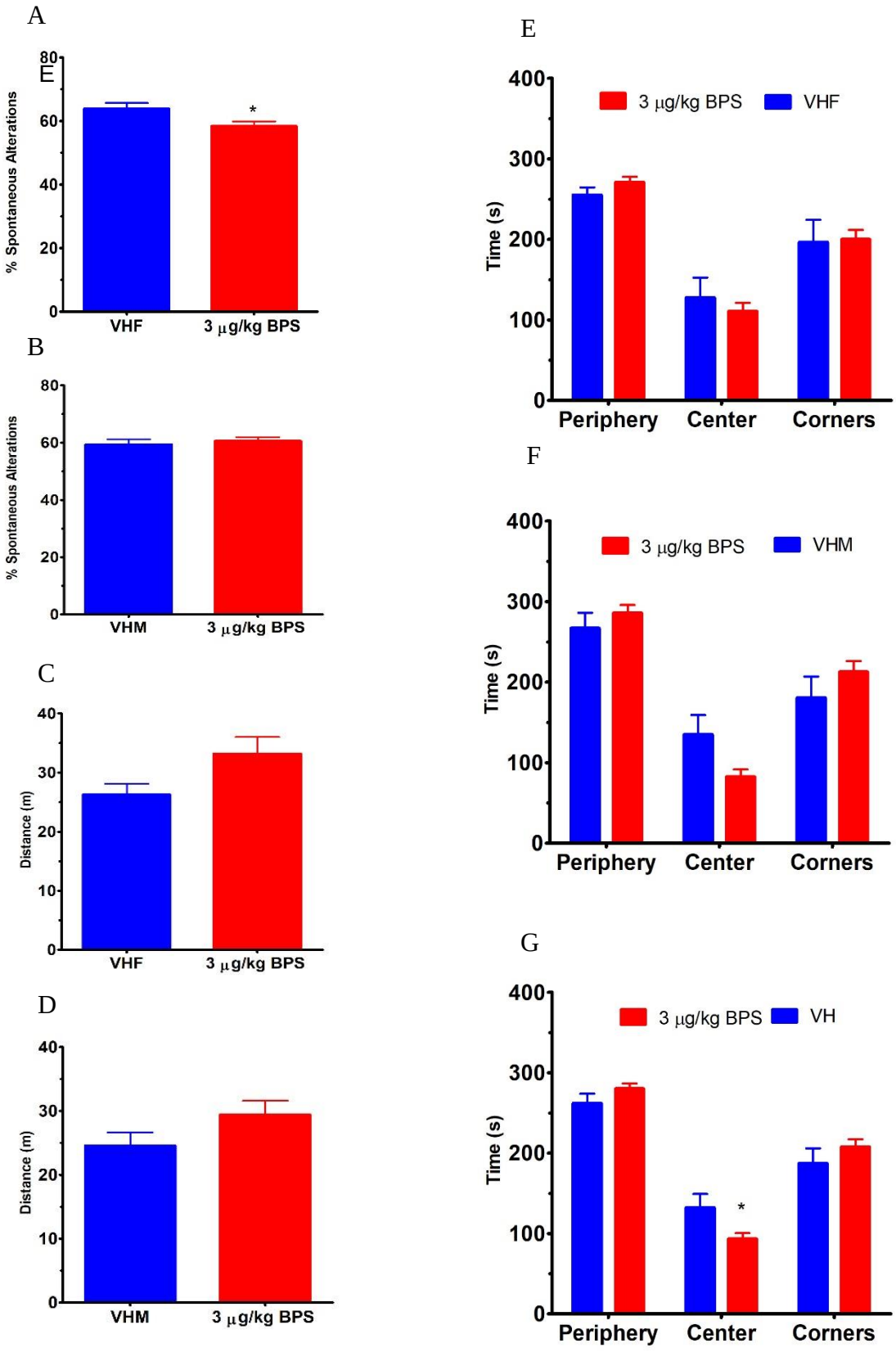

A
% Spontaneous Alterations
80
60
40
20
0
*
VHF
3 µg/kg BPS
B
% Spontaneous Alterations
80
60
40
20
0
VHM
3 µg/kg BPS
C
Distance (m)
40
30
20
10
0
VHF
3 µg/kg BPS
D
Distance (m)
40
30
20
10
0
VHM
3 µg/kg BPS
E
Time (s)
400
300
200
100
0
3 µg/kg BPS
VHF
Periphery
Center
Corners
F
Time (s)
400
300
200
100
0
3 µg/kg BPS
VHM
Periphery
Center
Corners
G
Time (s)
400
300
200
100
0
3 µg/kg BPS
VH
*
Periphery
Center
Corners

Figure 3.6. Y-maze and Open-field behavior tests in NODEF pups at 12 weeks of age. % Spontaneous alteration during the Y-maze in female (A) and male (B). (C) Distance traveled during the open-field test for female (C) and male (D) pups. Time spent in each section during the open-field test for female (E) and male (F) pups. Time spent in each section during the open-field test with both sexes combined (G) VHM, vehicle males. VHF, vehicle females. N = 5-6. The values are presented as mean ± SEM. *, $p < 0.05$.

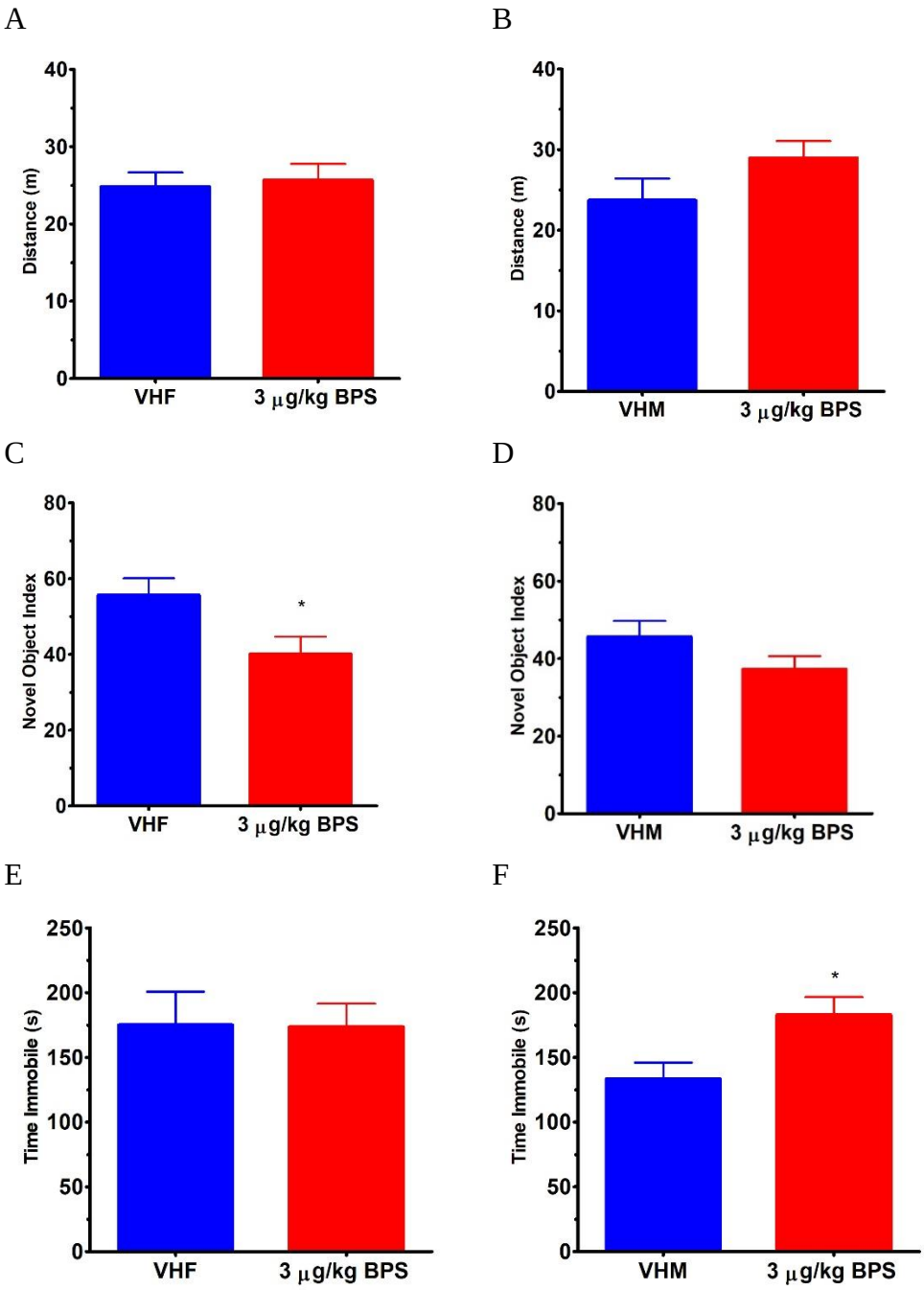
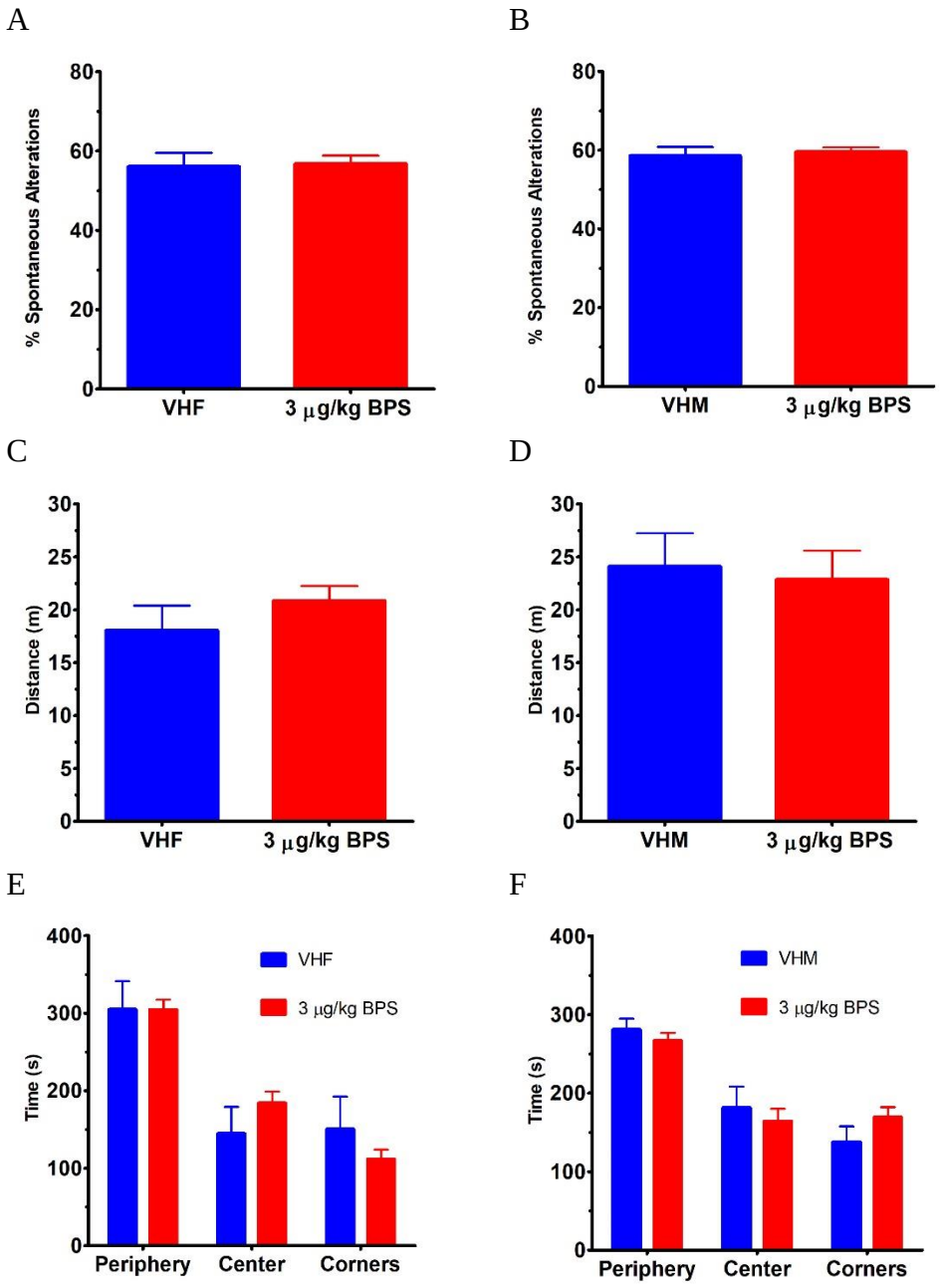


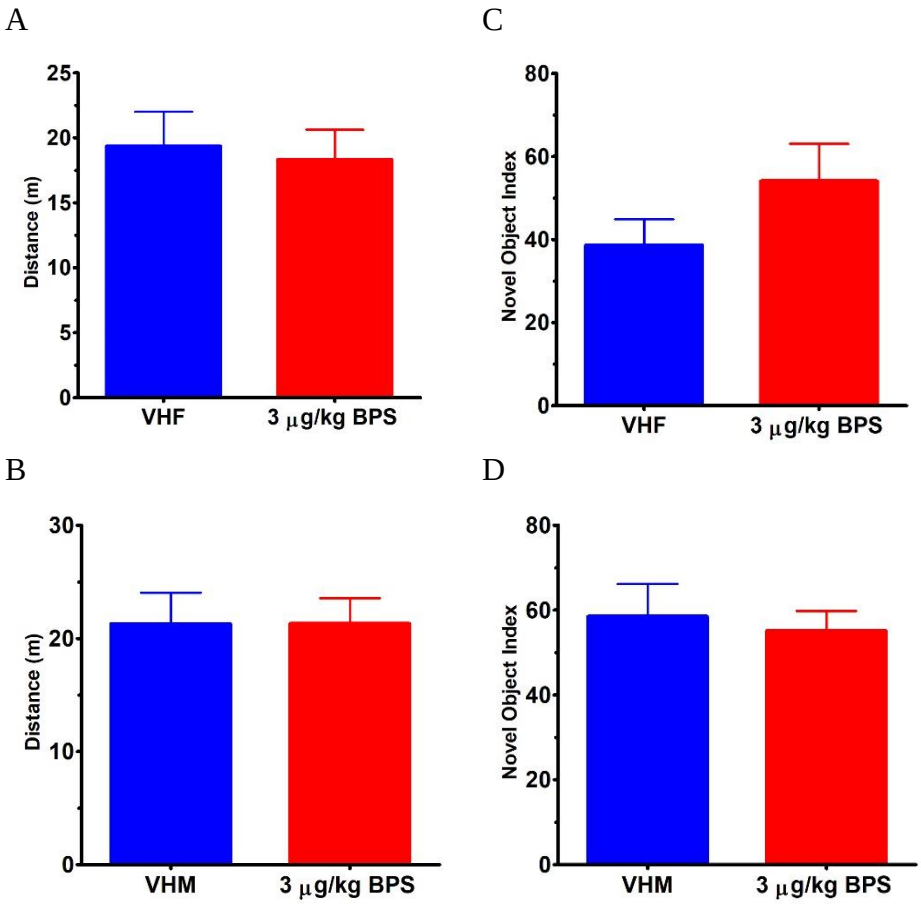
Figure 3.7. Behavior tests in NODEF pups at 12 weeks of age. Distance traveled during the novel object test for female (A) and male (B) pups. Novel object index for female (C) and male (D) pups. Time spent immobile for female (E) and male (F) pups during the tail suspension test.

VHM, vehicle males. VHF, vehicle females. N = 4-6. The values are presented as mean ± SEM.

*, $p < 0.05$.



Supplementary Figure 3.1. Behavior tests in NODEF pups at 3 weeks of age. % Spontaneous alteration during the Y-maze in female (A) and male (B). (C) Distance traveled during the open-field test for female (C) and male (D) pups. Time spent in each section during the open-field test for female (E) and male (F) pups. VHM, vehicle males. VHF, vehicle females. N = 5-6. The values are presented as mean ± SEM. *, $p < 0.05$.



Supplementary Figure 3.2. Behavior tests in NODEF pups at 3 weeks of age. Distance traveled during the novel object test for female (A) and male (B) pups. Novel object index for female (C) and male (D) pups. VHM, vehicle males. VHF, vehicle females. N = 4-6. The values are presented as mean ± SEM. *, $p<0.05$

CHAPTER 4

CONCLUSION AND FUTURE DIRECTIONS

Bisphenol A and bisphenol S are both endocrine disrupting chemicals that are able to cause reproductive toxicity, neurotoxicity, and immunomodulation. Despite their similar structures and possible mechanisms, there is an increase in the popularity of marketing products containing BPS as "a safer BPA-free" product. Companies are able to do this partially due to a recent FDA report stating that both BPA and BPS are safe for use in food packaging (Abbasi 2018). In addition, there are currently no restrictions surrounding the use of either bisphenol in the U.S (Abbasi 2018). Both BPA and BPS can exhibit a bimodal dose-response curve, signifying the need for testing both high and low concentrations of these bisphenols (Naderi et al. 2014; Eladak et al. 2015; LaPlante et al. 2017). Similar to BPA, BPS can bind to various receptors (Molina-Molina et al. 2013), modify an organism's epigenetics (Verbanck et al. 2017; Awada et al. 2019; Římnáčová et al. 2020), or disrupt cell signaling pathways (Viñas and Watson 2013; Awada et al. 2020) to exert their toxic effects.

In chapter 2, we examined the effect of sex and diet on bisphenol S exposure. Adult male nondiabetic mice fed a soy-based diet and exposed to 300 µg/kg BW BPS had significantly increased weekly blood glucose levels, suggesting a prediabetic state. In addition, these mice exhibited impaired glucose tolerance and an increase in proinflammatory markers. The increased % $CD4^{+}CD25^{-}$ cell populations in the male soy-based diet correlated with the increased anxiety-like behaviors seen during the novel object test. In male mice fed a Western diet, the BPS-exposed mice exhibited an increase in anxiety-like behaviors which were negatively correlated with decreased CD5 MFI of $CD24^{-}CD5^{+}$ cells. Female mice fed a phytoestrogen-free diet and exposed

to 30 or 300 µg/kg BW BPS exhibited decreased insulin resistance paired with decreased glucose tolerance and increased proinflammatory markers when compared to the control. In addition, these mice exhibited increased hyperactivity and anxiety-like behavior. Overall, BPS exposure in adult mice caused changes in behavior and immunomodulation in a sex dependent manner.

In chapter 3, we utilized *C. elegans* in addition to NODEF mice to determine the developmental, reproductive, neurological, and generational effects of BPS. We first tested 0.1, 1.0, 5.0 and 10.0 µM BPS exposure in *C. elegans.* Worms exposed to 5.0 or 10.0 µM BPS exhibited a generational effect with regards to reduced lifespan and number of offspring. In addition, worms exposed to 10.0 µM BPS for one generation continued to have a reduced lifespan in the third generation when compared to the control. All concentrations examined saw an accumulative effect on both reduced lifespan and number of offspring until a threshold was reached when worms were exposed for 2 or 3 generations. This was the first study to examine the generational effects of BPS in worms exposed for only one generation, to the best of our knowledge.

We next used the results of our *C. elegans* study to determine the appropriate concentration for a gestational exposure in NODEF. Dams were exposed to 0 or 2 µg/kg BW BPS throughout gestation. BPS-exposed male pups had significantly lower weaning weights and weaning BGLs when compared to control, suggesting a protective effect against obesity and T1D. BPS exposure had sex-dependent effects with regard to behavioral changes. There was an impairment of short-term and working memory in the BPS-exposed female mice. We observed an increase in depression-like/anxiety-like behaviors in the BPS-exposed male mice. In addition, when the results of the open field tests for both sexes were combined, there was a significant decrease in the time spent in the center of the apparatus, with male mice more affected. This suggests that BPS-exposure causes anxiety-like behaviors in both sexes, with male mice being more impacted.

When these studies are put together, it is clear that BPS is able to cause neurotoxicity, reproductive toxicity, and immunomodulation in both gestationally exposed and adult mice. These effects were dependent on the sex, diet, and window of exposure. Not surprisingly, mice exposed *in utero* were most significantly impacted, exhibiting memory impairments and increases in depression-like and anxiety-like behaviors. Additionally, our study with *C. elegans* demonstrated that worms can recover in the subsequent generations when a single generation is exposed and also have an accumulative effect until threshold is reached when multiple generations are exposed. While this thesis adds to the knowledge on the toxicity of BPS, more studies focusing on the mechanisms of BPS in comparison to BPA such as changes in microbiome are needed. Of particular focus should be the influence of BPS on the gut microbiome. Although several studies have shown changes in the microbiome as a potential mechanism of BPA (Javurek et al. 2016; Lai et al. 2016; DeLuca et al. 2018; Rinninella et al. 2019; Xu et al. 2019) there is currently only one study examining the effect of BPS on the microbiome (Catron et al. 2019).

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